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No barrier breakdown between human and cattle schistosome species in the Senegal River Basin in the face of hybridisation

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ABSTRACT

Schistosomiasis is widely distributed along the Senegal River Basin (SRB), affecting both the human population and their livestock. Damming of the Senegal River for irrigation purposes in the 1980s induced ecological changes that resulted in a large outbreak of *Schistosoma mansoni,* followed a few years later by an increase and spread of *Schistosoma haematobium* infections. The presence of hybrid crosses between the human and cattle schistosomes, *S. haematobium* and *Schistosoma bovis,* respectively, is adding complexity to the disease epidemiology in this area, and questions the strength of the species boundary between these two species. This study aimed to investigate the epidemiology of *S. haematobium*, *S. bovis* and their hybrids along the Senegal River basin using both microsatellite genetic markers and analysis of mitochondrial and nuclear DNA markers. Human schistosome populations with a *S. haematobium cox1* mtDNA profile and those with a *S. bovis cox1* mtDNA profile (the so-called hybrids) appear to belong to a single randomly mating population, strongly differentiated from the pure *S. bovis* found in cattle. These results suggest that, in northern Senegal, a strong species boundary persists between human and cattle schistosome species and there is no prolific admixing of the populations. In addition, we found that in the SRB *S. haematobium* was spatially more differentiated in comparison to *S. mansoni*. This may be related either to the presence and susceptibility of the intermediate snail hosts, or to the colonisation history of the parasite.

1. Introduction

Schistosomiasis is a major poverty-related disease caused by infection with parasitic flatworms of the genus *Schistosoma*. Worldwide, more than 200 million people are infected. More than 90% of the cases occur in sub-Saharan Africa with 112 million people infected with *Schistosoma haematobium* and 54 million with *Schistosoma mansoni,* causing urogenital and intestinal schistosomiasis, respectively (Brindley and Hotez, 2013). In northern Senegal, schistosomiasis is a major problem for both humans and livestock (Webster et al., 2013; Gower et al., 2017). In 1988 the Diama Dam was constructed in the lower delta of the Senegal River and a year later the Manantali Dam was built on the Bafing River, an upstream tributary of the Senegal River in Mali. The dams led to increased access to fresh water for irrigation but also triggered one of the largest epidemics worldwide of *S. mansoni*. A few years later the prevalence of *S. haematobium* also increased and new infection foci were described (Picquet et al., 1996; Southgate et al., 2001). Today, *S. mansoni* and *S. haematobium* are co-endemic in the Senegal River Basin (SRB) and along the shores of Lake Guiers, resulting in co-infections in the human populations (Meurs et al., 2012; Abdellahi et al., 2016). Despite mass treatment campaigns in the SRB, the prevalence of *S. haematobium* remained high in the Delta and the Middle Valley, while the prevalence of *S. mansoni* appears to be decreasing (Abdellahi et al., 2016).

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Schistosoma haematobium was already present in the SRB before dam construction; in villages close to the Senegal River ('walo' villages) and on the shore of Lake Guiers low prevalences of *S. haematobium* were found (Chaine and Malek, 1983). Higher prevalence was observed in the 'diéri' villages (unflooded villages) and two 'walo' villages, namely Lampsar at the Lampsar River and Guédé Chantier in the Middle Valley (Vercruysse et al., 1985). In addition, the animal schistosome species *Schistosoma bovis* and *Schistosoma curassoni* were found in local livestock and in slaughterhouses (Vassiliades, 1978). Most animals showed low infection intensities. Similar to the course of human schistosomiasis, Diaw et al. (1998) showed a sharp increase in bovine schistosomiasis and the appearance of new infection foci at the Senegal River Delta and Lake Guiers after dam construction. Also, a more recent study by Webster et al. (2013) found that many animals in the Richard Toll area in the SRB were infected with *S. bovis,* with large numbers of adult worms found in the mesenteric vessels of slaughtered livestock.

Investigating the population structure of *S. haematobium* along the SRB, using molecular markers, may help elucidate its dynamic epidemiology. However, molecular studies in certain areas of the SRB have revealed the presence of hybrid crosses between *S. haematobium* and *S. bovis* (Huyse et al., 2009; Webster et al., 2013), adding complexity to the epidemiology in this area. The observation was based on the discordant partial cytochrome oxidase subunit I (*cox1*) mitochondrial DNA (mtDNA) and nuclear ribosomal DNA internal transcribed spacers (ITS rDNA) genetic profiles of schistosome eggs and miracidia isolated from humans urine and stool samples (mito-nuclear discordance) (Huyse et al., 2009; Webster et al., 2013). The presence of these hybrids questions the strength of the species boundary between *S. haematobium* and *S. bovis* (Harrison and Larson, 2014). Hybridisation between two parental species groups can have different outcomes (Abbott et al., 2013). Firstly, selection and hybridisation are in a stable balance, with only some parts of the genome introgressed within the hybridising populations. In this case, the two parental populations remain differentiated, and there is no progress towards speciation or towards merging into one species. Secondly, barriers to gene exchange may break down entirely (panmixis) leading to a reduction or loss of differentiation. Finally, hybridisation may result in new populations of mixed ancestry that remain distinct from both parental populations ('hybrid speciation') (Abbott et al., 2013). Especially in the second scenario, this implies that parasite transmission (gene flow) between the human and animal host is extensive and that mammals are a potential reservoir for human schistosomiasis (Huyse et al., 2009; Webster et al., 2013). This would have important implications for the implementation of control programs (Leger and Webster, 2016).

In order to test whether one of the above scenarios holds, we studied the population genetic structure of *S. haematobium* at different levels. First we investigated the interspecific level by genotyping human and cattle schistosome populations using microsatellite genetic markers. This will help reveal the presence (or absence) of a reproductive barrier between human and cattle parasite species. In case of on-going gene flow, first generation hybrids are expected to cluster intermediate between the parental populations. Secondly, we looked for genetic differentiation between hybrids and 'pure' *S. haematobium* within one single village (Pakh). Hybrids are defined based on mito-nuclear discordance of the *cox1* subunit and the ribosomal ITS region. This analysis may show the potential influence of *S. bovis* mitochondrial introgression on the genetic constitution of *S. haematobium* populations, while excluding the confounding effect of geographical variation. Finally, we zoomed out and compared schistosome populations from the Lower and Middle Valley of the SRB to test for geographical patterns in schistosome transmission.

2. Material and methods

2.1. Ethics statement

Approval was obtained for all data used in this study from the review board of the Institute of Tropical Medicine (Antwerp, Belgium), the ethical committee of Antwerp University Hospital (Antwerp, Belgium), and 'Le Comité National d'Ethique de la Recherche en Santé' (Dakar, Senegal). For each of these studies, informed and written consent was obtained from all participants prior to inclusion in the study. At the end of the study all inhabitants of the participating villages were treated with praziquantel at 40 mg/kg as recommended by the World Health Organization (WHO, 2006).

