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Succinctus

# Hybridisation between the two major African schistosome species of humans

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ABSTRACT

It is generally accepted that *Schistosoma mansoni* and *Schistosoma haematobium*, causing intestinal and urinary schistosomiasis, respectively, are not able to hybridise, due to the high phylogenetic distance between them. Cloning of nuclear internal transcribed spacer rDNA and partial mitochondrial cytochrome *c* oxidase 1 fragments revealed two internal transcribed spacer rDNA genotypes within single eggs and miracidia, one identical to *S. mansoni* and the other identical to *S. haematobium*, suggesting hybrid ancestry. The cytochrome *c* oxidase 1 clones always belonged to only one of the parental species. This demonstrates that offspring of heterologous pairing between these two species is not (always) parthenogenetic.

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Schistosomiasis is a disease of great medical and veterinary importance in tropical and subtropical regions, caused by parasitic flatworms of the genus *Schistosoma* (subclass Digenea). The schistosome parasite is the only dioecious trematode, which creates an opportunity for interplay between male and female parasites in the definitive host where the sexual stage of the life cycle takes place. Laboratory studies have shown that closely related schistosome species (belonging to the same species group) can successfully hybridise for several generations (Taylor, 1970; Webster and Southgate, 2003) and some of these hybrids have also been found in nature (Tchuem Tchuenté et al., 1997; Steinauer et al., 2008; Huyse et al., 2009; Webster et al., 2013). When species belonging to a different species group are paired, the offspring is believed to be parthenogenetic (Taylor, 1970; Basch and Basch, 1984; Khalil and Mansour, 1995; Southgate et al., 1998). This has for example been demonstrated for the offspring of *Schistosoma mansoni* females and *Schistosoma intercalatum* males by means of iso-enzymes and cytogenetic analysis (Tchuem Tchuenté et al., 1994). Experimental crossing of *S. mansoni* and *Schistosoma haematobium*, the two most common human schistosome species, has been shown to lead to heterospecific pairing (Khalil and Mansour, 1995; Webster et al., 1999). The eggs isolated from the liver homogenates of the cross-specific infected hamsters contained fully developed miracidia with motile cilia and flame cells and they were able to hatch (Khalil and Mansour, 1995). The offspring was suggested to be parthenogenic, although this was not tested (cyto)genetically. Here we show, to our knowledge for the first time, that *S. mansoni* and *S. haematobium* can interbreed in human hosts, leading to hybrid offspring.

This study is part of a larger investigation on the epidemiology and control of schistosomiasis in Senegal, for which approval was obtained from the ethical committees of the Institute of Tropical Medicine, Antwerp, Belgium and the Ministry of Health, Dakar, Senegal. In short, schoolchildren were randomly selected in several villages along the shores of Lac de Guiers, the Lampsar River and Senegal River. From each child one stool and one urine sample were collected and examined by the Kato Katz method (two slides of 25 mg) and urine filtration (10 ml), respectively, and the data were analysed anonymously. At the end of the study all children were treated with praziquantel according to World Health Organization (WHO) guidelines at 40 mg/kg.

Eggs were filtered from 44 positive stool and 63 positive urine samples, concentrated per sample and hatched in bottled mineral water. With the aid of a binocular microscope, individual miracidia were pipetted onto Whatman FTA® classic cards in a volume of 3 μl of water, while unhatched eggs were pooled per sample in 1.5 ml of 70% ethanol. DNA extraction of all urine and stool samples and microsatellite PCR amplification (specific to *S. mansoni*) of all stool samples were performed following the method of Van den Broeck

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et al. (2011). During genotyping, attention turned to an outlier allele in the genetic profile of microsatellite marker SMD28 (GenBank accession number AF202966). Allele 218 of this locus appears to be fixed in Senegalese strains of *S. haematobium*, *Schistosoma bovis* and their hybrid cross (our unpublished data), and has to date not been found in Senegalese *S. mansoni* populations that are mostly homozygous for allele 239 (total range 224–239 bp; Van den Broeck et al., 2011). All urine samples were therefore genotyped using the same microsatellite marker (SMD28). Approximately 36% of the eggs and miracidia isolated from urine and 2% isolated from stools, displayed a 'hybrid' microsatellite profile 218:239 or 218:236. Ten of these eggs and miracidia (Table 1) were retained for further molecular characterisation in the present study. Adult *S. mansoni* and *S. haematobium* samples from the Schistosomiasis Collection at the Natural History Museum London, UK (SCAN) were included as controls (Emery et al., 2012).

A suitable marker to detect hybridisation is the internal transcribed spacer (ITS) rDNA region. It occurs in the nuclear genome of all eukaryotes as part of a tandem repeat multigene that is subdivided into three coding regions (18S, 5.8S and 28S) and four non-coding regions (ETS, ITS 1, ITS 2 and NTS). This region can retain both parental copies for several generations before they are homogenised by concerted evolution (Sang et al., 1995). It has previously been used to detect hybridisation events in schistosome species (Steinauer et al., 2008; Huyse et al., 2009; Webster et al., 2013).

