Detecting hybridization in African schistosome species: does egg morphology complement molecular species identification?

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SUMMARY

Hybrid parasites may have an increased transmission potential and higher virulence compared to their parental species. Consequently, hybrid detection is critical for disease control. Previous crossing experiments showed that hybrid schistosome eggs have distinct morphotypes. We therefore compared the performance of egg morphology with molecular markers with regard to detecting hybridization in schistosomes. We studied the morphology of 303 terminal-spined eggs, originating from 19 individuals inhabiting a hybrid zone with natural crosses between the human parasite *Schistosoma haematobium* and the livestock parasite *Schistosoma bovis* in Senegal. The egg sizes showed a high variability and ranged between 92·4 and 176·4 μm in length and between 35·7 and 93·0 μm in width. No distinct morphotypes were found and all eggs resembled, to varying extent, the typical *S. haematobium* egg type. However, molecular analyses on the same eggs clearly showed the presence of two distinct partial mitochondrial *cox*1 profiles, namely *S. bovis* and *S. haematobium*, and only a single nuclear ITS rDNA profile (*S. haematobium*). Therefore, in these particular crosses, egg morphology appears not a good indicator of hybrid ancestry. We conclude by discussing strengths and limitations of molecular methods to detect hybrids in the context of high-throughput screening of field samples.

Key words: hybridization, *Schistosoma haematobium*, *Schistosoma bovis*, egg morphometrics, barcoding, zoonosis, Senegal.

INTRODUCTION

Schistosomiasis is a major poverty-related disease infecting more than 207 million people worldwide, caused by parasitic flatworms of the genus *Schistosoma*. In sub-Saharan Africa 112 million people are infected with *S. haematobium*, which causes urinary schistosomiasis, and 54 million are infected with *S. mansoni*, which causes intestinal schistosomiasis (Brindley and Hotez, 2013). The life cycle of *Schistosoma* includes an asexual amplification in an intermediate snail host and sexual reproduction in a definitive human or mammal host. Schistosomiasis-related pathology is caused by the accumulation of eggs in tissues, leading to a typical inflammatory reaction, which can develop into fibrosis of the liver or urinary bladder (Gryseels *et al*. 2006). *Schistosoma haematobium* eggs have a terminal-spine and are excreted in the urine, whereas *S. mansoni* eggs have a lateral spine and are excreted in the feces. Diagnosis of schistosomiasis is mainly based on the presence and shape of parasite eggs in the urine or feces. Although today PCR-based diagnostic techniques are available, microscopy techniques are still the reference method used for disease mapping and mass drug administration programmes (Meurs *et al*. 2015; Weerakoon *et al*. 2015).

Sometimes eggs with lateral spine are found in urine, or opposite, terminal-spined eggs are found in stool samples. Such eggs are called ectopic eggs. This can be due to spill-over, caused by high infection intensities (Husting, 1965) or heterologous pairing (Ratard *et al*. 1991). Heterologous pairing between different schistosome species may result in the production of parthenogenetic or hybrid eggs (Southgate *et al*. 1998). Studies have shown that several schistosome species easily hybridize in nature (Rollinson *et al*. 1990; Webster and Southgate, 2003; Steinauer *et al*. 2008; Huyse *et al*. 2009, 2013; Webster *et al*. 2013; Boissier *et al*. 2015). The detection of hybrids in human individuals is of high importance as hybrid species may have an increased transmission potential, higher virulence and different drug sensitivity (Taylor, 1970; King *et al*. 2015). Early hybridization experiments by Taylor (1970), who crossed *S. mansoni, S. rodhaini, S. mattheei, S. haematobium* and *S. bovis*, showed

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that hybrid eggs often have a morphology intermediate to that of the parental species. Thus, the morphological analysis of eggs may have the potential to detect hybridization events. However, the morphology of eggs resulting from natural hybridization events in the field has hardly been studied.

In this study, we investigated the schistosome eggs resulting from natural hybridization between the human parasite *S. haematobium* and the cattle parasite *S. bovis*. This hybrid is common in the Senegal River basin (SRB) in Northern Senegal (Huyse *et al*. 2009), where complex interactions between schistosome species may stem from human-induced ecological changes and migration. Today, *S. mansoni* and *S. haematobium* are co-endemic in the SRB and around the Lac de Guière, resulting in mixed infections in humans (Meurs *et al*. 2012). *Schistosoma bovis*, closely related to *S. haematobium* (Webster *et al*. 2006), is prevalent in the SRB where it infects livestock (Webster *et al*. 2013). *Schistosoma haematobium* × *S. bovis* hybrid crosses were discovered through the discordance between species identification based on a nuclear and a mitochondrial marker (Huyse *et al*. 2009). This hybrid has also been found in other parts of Senegal (Webster *et al*. 2013), Niger (Brémond *et al*. 1993), Mali (Soentjens *et al*. 2016) and Corsica (Moné *et al*. 2015). In Senegal, the majority of hybrid eggs have typically a *S. haematobium* nuclear (ITS rDNA) and a *S. bovis* mitochondrial [cytochrome *c* oxidase subunit I (*cox*1)] profile. They are found in urine and stool samples, together with 'pure' eggs, from which both the nuclear and the mitochondrial profiles are identified as *S. haematobium*.