2.2. Study area and data collection

Data were obtained from samples collected from 13 sites (Fig. 1; Table 1): 10 rural villages, a town (Richard Toll) in northern Senegal, a village (Assoni) in the vicinity of Kédougou (southeastern Senegal) and in two villages along the Niger River Basin in Mali. Data were collected during several parasitological surveys in 2006, 2007, 2009, 2010 and 2014. The prevalence of urinary and intestinal schistosomiasis at the study sites can be found in Supplementary Table S1. We sampled parasite stadia (eggs, miracidia) from humans, cercariae from *Bulinus* snails and adult schistosomes from cattle. The majority (n = 529) of parasites were miracidia collected in Pakh (16°24′10″N, 15°48′42″W) in 2014 from 35 infected people (of all ages); also cercariae were collected from 13 *Bulinus* snails in Pakh. From the village of Diatar, adult *S. haematobium* worms (1993) were obtained from the Schistosomiasis Collection at the Natural History Museum (London, UK). This isolate originated from naturally infected snails; the adult worms were obtained after one passage through mice (SCAN; Emery et al., 2012). Adult *S. bovis* worms were collected in Richard Toll (2010) from four cows (64 samples in total) and extracted by Webster et al. (2013). The eggs from Mali were collected in Kalabogou and Kaladagan, and obtained through a previous study by Willemann Stecher et al. (2017).

Schistosoma eggs were isolated from urine samples by filtration through a 12 μm pore size filter (Isopore, USA) (data 2014) or sedimentation (years 2006–2010). Isolated eggs were hatched in Petri dishes with clean bottled water through exposure to sunlight. Individual miracidia were pipetted onto FTA® Classic cards (Whatman) in a volume of 2 μL (2014) or 3 μL (2009–2010). Unhatched eggs were transferred to a 1.5 mL microtube filled with 95% ethanol. Snails were collected in Pakh at four transmission sites during three surveys in March and April 2014. They were individually transferred to multi-well plates filled with bottled water and exposed to light to stimulate shedding. Individual cercariae were pippetted onto FTA® Classic cards in a volume of 3 μL of water.

2.3. DNA extraction and molecular analysis

DNA extraction of eggs and miracidia was performed following the method of Van den Broeck et al. (2011) for samples collected between 2006 and 2010. Eggs from Mali were isolated from the dried filters used for urine filtration and processed together with the Senegalese eggs. The DNA from miracidia and cercariae collected in 2014 was extracted following Webster et al. (2015) with a high-throughput and cost effective non-wash Whatman-FTA alkaline DNA elution protocol. DNA from adult *S. haematobium* worms was extracted using the Nucleospin® Tissue kit following the manufacturer's instructions (Macherey-Nagel, Belgium).

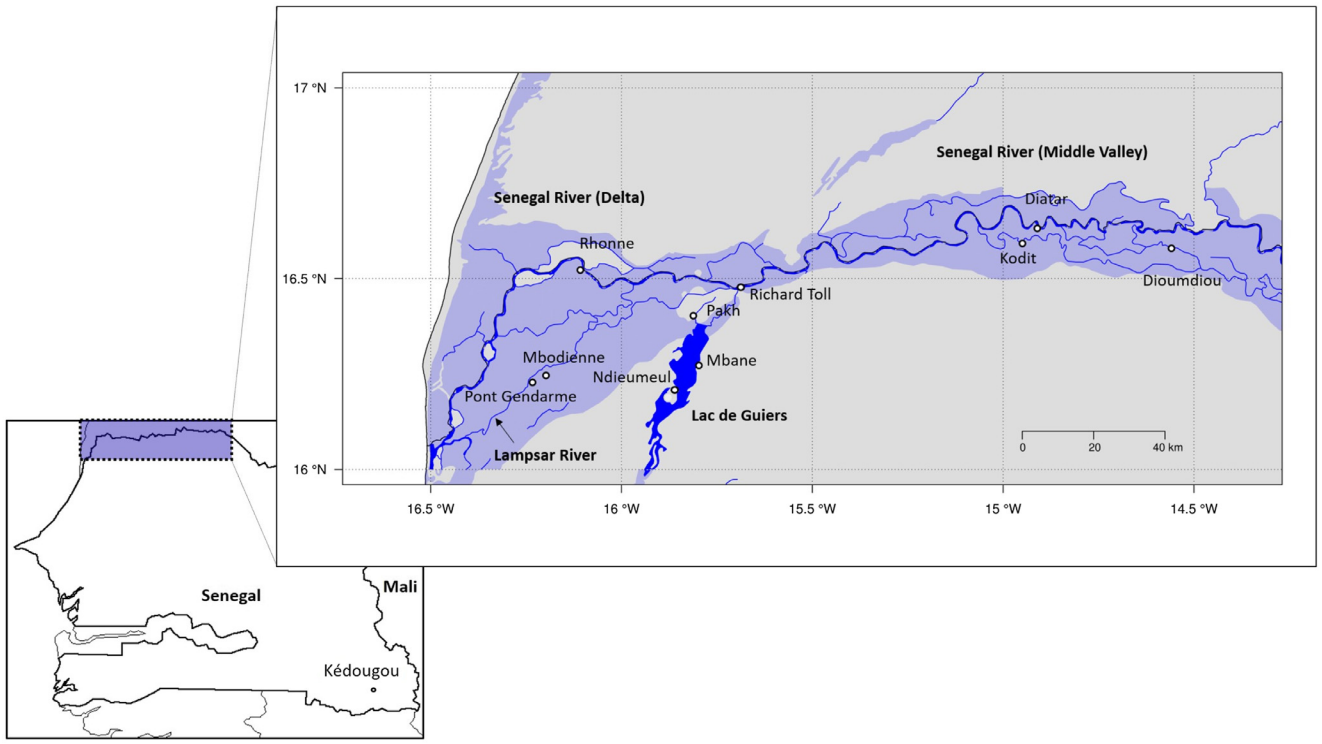

Fig. 1. Map with sample locations: Kédougou is located in southeastern Senegal. The detailed map of the Senegal River Basin in northern Senegal includes the Middle Valley with the villages of Diatar, Dioundiou, and Kodit; towards the coast, in the Senegal River Delta is the village of Rhonne; in the vicinity of Lake Guiers on the eastern shore is the village of Mbane; on the western shore, the village of Ndieumeul; and north of the lake the village of Pakh, and the city Richard Toll at the Senegal River; along the Lampsar River, a tributary of the Senegal River, are the villages of Mbodienne and Pont Gendarme.

Table 1
Number of schistosome parasites (792) successfully genotyped at the *cox1* and ITS rDNA loci per region and per year in Senegal and Mali.