The individual eggs and miracidia were subjected to PCR amplification of the complete ITS region (981 bp; primers ITS4: TCCTCCGCTTATTGATATGC and ITS5: GGAAGTAAAAGTCGTAACAAG (Barber et al., 2000)) and partial cytochrome *c* oxidase subunit I (*cox1*) mtDNA (585 bp; primers Asmit1 TTTTTTGGTCATCCT GAGGTGTAT (Bowles et al., 1992) and Schisto3′ TAATGCATMG-GAAA-AAAACA (Lockyer et al., 2003)), following the same protocol as in Huyse et al. (2009). PCR products were purified by means of GFX columns (Healthcare, UK) and cloned following the manufacturer's instructions (TA cloning kit, Life Technologies, USA). For each cloning reaction 20 colonies were selected for PCR with the M13 forward and reverse primer and visualised on a 1.5% ethidium bromide agarose gel. Four to 11 positive clones from each of the reactions were selected for sequencing with the M13 forward and reverse primers, using Big Dye Chemistry v1.1 in an ABI 3130 Genetic Analyser (Life Technologies, USA). The sequences were assembled and manually edited using Geneious 5.4.6 (http://www.geneious.com/). For the four miracidia isolated from urine samples, 13–17 additional colonies of the ITS cloning reaction were analysed by restriction fragment length polymorphism (RFLP) analysis by digesting the PCR fragments with the restriction enzyme *Alu*I. The ITS fragment of *S. mansoni* has one *Alu*I restriction site while those of *S. haematobium* and *S. bovis* have none.

**Table 1**
Number of sequences per sample (individual egg or miracidium) collected in northern Senegal and identified as *Schistosoma mansoni* and *Schistosoma haematobium* for the cloned internal transcribed spacer rDNA and cytochrome *c* oxidase subunit I fragments, and the microsatellite allele size of locus SMD28 for each sample.

| Village and Sampling year | Stage | Origin | ITS | | *cox*1 | | Allele size |
|---|---|---|---|---|---|---|---|
| | | | Sm | Sh | Sm | Sh | |
| Ndieumeul 2010 (Lac de Guiers) | Egg | Stool | 8 | 2 | 10 | | 218:239 |
| | Egg | Stool | 2 | 2 | | 11 | 218:239 |
| | Egg | Stool | 1 | 3 | 8 | | 218:239 |
| | Egg | Stool | 8 | 2 | 10 | | 218:239 |
| | Egg | Stool | 3 | 1 | 4 | | 218:239 |
| | Egg | Stool | | 4 | | 5 | 218:239 |
| NiétiYone 2006 (Lac de Guiers) | Miracidia | Urine | 1 | 10 + 13[a] | | 5 | 218:239 |
| | Miracidia | Urine | | 11 + 16[a] | | 9 | 218:239 |
| Mbodienne 2006 (Lampsar River) | Miracidia | Urine | | 9 + 14[a] | | 8 | 218:239 |
| | Miracidia | Urine | 2 | 8 + 17[a] | | 6 | 218:239 |
| *S. haematobium* 3122 (SCAN[b]) | Urine | Adult | | 4 | | 5 | 218:218 |
| *S. mansoni* 3108 (SCAN[b]) | Stool | Adult | 4 | | 1 | | 239:239 |

[a] Number of additional clones analysed by restriction fragment length polymorphism (RFLP) analysis.
[b] Reference sample from the Schistosomiasis Collection at the Natural History Museum London, United Kingdom.

Six samples and the reference *S. haematobium* had a *cox1* sequence identical to the *S. haematobium* GenBank sequence (Accession No. FJ588852), while the remaining four samples and the reference *S. mansoni* had a *cox1* sequence nearly identical to the *S. mansoni* GenBank sequence (Accession No. AY446106). All sequenced clones from the same sample were identical (no intra-individual variation); there were a few base-pair differences between the *S. mansoni* sequences from different samples.

All sequenced ITS rDNA clones of the reference material of *S. mansoni* and *S. haematobium* were identical to the GenBank sequences (Accession Nos. AY446082 and FJ588861, respectively); no intra-individual variation was found. In seven of the 10 suspected hybrid individuals, two copies of ITS were found, one identical to *S. mansoni* (Accession No. AY446082) and the other identical to *S. haematobium* (Accession No. FJ588861). All sequenced clones of the remaining three samples were identical to *S. haematobium*, even after additional colony screening through RFLP analysis (see Table 1 for exact numbers).

The occurrence of *S. mansoni* and *S. haematobium* ITS sequences in a single egg or miracidium indicates that both species successfully hybridise. Four of the seven samples with mixed ITS profiles had a *S. mansoni cox1* haplotype while three samples displayed a *S. haematobium* haplotype suggesting that hybridisation is bidirectional. There were three samples with a 'pure' *S. haematobium* profile for both *cox1* and ITS rDNA, even though they displayed the 'hybrid' 218:239 microsatellite profile. This could suggest that the 218:239 profile is not a diagnostic marker for hybridisation, or the signal of hybridisation is blurred due to biased homogenisation of the ITS copies towards one of the parental sequences. In some cases this can already occur in F2 hybrids or backcross generations due to asymmetrical backcrossing (Aguilar et al., 1999).