Crossing experiments between *S. haematobium* and *S. bovis* by Taylor (1970) and Brémond *et al*. (1993) resulted in eggs with intermediate sizes and shapes. Previous field observations of a limited number of eggs, found in patients infected with *S. haematobium* × *S. bovis* hybrids, suggested that different egg morphotypes are present (Huyse *et al*. 2009; Moné *et al*. 2015; Soentjens *et al*. 2016). However, despite being widely distributed, the egg morphology of this hybrid has never been thoroughly investigated. Here we studied the egg morphology of *S. haematobium* × *S. bovis* hybrids from a community in Northern Senegal. We address the question whether hybrids have indeed distinct egg morphotypes that can be recognized during microscopy and used for hybrid detection. The egg morphology of terminal-spined eggs found in the SRB is discussed in relation to *S. haematobium* and *S. bovis* egg morphologies from literature and compared with the egg morphology described in the experimental crosses by Taylor (1970) and Brémond *et al*. (1993). In addition, we assessed whether the morphology of the hybrid terminal-spined schistosome eggs with a *S. bovis* mitochondrial DNA (mtDNA) is different from the 'pure' eggs with a *S. haematobium* mtDNA profile. Finally, we discuss the usefulness of egg morphology as a characteristic to study and diagnose hybridization events, in comparison with traditional molecular markers.

MATERIAL AND METHODS

Study area

This study was performed in the village Pakh, Northern Senegal (16°24′9″N; 15°48′42″W). Data collection was conducted between February and June 2014 in the framework of a larger molecular-epidemiological study on schistosomiasis in this village. Approval was obtained from the review board of the Institute of Tropical Medicine (Antwerp, Belgium), the ethical committee of the Antwerp University Hospital (Antwerp, Belgium) and the Comité National d'Ethique de la Recherche en Santé (Dakar, Senegal).

Host information

Demographic parameters (gender, age and ethnicity) and the infection status (*S. haematobium* infection intensity and co-infection with *S. mansoni*) of participants were defined (see Table 1). From each participant at least two urine and two stool samples were collected. *Schistosoma haematobium* infection intensity was determined after urine filtration using a filter of 12 μm pore-size (Isopore, USA) and expressed as the number of eggs per 10 mL of urine (ep 10 mL). According to WHO standards, >49 eggs/10 mL corresponds to a high infection intensity, while <50 eggs/10 mL is considered a low infection intensity. Co-infection with *S. mansoni* was determined using the Kato-Katz technique (Katz *et al*. 1972). The presence or absence of ectopic eggs (*S. haematobium* eggs in stool samples or *S. mansoni* in urine samples) was recorded during microscopy.

Collection of schistosome eggs

Schistosoma eggs from urine samples were isolated by filtering urine through a filter of 12 μm pore-size (Isopore, USA). *Schistosoma* eggs from stools were isolated by first separating out large debris with water and a metal sieve and then filtering the remaining aqueous solution through a Pitchford and Visser funnel (Pitchford and Visser, 1975). Isolated eggs were put in Petri dishes filled with clean water and exposed to sunlight to induce hatching. Unhatched eggs were transferred to a 1·5 mL microtube filled with 95% ethanol.

Egg morphology

For morphological analysis 303 eggs were randomly selected from 19 individuals infected with

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Table 1. Host individual information (demographic factors and infection status) and sample information concerning the number of eggs measured and genotyped per host

Host individual	Age	Gender	Co-infection with *S. mansoni*	Ethnicity	*S. haematobium* infection intensity[a]	No. of eggs measured			No. of eggs genotyped		
						Total	From urine	From stool	Total	HH	HB
0106	12	Female	Yes	Wolof	Low	24	22	2	4	3	1
0208	11	Female	No	Wolof	Low	6	0	6	2	1	1
0506	10	Female	Yes	Wolof	Low	31	8	23	0	0	0
0507	7	Male	No	Wolof	High	30	28	2	0	0	0
0608	8	Male	Yes	Wolof	High	1	1	0	1	0	1
1013	11	Male	Yes	Wolof	High	45	25	14	3	2	1
1111	11	Male	No	Wolof	High	12	0	12	0	0	0
1208	5	Male	No	Wolof	High	3	3	0	1	1	0
1305	9	Male	Yes	Wolof	High	18	18	0	9	6	3
1409	12	Female	No	Wolof	High	10	10	0	6	4	2
1603	16	Male	No	Wolof	High	3	3	0	1	0	1
2106	11	Female	Yes	Wolof	High	27	16	11	0	0	0
2414	15	Female	No	Wolof	High	2	2	0	0	0	0
3408	5	Male	No	Wolof	High	23	23	0	14	10	4
3519	18	Male	No	Wolof	High	24	24	0	10	7	3
3908	9	Female	Yes	Wolof	High	15	0	10 (+5)[b]	5	3	2
5612	12	Female	No	Wolof	Low	14	8	0	3	3	0
7348	4	Female	No	Peul	High	24	24	0	5	5	0
7507	22	Female	Yes	Peul	High	3	3	0	2	1	0

HH eggs (ITS + *cox*1 = *S. haematobium*); HB eggs (ITS = *S. haematobium; cox*1 = *S. bovis*).
The number of eggs analysed per host is not related to infection intensity.
[a] WHO Categories: <50 eggs/10 mL = low infection intensity; >49 eggs/10 mL = high infection intensity.
[b] Five lateral spined eggs.