Village/region	Year	Number of *Schistosoma* stadia genotyped		
		Microsatellites (17 panel)	*Cox1*	ITS rDNA
Senegal River (Middle Valley)				
Diatar	1993/2007	12 miracidia/9 adults	21	18
Dioundiou	2006, 2007	12 miracidia	12	8
Kodit	2006	9 miracidia	9	9
Senegal River (Delta)				
Rhonne (Khenne)	2007	23 miracidia	9	16
Richard Toll	2010	62 adults *S. bovis*[a]	NA	NA
Lake Guiers				
Mbane	2006, 2007	19 miracidia, 4 cercaria	18	7
Saneinte Tack	2006, 2007	4 miracidia	0	1
Ndieumeul (Thiekenne)	2006 to 2010	13 miracidia, 12 eggs	14	2
Pakh	2009	16 miracidia	14	4
	2014	529 miracidia, 13 cercariae	437	76
Lampsar River				
Mbodienne	2006, 2007	16 miracidia, 3 cercariae	19	11
Pont Gendarme	2006	13 miracidia	12	12
Kédougou (Southern Senegal)	2011	11 miracidia	3	20
Mali Kaladagan and Kalabougou	2009	12 eggs	0	0
Total	*2006 to 2014*	*792*	*570*	*186*

[a] Collected from cattle; all other miracidia and worms originate from human urine samples. Cercariae originated from *Bulinus* snails. NA, data not available.

2.3.1. Microsatellite analysis

A multiplex panel of *S. haematobium* microsatellite loci, published by Webster et al. (2015), was slightly modified by adding one primer pair (Sha_104176_i13) designed in this study, and removing primer pairs 131 and Sh 7. In total 17 microsatellite loci were amplified in two separate multiplex PCRs using a volume of 10 µl containing 2 µl of DNA, 5 µl of Master Mix and 1 µl of Q-Solution (Multiplex PCR Kit Qiagen®). Primer conditions for multiplex 1, containing eight primers, were as follows: 0.2 µM of all primer pairs *Sh1* (VIC), *Sh3* (FAM), *Sh6* (NED), *Sh9* (FAM), *Sh14* (NED), *C102* (VIC), *C111* (PET) (Gower et al., 2011; Glenn et al., 2013) and 0.4 µM of the primer pair *Sha_104176_i13* (PET) (F: TTCTTGACGACTACTTCCAA; R: CGACAACATACCCACTCTTA, unpublished). Multiplex 2 contained nine primers: 0.2 µM of the primer pairs *Sh2* (NED), *Sh4* (FAM), *Sh5* (NED), *Sh8* (PET), *Sh10* (PET), *Sh11* (VIC), *Sh12* (PET), *Sh13* (FAM) and *Sh15* (VIC)

(Glenn et al., 2013). Parameters during thermal cycling were an initial denaturing step at 95 °C, followed by 40 cycles of 30 s at 94 °C, 1 min 30 s at 54 °C and 1 min 30 s at 72 °C followed by a final cycle at 60 °C for 30 min. PCR products were visualized on a 1.5% Midori Green agarose gel. After a 1:10 dilution in formamide, fragment analysis was performed in a 3130 Genetic Analyzer (Applied Biosystems) with the GeneScan™ 500 LIZ® size standard. Allele peaks were visualised in Geneious® v6.1.6 (http://geneious.com/) using the microsatellite plug-in.

To discriminate between *S. haematobium*, *S. bovis* and *S. mansoni*, a rapid diagnostic 'multiplex' one-step PCR (RD-PCR) was performed (Webster et al., 2010; Van den Broeck et al., 2011). The complete ITS rDNA region (981 bp) was amplified with primers ITS4: TCCTCCGCTTATTGATATGC and ITS5: GGAAGTAAAAGTCGTAACAAG (Barber et al., 2000). PCR products were analysed by restriction fragment length polymorphism (RFLP) with the restriction enzymes *Mbo*I (Fermentas) and *Alu*I (Fermentas). Digestion of the ITS fragment with *Mbo*I (Fermentas) distinguishes *S. haematobium* from *S. bovis* and *S. mansoni*. Samples showing a *S. bovis* or *S. mansoni Mbo*I pattern were subsequently digested with *Alu*I (Fermentas), which differentiates between *S. bovis* (no restriction; 981 bp) and *S. mansoni* (fragments of 545 and 421 bp). Both methods are described in detail in Boon et al. (2018).

2.4. Data analysis

2.4.1. Population genetic structure of S. haematobium and S. bovis

We calculated the percentage of missing data per locus and removed parasite individuals from which less than nine loci were successfully genotyped. The observed heterozygosity (Ho), the expected heterozygosity (He) and the number of alleles (A) were calculated for each locus with the R package *adegenet* (Jombart, 2008). To analyse patterns of population genetic structure in *S. haematobium* and *S. bovis* from northern Senegal, we performed three multivariate analyses (Figs. 2–4) using different datasets. The first analysis aimed to compare genetic structure per species (*S. haematobium* versus *S. bovis*) and to detect patterns of hybridisation between both. We applied a Principal Component Analysis (PCA) on a dataset including *S. haematobium* from all geographical locations (10 villages in northern Senegal, Kédougou in southern Senegal and one village in Mali) and *S. bovis* from northern Senegal (cercariae with a pure *S. bovis* profile based on *cox1* and ITS barcoding from *Bulinus* snails and adult schistosomes from cattle) (Fig. 2). The PCA was applied on allele frequencies per parasite using the R package *adegenet* (Jombart, 2008); allele frequencies were centred but not scaled. This PCA plot visualises admixing between the *S. haematobium* and the *S. bovis* populations. On this PCA plot we should be able to detect first generation hybrids between both populations because in an admixed population, the expected allele frequency of an individual is a linear mix of the frequencies in the parental populations that can be visualised by a PCA (Patterson et al., 2006). Secondly, we performed a PCA on a dataset including only miracidia isolated from human urine samples from the village of Pakh (Fig. 3). This analysis aims to detect the potential influence of hybridisation on the genetic constitution of *S. haematobium* populations. It investigates any genetic clustering of parasites per individual host and per *cox1* mitochondrial haplotype (with *S. bovis cox1* haplotype and mito-nuclear discordance as a proxy for