The increasing use of molecular techniques in schistosomiasis research has resulted in a growing number of reports on hybridisation and introgression in schistosomes (Steinauer et al., 2008; Huyse et al., 2009; Webster et al., 2013). The detection of hybridisation has been problematic in the past due to the lack of adequate sampling and parasite identification techniques. Laboratory passage of the parasites might prevent the detection of hybridisation events due to selection and genetic bottlenecking that the populations are subjected to in the laboratory system (e.g. due to the use of less suitable rodent or snail hosts in experimental infections (Khalil and Mansour, 1995 and references therein). New molecular markers in combination with better storage protocols for larval schistosomes now enable the genotyping of large numbers of individual samples isolated directly from the field (Huyse et al., 2009; Webster et al., 2013). Another crucial factor is the combination of nuclear and mtDNA markers; a single-locus approach such as barcoding would fail to detect hybridisation or introgression events (Huyse et al., 2009).

Four species of schistosomes are prevalent in the Senegal River Basin: *S. haematobium*, *S. mansoni*, *S. bovis* and *Schistosoma curassoni*, the latter two being important veterinary parasites (Webster et al., 2013). There are several studies suggesting that these species interact with each other. Recent molecular genotyping studies demonstrated the occurrence of hybrid crosses between *S. haematobium* and *S. bovis* infecting Senegalese children (Huyse et al.,

2009), and between *S. haematobium*, *S. bovis* and *S. curassoni* (Webster et al., 2013). An epidemiological study by Meurs et al. (2012) in two northern Senegalese communities showed that 53% of infected subjects had mixed *S. mansoni* and *S. haematobium* infections, of which 15% showed ectopic egg elimination. This is the excretion of eggs via an unusual route, i.e. *S. mansoni* eggs in urine or *S. haematobium* eggs in feces, which can be the result of heterospecific pairing (Southgate et al., 1998). Ernould et al. (1999) reported levels up to 31% of ectopic *S. mansoni* eggs in urine samples, suggesting that heterospecific pairing between *S. mansoni* and *S. haematobium* might be relatively frequent in northern Senegal. Here we provide evidence that such pairings indeed take place and that they can result in genetic recombination between both species.

Hitherto it was always assumed that heterospecific pairing between schistosome species belonging to different species groups would result in parthenogenesis. In experimental crossings insemination was clearly shown but it was suggested solely to be a trigger for parthenogenesis without fusion of the sperm and egg nucleus (pseudogamy; see the review of Jourdane et al. (1995)). Generative parthenogenesis (yielding haploid offspring through oogenetic reduction division) is described as the most frequent type of parthenogenesis in schistosomes (Jourdane et al., 1995). This would lead to haploid offspring and thus homozygous genotypes, which have not been recovered in this study (usually at least one of the microsatellite markers is heterozygous). A unisex infection experiment in hamsters showed that female *S. haematobium* can produce eggs parthenogenetically, but these eggs were small and only a few contained motile embryos (Khalil and Mansour, 1995). In contrast, when they mated with *S. mansoni* males the eggs isolated from the liver had a length that was intermediate between both parental species and contained fully developed miracidia. Besides egg size, nothing is known about the phenotypic characteristics of these hybrid crosses. There have been no experiments that tested the cercarial infectivity of the offspring or anything beyond this stage.

However, experimental studies have been performed on a putative hybrid *S. mansoni* strain that was passed more frequently in the urine than in the stools of patients in Beni-Suef in upper-middle Egypt (Soliman et al., 1986). These isolates were cultured in the laboratory and compared with material from Giza, Egypt where all *S. mansoni* were passed in faeces. The Beni-Suef strain had a lower worm recovery, a longer maturation time and lower fecundity compared with the Giza strain, and showed more resemblance with *S. haematobium* in the number of testes and in the egg distribution patterns in tissues of infected hamsters. The authors suggested this could point to a hybrid origin since the patients had mixed infections with *S. haematobium* and *S. mansoni* (Soliman et al., 1986). It is impossible to verify this postulation without the use of molecular markers, but if true, it demonstrates that genetic recombination between *S. haematobium* and *S. mansoni* also occurs outside Senegal and that these hybrids have different morphological and biological characteristics compared with pure *S. haematobium* or *S. mansoni*.

Based on the current data, we are unable to infer anything about the viability of the hybrid offspring. The presence of mixed ITS profiles in two of the four miracidia, albeit in low copy numbers, might suggest that these are further generation or backcross hybrids but more data are needed to confirm this observation. Field studies are therefore needed to verify whether and how often viable offspring occur in nature and by which snail species they are transmitted, while experimental infection studies in laboratory animals are needed to study both the biological and the genetic characteristics of *S. mansoni* and *S. haematobium* crosses. This will allow determination of the magnitude of this phenomenon and any impact it may have in the epidemiology and transmission of schistosomiasis in African regions where *S. mansoni* and *S. haematobium* are co-endemic.

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