S. haematobium. Eggs were temporarily mounted in milli-Q water under a coverslip. Photographs were taken using a Leica DMLB microscope and a Qimaging camera (Fast 1394 or MicroPublisher 5·0 RTV). Measurements were taken using the software programs Auto-Montage Pro (Synoptics Ltd.) or Surveyor (Objective Imaging Ltd.). The egg size (length and width, in μm) and the spine morphology (terminal or lateral) were recorded. As a measure of egg shape, the ratio of egg length divided by egg width was also calculated. For each measurement the mean, median, standard deviation (SD), minimum and maximum was calculated (Table 2) and recorded separately per spine morphology (terminal-spined *vs* lateral-spined) and per source (eggs from urine *vs* stools) (Table 2). Lateral-spined eggs were excluded for further analysis. For terminal-spined eggs, the length and length/width ratio per host individual were reported as boxplots, for individuals with minimum ten eggs measured (see Fig. 1). Density histograms were made to visualize the distributions of length and width in terminal-spined eggs and a scatter plot with regression line to illustrate their association (Figs 2 and 3). Analysis of variance (ANOVA) was used to test whether measurements of terminal-spined eggs differed according to source (urine or stool) or host individual. The R package *lme4* (Bates *et al*. 2015) was used to perform a linear mixed effect analysis of the relationship between egg length, width and ratio with host characteristics. As egg measurements are grouped per host, the variable host individual was included as a random effect in a random intercept model.

Molecular analysis

DNA extraction was performed on 207 out of the 303 eggs following the proteinase *K* extraction method described in Van den Broeck *et al*. (2011). Eggs were genotyped with a mitochondrial (mtDNA) and a nuclear marker (ITS rDNA).

mtDNA genotyping: To discriminate between *S. haematobium, S. bovis* and *S. mansoni* a rapid diagnostic 'multiplex' one-step polymerase chain reaction (RD-PCR) was performed (Webster *et al*. 2010; Van den Broeck *et al*. 2011). Parasite DNA was subjected to PCR amplification of a partial *cox*1 mtDNA fragment in a total reaction volume of 25 μL consisting of 1 × PCR buffer, 1·5 mM $MgCl_2$, 200 μM of each dNTP, 0·4 μM Asmit 1 (Eurogentec, forward primer TTTTTTGGTCATCCTGAGGTGTAT, Bowles *et al*. 1992), 0·2 μM of each reverse primer [Eurogentec; *S. haematobium* (Sh.R.) TGATAATCAATGACCCTGCAATAA 543 bp (Webster *et al*. 2010), *S. bovis* (Sb.R) CACAGGATCAGACAAACGAGTACC 306 bp (Webster *et al*. 2010), *S. mansoni* (Sm.R)

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Table 2. Egg measurements from urine and stool samples

	Length (in μm)	Width (in μm)	Ratio (length/ width)	Length (in μm)	Width (in μm)	Ratio (length/ width)
Spine morphology	*Terminal-spined eggs* ($n = 303$)			*Lateral-spined eggs* ($n = 5$)		
Minimum	92·4	35·7	1·47	111·6	50·2	1·92
Maximum	176·4	93·0	3·28	143·1	74·6	2·22
Mean	136·6	62·4	2·23	130·4	64·7	2·04
Median	136·5	62·7	2·20	139·4	66·7	2·05
Standard deviation	14·6	10·1	0·33	12·3	8·9	0·12
Source	*Terminal-spined eggs from urine* ($n = 218$)			*Terminal-spined eggs from stool* ($n = 85$)		
Minimum	92·4	35·7	1·47	110·4	42·0	1·59
Maximum	176·4	93·0	3·28	173·2	86·5	3·03
Mean	136·5	61·8	2·25	136·9	63·9	2·18
Median	136·1	62·5	2·22	138·0	64·1	2·17
Standard deviation	15·2	10·5	0·34	12·8	9·1	0·31
Genotyped eggs	*HH terminal-spined eggs* ($n = 46$)			*HB terminal-spined eggs* ($n = 18$)		
Minimum	109·1	40·4	1·47	116·0	51·3	1·62
Maximum	173·2	93·0	3·26	159·8	79·2	2·68
Mean	137·3	64·8	2·19	138·5	65·5	2·14
Median	137·6	66·1	2·08	136·1	65·5	2·13
Standard deviation	15·7	10·6	0·34	13·8	7·2	0·15

HH eggs (ITS + *cox*1 = *S. haematobium*); HB eggs (ITS = *S. haematobium; cox*1 = *S. bovis*).

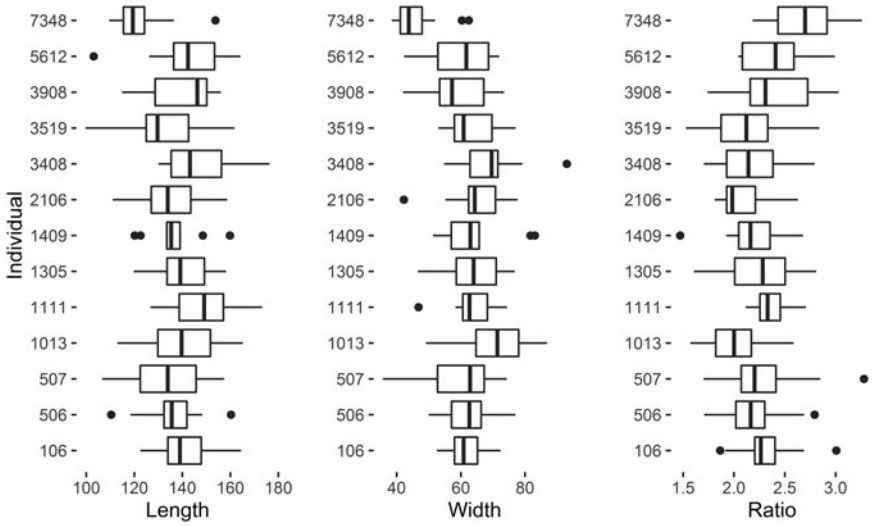

Fig. 1. Boxplots for egg length, width and ratio per host individual. Only individuals with at least ten measured eggs are shown. The boxplots are based on median values.