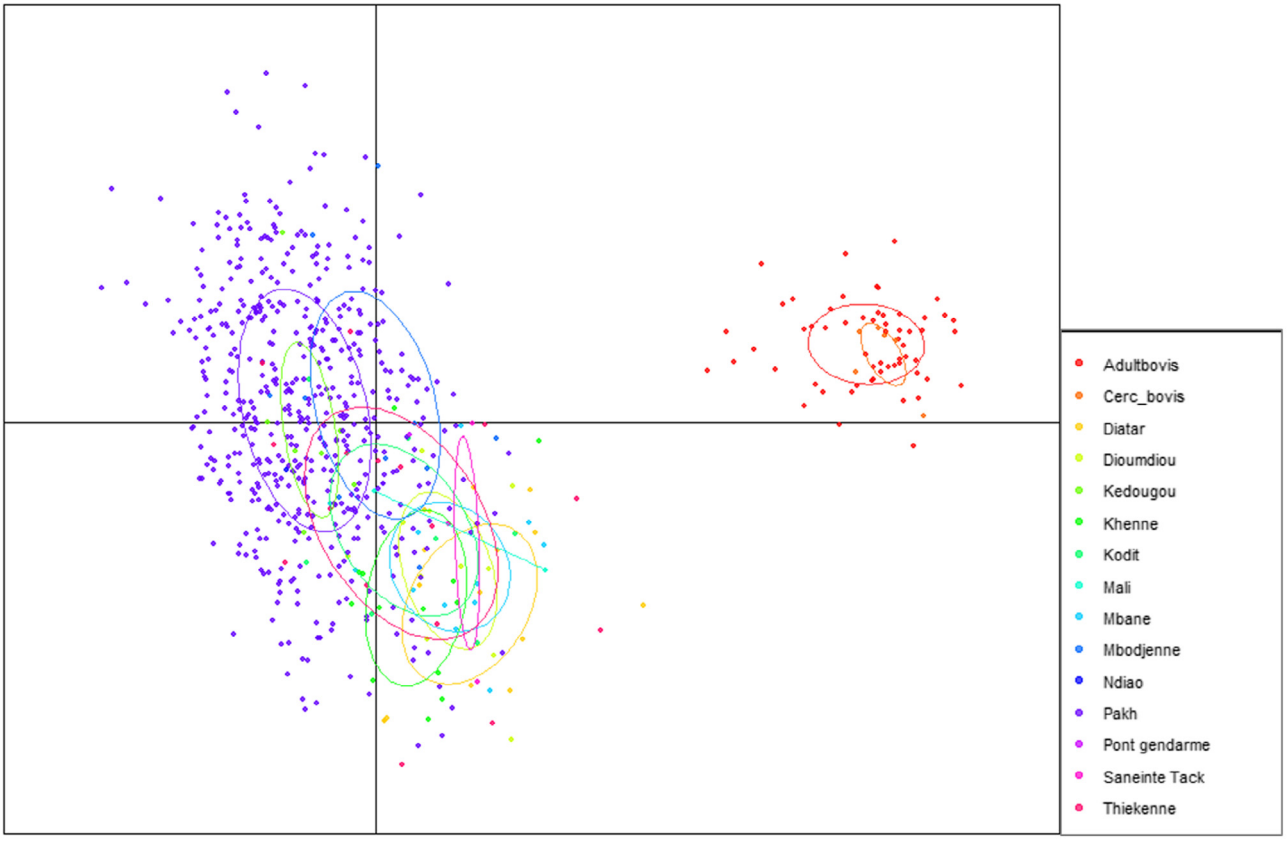

Fig. 2. Biplot of a Principal Component Analysis on the allele frequencies (using 12 microsatellite loci) of *Schistosoma* parasites successfully genotyped at more than seven of the 12 loci (668 observations). The cumulative proportion is 16.4% for the first two principal components (PC1: 11.0%; PC2: 5.4%). Observations are coloured per village; Diatar, Dioundiou, Kodit, Rhonne (Khenne), Ndiao (only one observation), Mbane, Saneinte Tack (hamlet of Mbane), Ndieumeul (Thiekenne), Pakh, Mbodienne and Pont Gendarme in northern Senegal, Kédougou in southeastern Senegal and Mali. *Schistosoma bovis* adult worms are denoted in red while *S. bovis* cercariae with a pure *S. bovis* profile (based on *cox1* and ITS barcoding) are denoted in orange; they are both in the top right section of the figure. These should not be confused with *Schistosoma haematobium* from Ndieumeul (Thiekenne) and Seneinte Tack. The inertia ellipses contain 2/3 of the observations.

'hybrids'). This was also tested by performing a Discriminant Analysis of Principle Components (DAPC, see below) with a *cox1* mitochondrial haplotype as a prior cluster (results not shown). Finally we applied a DAPC (Jombart et al., 2010) on a *S. haematobium* (and hybrids) dataset including all geographical locations (Fig. 4) to test for geographic barriers to gene flow. In this dataset most observations of Pakh 2014 were removed to have comparable numbers of observations for each village; only the miracidia from Pakh collected in 2009 and the *S. haematobium* cercariae were included. DAPC is a multivariate method that analyses between-group variability of predefined clusters; it maximizes variance between and minimizes variance within groups. We predefined clusters per village. To test whether the genetic distances (Nei's pairwise F_{ST}) were correlated with Euclidean geographic distances, we performed a Mantel test on the samples from Senegal with the R package *vegan* (Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H. H., Wagner, H., 2013. Package 'vegan.' R Packag. ver. 2.0–8). A Monte Carlo permutation was performed as a test statistic.

2.4.2. Genetic diversity and inbreeding of S. haematobium and S. bovis populations

Average pairwise F_{ST} (Weir and Cockerham) were calculated to compare the size order of genetic distance at the different levels of analysis in this study (species level, between villages, between human populations within the village of Pakh and between parasite populations with different *cox1* haplotypes; also within the village of Pakh). The genetic diversity (Ho, He), inbreeding level (F_{IS}, Weir and Cockerham) and allelic richness (AR) rarefied to a sample size of five, were calculated per village and for each *S. bovis* population (the adult schistosomes collected in Richard Toll, and the cercariae collected in Pakh). In the package *hierfstat* (Goudet and Jombart, 2015) we bootstrapped (10,000) over the loci of populations to create a 95% confidence interval for F_{IS}. In all tests a village was considered to be a population.

3. Results

3.1. Dataset and cox1 and ITS rDNA genotyping

Among the 792 *Schistosoma* stadia genotyped with the microsatellite markers, 570 were also successfully genotyped for the *cox1* marker and 186 were successfully genotyped for the ITS rDNA marker (Table 1). Of the 570 larvae successfully genotyped for *cox1*, 440 parasite larvae (77.2%) had a *S. haematobium cox1* mtDNA profile and 129 (22.6%) a *S. bovis cox1* mtDNA profile. Of the 186 larvae successfully genotyped for ITS, the ITS rDNA profile was *S. haematobium* for 164 (88.2%) of the parasite larvae, six cercariae out of 20 cercariae had a *S. bovis* profile (they were considered pure *S. bovis* because they had also a *S. bovis cox1* mtDNA profile) and one miracidium (from Kodit) had a double *S. haematobium* and *S. bovis* ITS rDNA profile and a *S. haematobium cox1* mtDNA profile. At Rhonne, one miracidium had a double *S. haematobium* and *S. mansoni* profile, and one miracidium had a *S. mansoni* profile. The latter two were excluded from further analysis.