TGCAGATAAAGCCACCCCTGTG 375 bp (Van den Broeck *et al*. 2011)], 1 unit *Taq* polymerase (Eurogentec), milli-Q water and 2 μL of DNA. PCR parameters during thermal cycling were 3 min at 95 °C, followed by 40 cycles of 30 s at 94 °C, 1 min 30 s at 58 °C and 1 min 30 s at 72 °C followed by a final cycle at 72 °C for 7 min. PCR products were visualized on a 1% Midori Green agarose gel.

ITS rDNA genotyping: Samples were subjected to PCR amplification of the complete ITS rDNA region [981 bp; primers ITS4: TCCTCCGCTT ATTGATATGC and ITS5: GGAAGTAAAAGT CGTAACAAG (Barber *et al*. 2000)]. PCR amplification was performed in a volume of 25 μL containing 1 × PCR buffer, 2 mM $MgCl_2$, 200 μM of each dNTP, 2 mM of each primer, 1 unit *Taq* polymerase (Eurogentec), milli-Q water and 2 μL of DNA. Thermal cycling started with 3 min at 95 °C, followed by 40 cycles of 30 s at 94 °C, 45 s at 54 °C and 1 min 30 s at 72 °C followed by a final cycle at 72 °C for 7 min. PCR products were analysed by restriction fragment length polymorphism (RFLP) and visualized on a 1% Midori Green agarose gel. Digestion of the ITS fragment with *MboI* (Fermentas) distinguishes *S. haematobium* from *S. bovis* and *S. mansoni*. *Schistosoma haematobium* PCR products are digested in fragment sizes of (in bp) 280, 147, 130, 100, 85 and 36; *S. bovis* in fragments sizes of 380, 174, 167, 130, 83 and 36; and *S. mansoni* in sizes of 380, 176, 174, 130, 81, 16 and 9 bp.

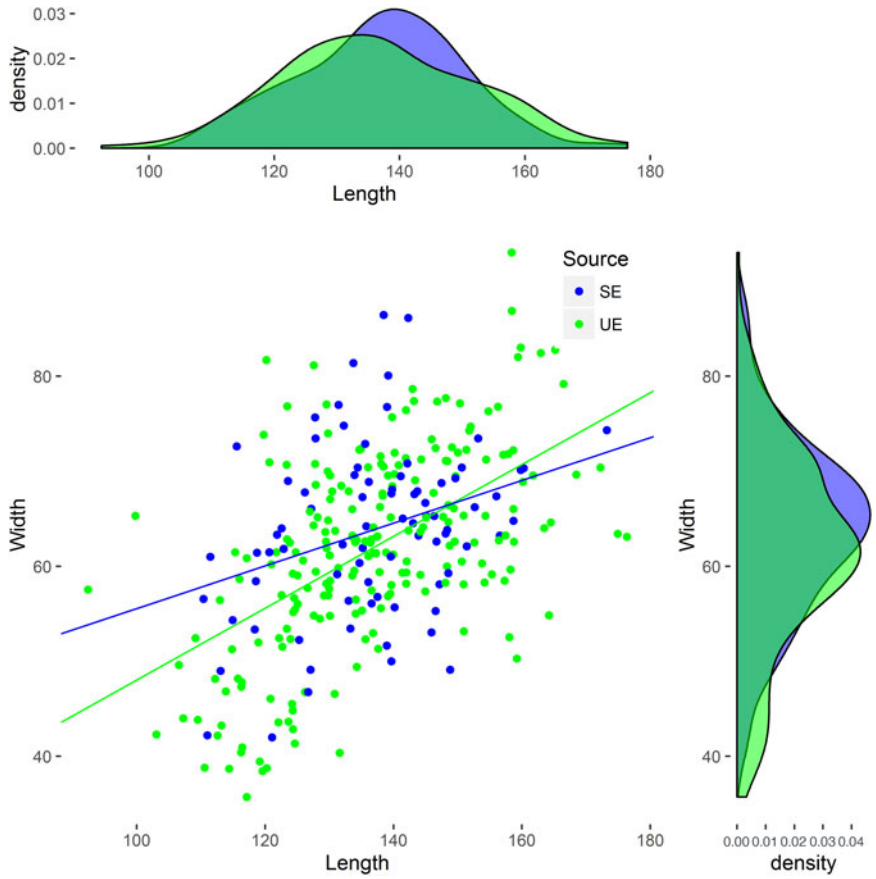

Fig. 2. Scatter plot showing the length and width of terminal-spined eggs. The relation between egg length and egg width is indicated by a regression line. Kernel density histograms are shown for egg length and egg width. Eggs originating from urine samples (UE) are shown in green, eggs originating from stool samples (SE) are in blue.

Samples showing a *S. bovis* or *S. mansoni* MboI pattern were subsequently digested with AluI (Fermentas), which differentiates between *S. bovis* (no cut; 981 bp) and *S. mansoni* (fragments of 545 and 421 bp). To confirm the genotype based on restriction enzymes, for a random subset of eggs ($n = 11$) the ITS rDNA region was sequenced by Sanger sequencing. The PCR products were purified using the E.Z.N.A Cycle Pure kit (Omega Bio-tek, Norcross, USA). Sequencing was done using a Big Dye Chemistry Cycle Sequencing kit (version 3.1) in a 3130 Genetic Analyser (Applied Biosystems). Sequences were assembled and manually edited using Geneious® 6.1.6. (http://geneious.com/).

Egg morphology in relation to genotype

The measurements of terminal-spined eggs were related to their genotype. Genotype classes were based on the genotype of *cox*1 mtDNA [*S. haematobium* (H), *S. bovis* (B) or *S. mansoni* (M)] and the ITS rDNA genotype (H and/or B and/or M). A scatter plot and density histograms were generated to visualize the length and width of genotyped eggs per genotype-class (Fig. 3). ANOVA was used to compare the length, width and ratio of eggs among genotype groups. R version 3.1.3 (R Development Core Team, 2015) was used for all statistical analyses.

RESULTS

Dataset and host information

We measured 303 eggs from 19 different individuals. 218 eggs originated from urine of 16 patients, 85 eggs were extracted from stool samples of eight patients (see Tables 1 and 2).

Table 1 provides host demographic information and infection status. Individuals were between four and 22 years old, nine males and ten females. The majority of individuals (13) had a high *S. haematobium* infection intensity (>49 eggs/10 mL) whereas six individuals had a low infection intensity (<50 eggs/10 mL). Nine individuals were co-infected with *S. mansoni*; the *S. mansoni* infection intensities were low (1–99 epg). In eight of the 19 selected individuals ectopic *S. haematobium* eggs were reported from stools during microscopic analysis of Kato–Katz slides.

Egg morphology

All eggs extracted from urine samples were terminal-spined, i.e. no ectopic *S. mansoni* eggs were present

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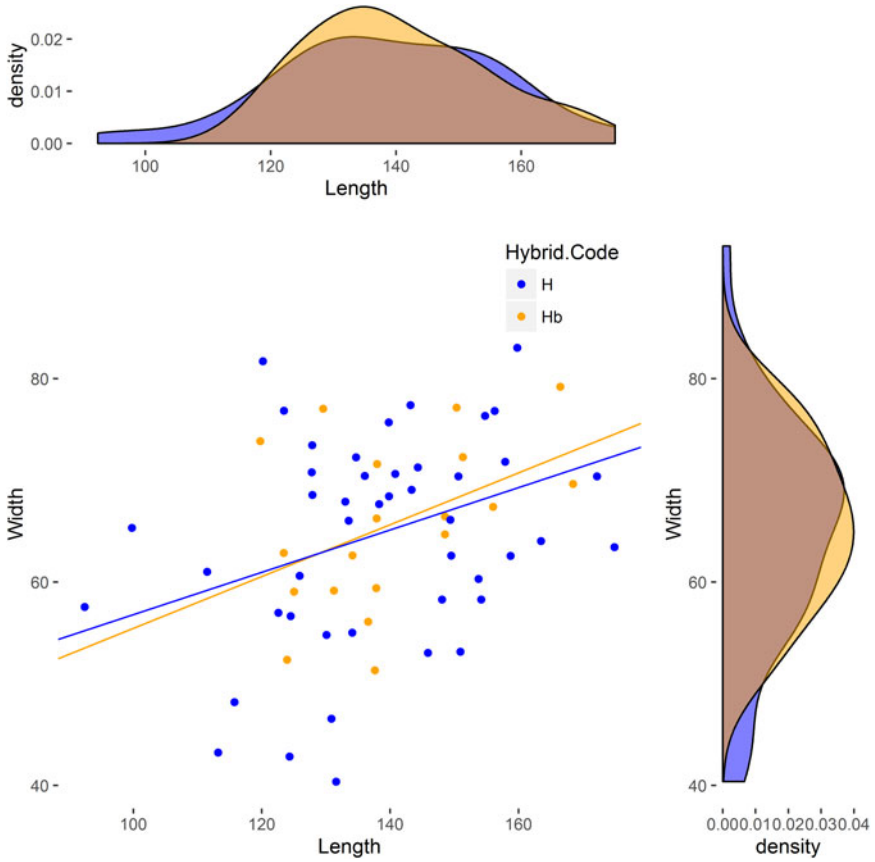

Fig. 3. Scatter plot showing the length and width of genotyped, terminal-spined eggs. The relation between egg length and egg width is indicated by a regression line. Kernel density histograms are shown for egg length and egg width. Eggs genotyped as HH (*S. haematobium*) are shown in blue, HB eggs (*S. haematobium* × *S. bovis*) in orange.

in urine samples. Five eggs originating from one stool sample had a lateral spine typical for *S. mansoni* and were excluded from further analyses. All other eggs from stools (94%) were ectopic, i.e. terminal-spined. The eggs showed only minor variations in shape. Most eggs were round-to-oval, resembling typical *S. haematobium* eggs (e.g. Fig. 4B, F–J andL). A few eggs appeared very slightly spindle-shaped (e.g. Fig. 4C). However, no distinct classes of egg shape could be distinguished. Egg size showed a high variability. Terminal-spined eggs had a length ranging from 92·4 to 176·4 μm (mean 136·6 μm) and a width ranging from 35·7 to 93·0 μm (mean 62·4 μm). The length/width ratio ranged from 1·47 to 3·28 (see Table 2). The distributions of egg length and egg width were continuous and unimodal as shown by density histograms (Figs 2 and 3).

The mean size of terminal-spined eggs from stool samples ($n = 85$) was not significantly different from those from urine samples ($n = 218$). The mean size of eggs was significantly different between host individuals (ANOVA, $P < 0{\cdot}001$). Linear mixed models (LMM) indicated that a correlation was present of egg size within individuals. On average 25% of the variation in egg length was present between individuals, 75% of the variation in egg length was present within an individual. The correlation of egg width within individuals was slightly higher than egg length: 30% of the variation was present between individuals, 70% of the variation within individuals. For the length/width ratio 23% was present between individuals and 77% within individuals. Figure 1 shows a visual representation of egg length, egg width and the length/width ratio for each host individual with at least ten eggs measured. We did not find a consistent relation between egg size and host characteristics (age, gender, ethnicity, infection intensity and co-infection with *S. mansoni*).