The majority of miracidia (n = 529) that were genotyped originated from Pakh (2014). The ITS rDNA profile was genotyped for 67 miracidia and always matched *S. haematobium*. A total of 112 out of 437 (25.6%) genotyped miracidia displayed a *S. bovis cox1* profile while 325 miracidia (74.4%) had a *S. haematobium cox1* profile. The remaining 103 miracidia were only genotyped with the microsatellite markers.

Bulinid snails were found at the two first water contact sites in Pakh. In total 13 out of 380 bulinid snails were infected with *Schistosoma* cercariae (4% of the snails in site 1A and 5% of the snails in site 1C). Both transmission sites are connected because they are located at the same irrigation canal. From each of the 13 infected snails, five cercariae were genotyped. Since only one parasite genotype was present per snail, i.e. clones, only one cercarial genotype per snail was used in further analysis. Based on the barcoding with *cox1* and ITS rDNA, the cercariae isolated from six *Bulinus globosus* specimens (site 1C) showed a pure *S. haematobium* profile while cercariae from five *Bulinus truncatus* specimens (site 1A) presented a pure *S. bovis* profile; two *B. globosus* specimens (site 1A and 1C) were shedding cercariae that presented a hybrid profile with a *S. haematobium* ITS rDNA genotype and *S. bovis cox1* haplotype.

3.2. Microsatellite analysis

Only miracidia with at least nine out of 17 of the loci successfully scored were included in the analyses, leading to a total of 708 miracidia included (89.3%). Four loci (*C102*, *C111*, *Sh10*, *Sh8*) did not amplify in the *S. bovis* samples (the adult worms from cattle and the *S. bovis* cercariae from the bulinid snails) and one locus (*Sh5*) amplified poorly (see Supplementary Table S1). This is because the loci were designed specifically for *S. haematobium*. This supports the genetic differentiation between *S. haematobium* and *S. bovis*. For technical and statistical reasons the five above-mentioned loci were excluded from further analyses.

3.3. Population genetic differentiation between S. haematobium and S. bovis

A PCA biplot based on allele frequencies shows two distinct genetic clusters (Fig. 2). The cluster at the right side of the first axis (the horizontal axis) includes the adult *S. bovis* worms from cattle and the cercariae with a *S. bovis* ITS rDNA profile and a *S. bovis cox1* profile. The cluster at the left side of the first axis includes parasites originating from human urine samples or cercariae. This cluster includes both 'pure' *S. haematobium* larvae as defined by a *S. haematobium* ITS profile in combination with a *S. haematobium cox1* profile, and the 129 hybrid parasites, as defined by the discordant *S. haematobium* ITS and *S. bovis cox1* profile. Note that the differentiation of parasite genetic variation between each species cluster is in reality stronger because only loci that amplified for both species were used.

3.4. Population genetic structure of S. haematobium and hybrids within the village of Pakh

We could not infer any significant genetic clustering when individual parasites were grouped according to their *cox1* haplotype (*S. haematobium* versus *S. bovis*; Fig. 3), nor when they were grouped according to their human host (results not shown). This indicates that these parasite populations are in panmixia. The same pattern was found with discriminant analysis where the *cox1* haplotype was used to perform prior clustering (results not shown).

3.5. Population genetic structure of S. haematobium and hybrids across the Senegal River Basin

The DAPC plot highlights the genetic differentiation of parasite populations between villages (Fig. 4). Within northern Senegal, the *Schistosoma haematobium* parasites of Diatar, and to a lesser extent those from Dioumdiou, were clearly differentiated from the other villages of the SRB. The DAPC showed that parasites from Diatar showed a higher genetic differentiation from parasite populations bordering Lake Guiers (e.g., Pakh, Ndieumeul (Thiekenne) and Mbane) than the *S. haematobium* populations originating from Kédougou (southern Senegal). This is true for both the samples from 1993 and those from 2007; they cluster closely together

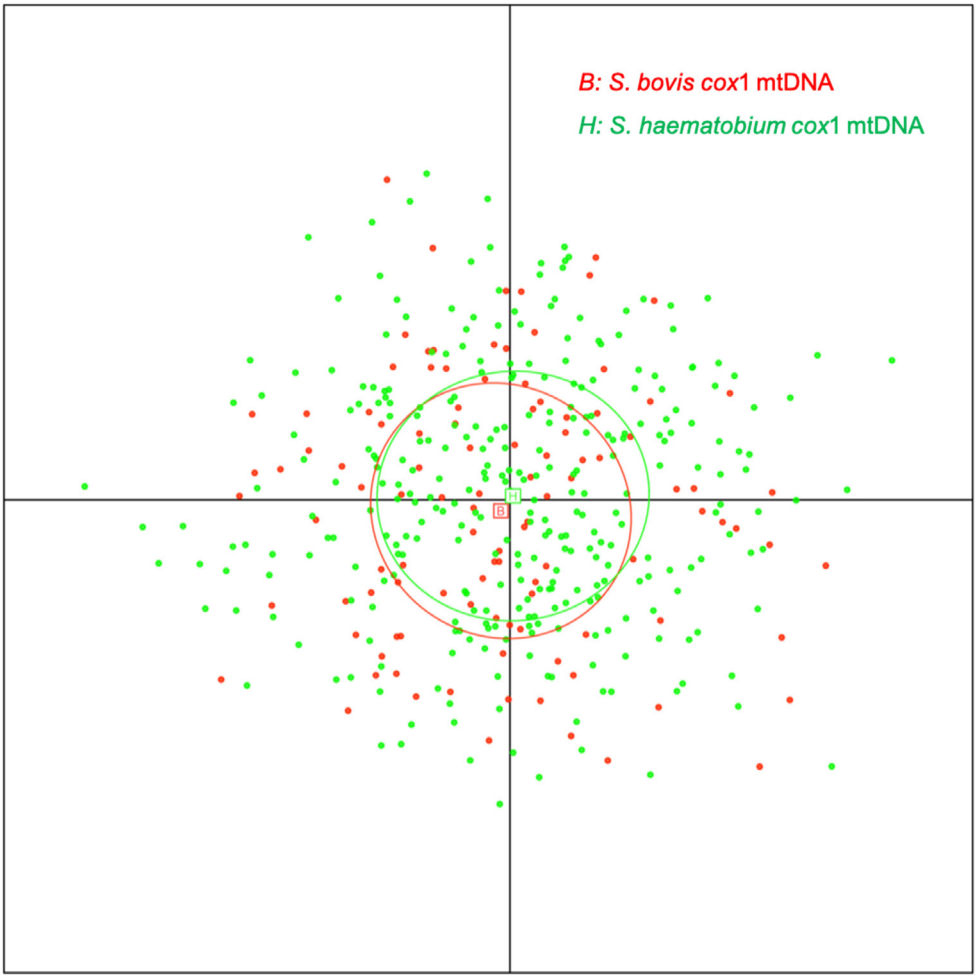

Fig. 3. Biplot of a Principal Component Analysis on the allele frequencies (using 17 microsatellite loci) of *Schistosoma haematobium* and hybrid parasites from the village Pakh (2014; northern Senegal) successfully genotyped at more than nine loci. Observations (n = 419) were coloured according to the *cox1* profile; *S. haematobium* (n = 313), *Schistosoma bovis* (n = 108). The cumulative proportion is 10.1% for the first two principal components (PC1: 5.4%; PC2: 4.7%).

when added to the DAPC as separate populations (details not shown). The village Kodit does not cluster with Diatar and Dioumdiou, even though they are all located in the Middle Valley of the SRB. A Mantel test did not find evidence for isolation-by-distance (P = 0.1). This marginal insignificant value indicates that, although geographical differentiation is present between parasite populations, the genetic distance between parasite populations does not correlate well with the geographical distance. For instance, the village Kodit is located in the neighbourhood of Diatar (Fig. 1) but is genetically highly differentiated from Diatar (Fig. 4).