Molecular analysis

We performed DNA extraction and molecular analysis on 166 terminal-spined eggs from urine, 38 terminal-spined eggs from stool and three lateral-spined eggs from stool. The success rate of the PCR reactions was rather low, especially for eggs from stools. In total, 66 terminal-spined eggs (32%; 59 from urine, seven from stool) were successfully genotyped for both *cox*1 and ITS rDNA. Based on the RFLP profiles of ITS rDNA and *cox*1, 46 eggs were identified as *S. haematobium* (HH; ITS = *haem, cox*1 = haem) and 19 eggs (29%) as *S. haematobium* × *S. bovis* hybrids (HB; ITS = *haem, cox*1 = *bovis*). No

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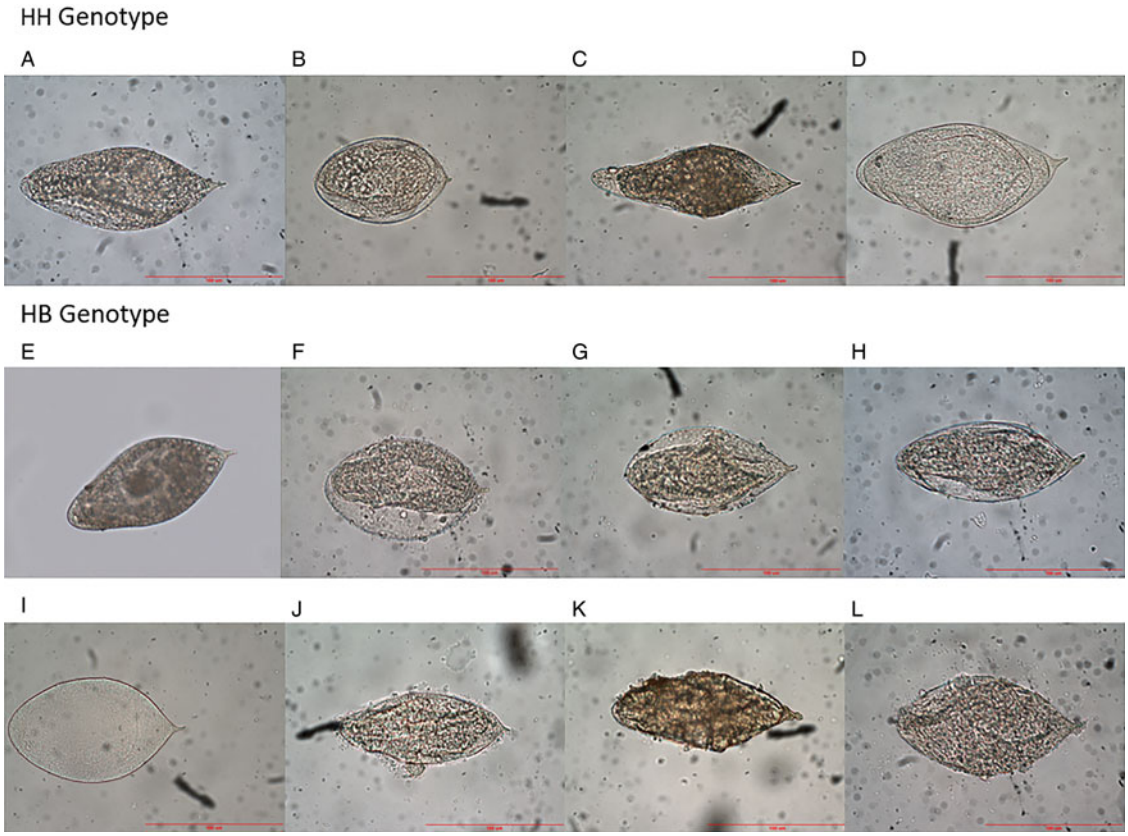

Fig. 4. Photographs of a selection of terminal-spined eggs measured in this study. Eggs A–D were genotyped as HH (*S. haematobium*) based on ITS and *cox*1, the eggs E–L were genotyped as HB (*S. haematobium* × *S. bovis*).

eggs were found with a reverse profile (BH; ITS = *bovis*, *cox*1 = *haem*), nor with a pure *S. bovis* profile or a hybrid ITS profile with fragment sizes of both species. To confirm the RFLP results the ITS region was sequenced for 11 randomly chosen eggs. All 11 sequences were identical to the *S. haematobium* isolate from Northern Senegal (Accession no. FJ588861) described in Huyse *et al.* (2009). HB eggs were found in 10 out of 15 individuals with at least one egg genotyped.

Egg morphology in relation to genotype

Hybrid *S. haematobium* × *S. bovis* eggs (HB) were slightly larger and varied less in size and shape (length/width ratio) than 'pure' *S. haematobium* eggs (HH) (Table 2). Differences were however not significant (Fig. 3). HB were present in stools (=ectopic eggs) and urine. Seven ectopic eggs were successfully genotyped; four of these had a HH genotype and three had a HB genotype. From the 59 terminal-spined eggs in urine 16 eggs were HB and 42 were HH. Egg measurements were not associated with egg genotype (Fig. 3), nor did egg shape correlate with egg genotype. Several HB eggs showed the round-to-oval shape typical for pure *S. haematobium* (Fig. 4E–L), and one HH egg was somewhat spindle-like shaped, more typical for *S. bovis* (Fig. 4C) (Pitchford, 1965).

DISCUSSION

The morphology of terminal-spined eggs from the SRB

This study is the first to thoroughly study the egg morphology of *S. haematobium* × *S. bovis* hybrids collected from human samples. Although the eggs of both parental species, *S. haematobium* and *S. bovis*, are terminal-spined, they differ in several important aspects. Typically, *S. haematobium* eggs are deposited in urine, are round-to-oval in shape and range in length from 83 to 187 μm (Pitchford, 1965; Table S1). In contrast, *S. bovis* eggs are deposited in stool and are spindle-shaped, consisting of a broad middle portion and drawn-out rod-like ends, one bearing a well differentiated spine, the other evenly rounded (Taylor, 1970; Touassem, 1987). *Schistosoma bovis* eggs are also larger than those of *S. haematobium*, their length usually exceeding 200 μm and reaching up to 300 μm (Taylor, 1970; Touassem, 1987; Table S1). The egg sizes obtained in this study (136·6 ± 14·6 μm × 62·4 ± 10·1 μm) correspond very well with *S. haematobium* egg sizes but not with *S. bovis* egg sizes (Table S1, Khalil, 1924; MacHattie *et al.*, 1933; Alves, 1949; Taylor, 1970; Loker, 1983; Southgate *et al.*, 1985 and Touassem, 1987). The range of the egg lengths (92·4–176·4 μm) falls within the range of reported *S. haematobium* egg lengths. The mean egg width obtained in this study (62·4 ± 10·1 μm) was rather large in comparison

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with other studies. However, only few authors have reported the width of *S. haematobium* eggs from humans. De Clercq *et al.* (1994) reported a mean width of 55·0 ± 5·0 μm from the Bankass district in Mali, and Pitchford (1959) found a mean of 51·4 μm for eggs from South Africa. Vercruysse *et al.* (1984) measured the egg width of a strain that originated from children from Guédé Chantier (SRB) and that was subsequently cycled in mice. These eggs had almost the same width (62 ± 12 μm) as those examined in this study but the length was larger (153 ± 11 μm).

The mean egg length/width ratio found in this study (2·23) also corresponds with the mean ratios of *S. haematobium* eggs reported in other studies (2·47 to 2·53). The range of the ratio of the eggs was rather broad (1·47–3·28). A ratio of 3·28 approaches the mean ratio of *S. bovis* eggs, which are more elongated than *S. haematobium* eggs (3·41–3·85, Table S1). This suggests that some eggs in this study, despite being smaller in size, may have a shape resembling *S. bovis*. However, we found only one egg with a somewhat spindle-like shape (Fig. 4C). Evidently, the morphological methods used in this study (visual inspection of photographs and calculation of the length/width ratio) can capture only some aspects of the egg shape. In theory, the egg shape could be determined more precisely by taking additional measurements or by using geometric morphometrics. However, such methods are time-consuming and hence would be impractical in the context of the high-throughput screening of samples, especially in the field. Besides, egg size and shape (length/width ratio) were highly variable; about 70% of this variation was present within a single patient. Intraspecific variability is known to impede species identification (Almeda *et al.* 1996) and therefore complicates the differentiation between pure and hybrid species. Finally, it should be noted that none of the studies mentioned above used molecular methods to verify species status. Hence, some of these measurements could include HB rather than pure *S. haematobium* or *S. bovis* eggs.

The HB eggs from Senegal do not closely resemble the eggs from previous experimental hybridization studies. Taylor (1970) performed experimental crosses between *S. bovis* ♀ (from Iran) and *S. haematobium* ♂ (from Iran and Nigeria). Parental *S. bovis* eggs were bigger and had always a spindle shape in contrast to *S. haematobium* eggs, which suggests that Taylor probably used pure parental species for his experiments. First generation hybrid (F1) eggs of the crosses were typical of *S. bovis* in morphology. This is in line with expectations as the egg shape of schistosomes is supposed to be determined by the female ootype (Smyth and Clegg, 1959). In this large, egg-shaped chamber that is part of the oviduct, the eggshell is formed. F2 eggs were intermediate between *S. bovis* and *S. haematobium*, and similar in size to *S. intercalatum* (length 175·0 ± 8·5 μm; width 61·7 ± 2·6 μm) or *S. mattheei* (173·0 ± 14·0 μm; 52·6 ± 4·5 μm) (Taylor, 1970). F3 eggs were similar in shape to those of the F2 generation but less viable. Similar results were obtained by Brémond *et al.* (1993) who performed experimental crossings between *S. bovis* and *S. haematobium* from Niger, resulting in hybrids with intermediate egg morphology. It should be noted that both Taylor (1970) and Brémond *et al.* (1993) only studied F1–F3 crosses. Such crosses cannot be directly compared with the HB from this study, which are probably the result of back-crossing with parental species as suggested by the dominance of *S. haematobium* ITS profiles.

Recently an outbreak of human urinary schistosomiasis was reported from Corsica (France). Moné *et al.* (2015) sequenced eggs isolated from infected tourists and identified, based on partial *cox*1 and ITS rDNA sequencing, both 'pure' *S. haematobium* and hybrid crosses between *S. haematobium* and *S. bovis*. They described two morphotypes of eggs, typical round-to-oval eggs (four out of 15 eggs) and more elongated eggs referred to as 'non-typical' *S. haematobium* eggs. The 15 eggs measured 106·5 ± 6·2 μm × 42·8 ± 5·4 μm, which is smaller than the mean egg sizes we found in Senegal (136·6 ± 14·6 μm × 62·4 ± 10·1 μm) and almost half the length of 'typical' *S. bovis* eggs (Table S1). Unfortunately, morphological and molecular analyses were not performed on the same eggs, so the link between morphotype and genotype could not be studied. Similar to our study, eggs resembling typical *S. bovis* shape were not found (Moné *et al.* 2015). Soentjens *et al.* (2016) studied a case of *S. haematobium* × *S. bovis* infection in travellers from Mali. Eggs were suspected to be hybrids because of the lozenge shape, which was intermediate between the typical round-to-oval *S. haematobium* eggs and the spindle-shaped *S. bovis* eggs. The length of the eggs varied between 110 and 175 μm. DNA sequencing of two eggs confirmed their hybrid constitution. The eggs in these two studies show the same discordance between the nuclear (*S. haematobium*) and mitochondrial (*S. bovis*) genotype Although the reported egg sizes differ in the study of Moné *et al.* (2015) the descriptions of atypical egg shapes show resemblance with the few atypical eggs observed in this study (Fig. 4C).