In PCA and DAPC analyses missing values were replaced by the average allele frequencies of all populations. This may artificially increase the similarity between parasites of Mali (only two observations) and the Senegalese *S. haematobium* populations because few Malinese parasites amplified at all loci.

3.6. Genetic differentiation and diversity estimates

Pairwise genetic distances (F_{ST}) had the highest value at the species level (between *S. haematobium* and *S. bovis*), and at the geographical level. The pairwise difference between villages was higher when including *S. bovis* populations from cattle (F_{ST} = 0.163) (Table 2). Parasite populations from different villages were on average more differentiated (F_{ST} = 0.060) than parasite populations from different individual hosts within the same village (F_{ST} = 0.019 in Pakh). The average genetic differentiation between parasites grouped according to the *cox1* haplotype was very low (F_{ST} = 0.002). The two *S. bovis* populations, adult worms from cattle and cercariae from snails, had a higher genetic diversity (Ho, He and AR) than the *S. haematobium* + hybrid populations (Table 3) (significant differences based on a Wilcoxon rank test). Hybrid parasites from Pakh (with *S. haematobium* ITS rDNA and *S. bovis cox1* mtDNA) had the same genetic diversity as parasites with a *S. haematobium cox1* mtDNA. The ITS rDNA was genotyped for 66 out of 529 miracidia in Pakh (it was always *S. haematobium*). For the others we based the analysis on information of the *cox1* mtDNA only.

4. Discussion

The SRB is well suited to study the three possible outcomes of hybridisation (Abbott et al., 2013; see section 1) because in many areas both parental species overlap in time and space. In Pakh we found snails shedding either *S. bovis* or *S. haematobium* cercariae at the same time at the same water contact site, which was frequented by both humans and cattle. This observation qualifies Pakh as a "contact zone" between *S. haematobium* and *S. bovis* populations. Inhabitants and their livestock are thus at risk of infection with both schistosome species, creating the opportunity for inter-species interactions and the generation of first generation

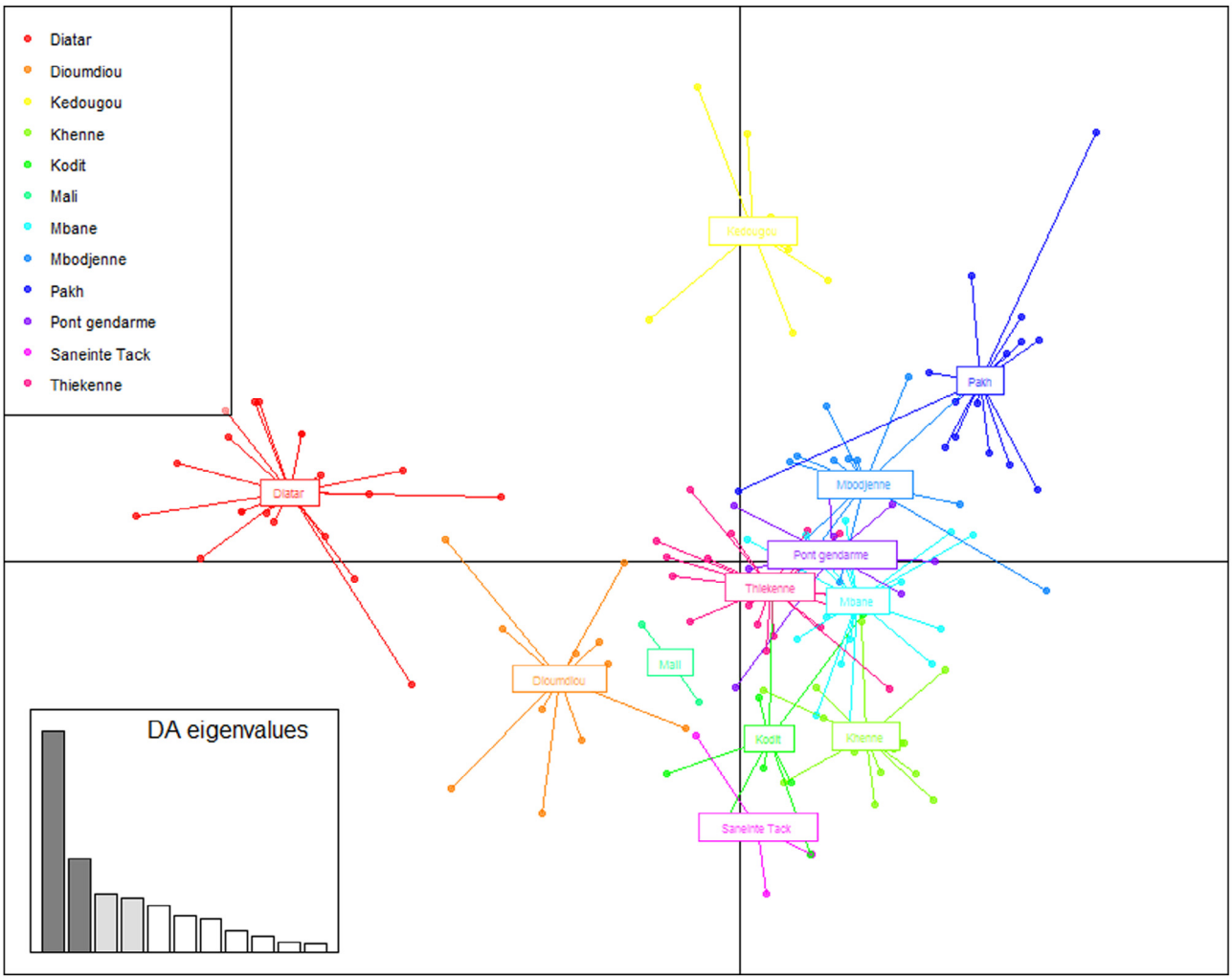

Fig. 4. Scatterplot of the first two components of the Discriminant Analysis of Principle Components analysis (DAPC) based on the allele frequencies (using 17 microsatellite loci) of *Schistosoma haematobium* successfully genotyped at more than seven of the loci (142 observations). The first 60 axes of the Principal Component Analysis (PCA) of the DAPC analyses were retained, which represents 94.6% of the total variation in allele frequency. The cumulative proportion is 51.9% for the first two DA eigenvalues (DA1: 36.4%; DA2: 15.4%). Observations were coloured per village (Diatar, Dioundiou, Kodit, Rhonne (Khenne), Mbane, Saneinte Tack (hamlet of Mbane), Ndieumeul (Thiekenne), Pakh, Mbodienne and Pont Gendarme in northern Senegal; Kédougou in southeastern Senegal and Mali) (Table 1, Fig. 1).