Link between egg morphology and genotype?

The genetic constitution of the eggs confirmed the presence of natural crosses between *S. haematobium* and *S. bovis* in Pakh. Almost 30% of the eggs (n = 19) had a *S. bovis cox*1 genotype, while the ITS genotype was always identical to *S. haematobium*. Eggs with a 'pure' *S. bovis* genotype were absent in this study, as were eggs with a typical *S. bovis* size and spindle shape. In our study, the egg size showed a high variability. This variation was not

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linked to the genotype (ITS and *cox*1), neither to the origin of the eggs (urine or stool). Although 70% of the variation was present within individuals, egg sizes differed significantly between individuals and 30% of the variation in egg size was attributable to differences between individuals. We could however not link this variation to specific host characteristics (age, sex, infection intensity), neither to the presence of hybrid eggs.

Different schistosome species or strains have typically different mean egg sizes or different maxima when plotting density curves (Pitchford, 1965; Almeda *et al*. 1996). The unimodal distribution of egg-measurements in this study and the absence of morphological differences between HH eggs and HB eggs suggest that both genetic classes of eggs belong to one random mating schistosome population, without mating restrictions in the next generation. The observation that HH and HB eggs were found together in almost all studied host individuals, supports the idea of random mating. This leads to the hypothesis that the majority of parasite stages found in Pakh stem from a (historical) hybridization event. Repeated backcrossing with *S. haematobium* not only results in a dilution of the introduced *S. bovis* mitochondrial genes, but also in a mixing and spreading of these genes into the population. As a consequence, a varying degree of introgression with *S. bovis* genes is likely to be present at the nuclear level of both the hybrid and the so-called 'pure' eggs, which is not detectable with traditional ITS barcoding. This is also suggested by the HH genotype of the egg with the spindle-shape, typical of *S. bovis* eggs (Fig. 4C).

The strengths and limitations of molecular markers for hybrid detection

In combination with a mtDNA marker, the internal transcribed spacer rDNA (ITS) is considered a good marker to detect hybridization as it retains both parental copies for several generations (Sang *et al*. 1995; Steinauer *et al*. 2008). However, due to concerted evolution, a phenomenon that removes paralogous sequences (for example ITS copies) from the genome and eliminates intra-individual polymorphism (Garcia *et al*. 2011) a biased homogenization towards one of the parental sequences can already occur in F2 hybrids (Fuertes Aguilar *et al*. 1999). As a consequence, in case of unequal back-crossing with only one of the parental species (e.g. *S. haematobium*), the signal of the other parental species (e.g. *S. bovis*) will fade out. When both the mtDNA and the ITS rDNA sequences are identified as *S. haematobium* we can therefore not reliably assign them as 'pure' since we cannot rule out crossing with *S. bovis* in previous generations, nor can we rule out introgression of other nuclear *S. bovis* genes (e.g. maintained through positive selection). As such, the current method (combining *cox*1 barcoding and ITS1) is limited in regard to detecting historical hybridization events and will therefore underestimate the presence of hybrids.

The presence of a *S. bovis cox*1 profile easily reveals (historical) hybridization and we therefore advocate the use of the rapid diagnostic PCR assay used in this study. We do need however more sensitive nuclear markers to identify hybridization at the nuclear level. Microsatellite markers will simultaneously sample more genomic regions and therefore increase the chance to pick up introgressed regions, but they too appeared limited in detecting hybrids between *S. mansoni* and *S. rodhaini* (Steinauer *et al*. 2008). The best method until now appears to be cloning different ITS copies to detect both parental ITS variants, or single-stranded conformational polymorphism (SSCP) analyses (see Webster *et al*. 2007; Steinauer *et al*. 2008). While the former method is very labour-intensive, the latter method only consists of PCR analysis and gel electrophoresis. SSCP analysis can pick up different ITS variants because single base-pair changes affect the migration on a gel.

Concluding remarks

Due to the absence of distinct egg morphotypes and the large intra-individual variation in egg sizes, egg morphology appears of limited value to detect hybridization events between *S. haematobium* and *S. bovis* in natural field isolates. Despite being efficient for high-throughput screening of field samples, traditional barcoding cannot detect introgression of *S. bovis* genes at the nuclear level. As this may result in an underdetection of hybrid schistosomes, other detection methods are needed. The detection of hybrids is essential to control the transmission of schistosomiasis and to select the best treatment for infected communities (Leger and Webster, 2016). Whole genome analysis of natural field stages is therefore needed to understand the introgression of genes from one species into the other in natural conditions (Twyford and Ennos, 2012), while hybridization experiments are needed to study the phenotype and fitness of different hybrid crosses compared to the pure parental strains.

SUPPLEMENTARY MATERIAL

The supplementary material for this article can be found at https://doi.org/10.1017/S0031182017000087.

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