Table 2
Average pairwise F_{ST} (Weir and Cockerham) between the schistosome populations used in this study (see Table 1 for groups).

F_{ST} between villages SRB, Mali, including *S. bovis*	0.163
F_{ST} between villages SRB and Mali, excluding *S. bovis*	0.060
F_{ST} between parasite infrapopulations from individuals living in Pakh	0.019
F_{ST} between parasite populations with different *cox1* haplotypes (data from Pakh)	0.002

SRB, Senegal River Basin.

hybrids. Both species have also been studied intensively in this region, as well as in humans, cattle and rodents (Webster et al., 2013; Catalano et al., 2018).

The distinct allele frequencies that we found for *S. bovis* and *S. haematobium* (also from the cercariae shed within the same contact zone in Pakh) leads to a clear clustering of parasite individuals per host species (Fig. 2). Admixed individuals (hybrids) should align on the PCA plot along a gradient joining the centres of the parental populations (Patterson et al., 2006), which is not the case here. The clear separation between *S. bovis* on the one hand, and the *S. haematobium* and hybrid populations on the other, is in fact stronger as this analysis is only based on loci that amplified in both species. This therefore suggests a strong reproductive barrier between cattle and human *Schistosoma* populations that appears to exist in this endemic setting. If mixing was frequent, hybrids between the *S. bovis* and *S. haematobium* populations should have fitted in a cluster of first generation (F1) hybrids and in two clusters of F2 backcross hybrids. Our study lacks such clustering of parasites and hence does not suggest recent or ongoing hybridisation. The miracidium with a hybrid ITS rDNA profile also clustered with *S. haematobium*, pointing to an older backcross or further generation hybrid, but not a F1 hybrid. Previous studies have already shown that signals of hybridisation are retained longer in the ribosomal copies of the ITS region compared with microsatellite markers (Steinauer et al., 1998). That study showed that the detection power of microsatellites declined very quickly after F1 backcross generations. The ITS region on the other hand, which is part of a tandem repeat multigene, can retain both parental copies for several generations before they are homogenized by concerted evolution (Sang et al., 1995).

Altogether these results strongly suggest that, despite the high prevalence of parasites with *S. bovis cox1* mtDNA, contemporary hybridisation events with *S. bovis* seem rare (at least in the populations that we studied) and no barrier breakdown between *S.*

Table 3
Genetic diversity (measured as inbreeding coefficient F_{IS}, observed Ho and expected Hs heterozygosity and allelic richness (AR)) of *Schistosoma haematobium* and *Schistosoma bovis* collected in Senegal and Mali based on 17 microsatellite markers.

Population	n[a]	F_{IS} (95% CI)	Ho[a]	Hs[a]	AR[b]
Diatar	20	−0.034 to 0.158	0.61	0.65	2.80
Dioumdiou	11	0.104 to 0.326	0.51	0.65	2.88
Kédougou	7	−0.060 to 0.156	0.60	0.63	2.71
Khenne	15	−0.003 to 0.202	0.60	0.66	2.93
Kodit	8	−0.049 to 0.243	0.58	0.64	2.83
Mali	2	−0.909 to 0.591	NA	NA	NA
Mbane	17	0.001 to 0.213	0.60	0.67	2.97
Mbodjenne	13	0.031 to 0.213	0.55	0.62	2.66
Pakh (2009)	16	0.021 to 0.201	0.53	0.60	2.64
Pont Gendarme	9	−2.953 to −0.094	0.63	0.63	NA
Saneinte Tack	3	0.010 to 0.393	NA	NA	NA
Thiekenne	21	0.077 to 0.252	0.54	0.64	2.77
Pakh (2014); *cox*1 = *S. bovis*	108	0.067 to 0.220	0.54	0.63	2.76
Pakh (2014); *cox*1 = *S. haematobium*	311	0.051 to 0.201	0.55	0.64	2.74
S. bovis adult worms[d]	59	0.034 to 0.179	0.67	0.75	3.34
S. bovis cercariae[d]	6	−0.092 to 0.330	0.68	0.76	3.40

[c]Average over all loci.
Ho, observed heterozygosity; He, expected heterozygosity; 95% CI, 95% confidence interval; NA, not applicable.
[a] Number of observations with at least nine loci amplified.
[b] Rarefied to five individuals.
[d] Based on 12 microsatellite markers.

haematobium and *S. bovis* could be detected. It seems that the hybridisation between *S. haematobium* and *S. bovis* is best represented by the first scenario: historically parts of the genome of *S. bovis* have introgressed into *S. haematobium* but the two parental populations (*S. bovis* and *S. haematobium*) remain differentiated. This suggests that animals do not represent a real reservoir for human schistosomiasis, at least not directly. Indeed, the presence of animal species infected with *S. bovis* will fuel hybridization with *S. haematobium* in rodents (Catalano et al., 2018) and can thereby have an indirect effect on schistosomiasis epidemiology. Since these hybrids may have altered life history traits, including increased intermediate host range, they can boost transmission of urogenital schistosomiasis in the SRB. For example, laboratory experiments have shown that F1 and F2 hybrids between *S. haematobium* and *Schistosoma guineensis* exhibited a greater infectivity for snail intermediate hosts than the parental schistosome species (Webster and Southgate, 2003a,b).

Despite the differentiation between both parental populations, hybridization can create a 'bridge' between two species, allowing exchange of genetic material including adaptive genetic variation (Fontaine et al., 2015). It might therefore be an important mechanism in schistosomes to increase their evolutionary potential, allowing them to adapt to changing environments. We tested whether allelic variation is associated with the introgression of *S. bovis cox1* mtDNA. To this end, parasites isolated from children in the village of Pakh ($n = 419$) were genotyped with 17 microsatellite markers and a mitochondrial *cox1* marker. We found no clustering of parasites according to *cox1* haplotype (*S. haematobium* versus *S. bovis*) (Fig. 3). This suggests that within Pakh and within individual hosts, one panmictic parasite population is present (Gorton et al., 2012). It also shows that based on these 17 microsatellite markers we cannot differentiate parasites with an introgressed mitochondrial genome from those with a *S. haematobium* mitochondrial genome. This corresponds with the results of a study on the morphology of schistosome eggs that were collected during the same field study, in Pakh in 2014 (Boon et al., 2017). In this study, the unimodal distribution of egg sizes and the absence of morphological differences between hybrid and non-hybrid eggs (as based on mito-nuclear genotyping) also suggested that both genetic classes of eggs belong to one random mating schistosome population without a reproductive barrier (Boon et al., 2017). Repeated backcrossing of hybrids with *S. haematobium* may have captured the *S. bovis* mitochondrial genome into a dominantly nuclear *S. haematobium* background (see Boon et al., 2018). However, ancient admixture cannot be detected with a limited number of microsatellites because all parasite individuals have the same ancestral proportion (Patterson et al., 2006). To detect backcrosses or traces of ancient introgression at the nuclear genome, a genome scan of hybrids and pure parental species is required. Platt et al. (2019) recently studied the exomes of single field-caught *S. haematobium* miracidia from Niger and Zanzibar. Their data did not reveal evidence for contemporary hybridization between *S. bovis* and *S. haematobium* either, but they demonstrated ancient introgression from a few *S. bovis* alleles into the genome of *S. haematobium* from Niger. Kincaid-Smith et al. (Kincaid-Smith, J., Tracey, A., de Carvalo Augusto, R., Bulla, I., Holroyd, N., Rognon, A., et al. 2018. Whole genome sequencing and morphological analysis of the human-infecting schistosome emerging in Europe reveals a complex admixture between *Schistosoma haematobium* and *Schistosoma bovis* parasites. bioRxiv 387969; doi: https://doi.org/10.1101/387969) sequenced the complete genome of the Corsican schistosome strain, which is a hybrid cross between *S. haematobium* and *S. bovis*. The adult parasite DNA was obtained by passaging miracidia, isolated from a urine sample from a patient infected in Corsica, in laboratory snails and hamsters. The mapping against *S. haematobium* and *S. bovis* reference genomes revealed a complex admixture between both parental genomes with a proportion of 76.9% of sequences mapped to *S. haematobium*, and 23.1% mapped to *S. bovis*.

The higher genetic diversity of *S. bovis* populations in comparison to *S. haematobium* and hybrid populations in Senegal corresponds with the results from Djuikwo-Teukeng et al. (2019). They showed that *S. bovis* populations from Cameroon had a higher allelic richness and diversity than *S. haematobium* populations from Niger and Zanzibar.

Due to the near identical (nuclear) genetic constitution of *S. haematobium* populations with and without *S. bovis cox1* introgression (see above), we assume that hybridisation does not have a strong influence on the population genetic analysis using the respective microsatellite panels. We also did not encounter any signal from *S. curassoni* in our sequences or population genetic analyses. This species can also hybridise with *S. bovis* and *S. haematobium*, but its distribution is much more restricted in Senegal compared with *S. bovis*. Webster and Southgate (2003a,b) only

found hybrids between *S. curassoni* and *S. haematobium* in Tambacounda and the Vallée du Ferlo, which is outside our study area.

Our analyses showed that parasite genetic variation of *S. haematobium* populations (including hybrids) was not randomly distributed across villages in the SRB. Parasite transmission (gene flow) appeared restricted between the Middle Valley and the other regions (The Delta, Lake Guiers and the Lampsar River). Parasites also clustered according to villages, albeit genetic differentiation between villages was not correlated with geographical distance. *Schistosoma haematobium* populations from the southeast of Senegal (Kédougou) were for example less differentiated from villages in the vicinity of Lake Guiers (±700 km) than the parasite population from Diatar (±100 km from Lake Guiers). This was the case for populations sampled in 1993 and 2007. Parasites from Kodit, also located in the Middle Valley, clustered however with parasites from villages located in the Delta (Khenne) and Lake Guiers. This suggests that genetic differentiation between parasite populations is not merely driven by isolation by distance.

Few other studies explored the population genetic structure of *S. haematobium* at a regional scale, which hampers comparisons. A strong genetic differentiation of *S. haematobium* populations was found between sub-Saharan countries (Glenn et al., 2013; Gower et al., 2013). But at the village level, almost no genetic differentiation was found between parasite populations of 47 children at two schools in the Ségou region in Mali (Gower et al., 2011, 2013).

Parasite population structure can be influenced by different factors, including the mobility of the final and intermediate hosts. Parasite transmission depends on the presence of a suitable snail species, and the genetic constitution of the snail population. Previous experimental studies have shown varying degrees of compatibility between populations of *S. haematobium* and populations of *Bulinus* intermediate host species in the SRB. *Bulinus globosus* is the main local intermediate host of *S. haematobium* in the Lower Valley of the SRB, while *B. truncatus* is the main host for *S. haematobium* in the Middle Valley (Sène et al., 2004). In Diatar *B. globosus* is scarce while *B. truncatus* and *Bulinus senegalensis* are common (Southgate et al., 2000). Snail infection experiments indicated that only *S. haematobium* from Diatar (Middle Valley) is compatible with *B. senegalensis* (Southgate et al., 2000). Our study now proves that this compatibility could partly be explained by the genetic constitution of this particular parasite population, as it clearly clusters apart from all other *S. haematobium* populations. For example, it has been shown that *B. truncatus* in the Middle Valley (Podor) is naturally infected with *S. haematobium*, unlike *B. truncatus* from the Lower Valley (Richard Toll) (Sène et al., 2004). These observations show the need for molecular studies on the snail intermediate host to understand the role of host and parasite genetic variation in compatibility studies.

Also, human mobility affects parasite transmission and thus differentiation. A recent study on *S. mansoni* in the same area showed that labour-related migration to Richard Toll to work in the sugar cane industry probably led to efficient admixture between the introduced *S. mansoni* populations (Van den Broeck et al., 2015). A single population of *S. mansoni* dominated almost the entire SRB. The fact that this migration had not the same impact on *S. haematobium* populations might be linked to the fact that *S. haematobium* was already present in the SRB before dam construction. As such, these infection foci might have evolved independently from each other, leading to differentiation. The lower abundance of *Bulinus* snails and the lower densities of human populations before dam construction may have hindered parasite transmission between villages or between the four water basins.

To conclude, no differentiation was found between human schistosome populations with a *S. haematobium cox1* mtDNA profile and those with a *S. bovis cox1* profile, using microsatellite markers. Both appear to belong to a single randomly mating population strongly differentiated from *S. bovis* found in cattle. These results suggest that, under natural conditions, a strong species boundary persists between human and cattle schistosome species, despite the opportunity for hybridization. In addition, the transmission potential of *S. haematobium* in northern Senegal appeared restricted between the Middle Valley and elsewhere in the SRB. *Schistosoma haematobium* was spatially more constrained in comparison to *S. mansoni*. This may be related either to the presence and genetic constitution of the intermediate snail hosts, or to the colonisation history of the parasite.

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Appendix A. Supplementary data

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