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Molecular characterization of schistosome cercariae and their *Bulinus* snail hosts from Niakhar, a seasonal transmission focus in central Senegal

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ABSTRACT

Bulinus senegalensis and *Bulinus umbilicatus*, two sympatric freshwater snails found in temporal ponds in Senegal, were thought to be involved in the transmission of *Schistosoma haematobium* and/or *Schistosoma curassoni*. To better understand the role of these *Bulinus* species in the transmission of human and animal *Schistosoma* species, *B. senegalensis* and *B. umbilicatus* were collected in 2015, during a malacological survey, from a temporal pond in Niakhar, central Senegal. Snails were induced to shed cercariae on two consecutive days. Individual cercariae from each snail were collected and preserved for molecular identification. Infected snails were identified by analysis of a partial region of the cytochrome *c* oxidase subunit 1 (*cox*1) gene. Six individual cercariae shed from each infected snail were identified by analyses of the *cox*1, nuclear ITS and partial 18S rDNA regions. Of the 98 snails collected, one *B. senegalensis* had a mixed infection shedding *S. haematobium*, *S. bovis* and *S. haematobium-S. bovis* hybrid cercariae and one *B. umbilicatus* was found to be shedding only *S. haematobium*. These data provide molecular confirmation for *B. senegalensis* transmitting *S. bovis* and *S. haematobium-S. bovis* hybrids in Senegal. The multiple *Bulinus* species involved in the human urogenital schistosomiasis in Senegal provides a high force of transmission warranting detailed mapping, surveillance and regular treatment of at-risk populations.

1. Introduction

Human schistosomiasis manifests as an intestinal or urogenital disease, causing significant levels of morbidity and mortality worldwide, with approximately 280,000 deaths per year, 258 million infected and 779 million additional people at risk of infection (Toor et al., 2018; Molehin, 2020). The disease, schistosomiasis, is due to infection with parasitic flatworms belonging to the genus *Schistosoma*. In Africa, the most important human-infecting *Schistosoma* species in terms of prevalence and morbidity are *Schistosoma haematobium* and *Schistosoma mansoni*, causing urogenital and intestinal schistosomiasis, respectively (Molehin, 2020).

Definitive hosts become infected when their skin comes into contact with water bodies containing larval forms (cercariae) of *Schistosoma* spp. released into the water by specific freshwater snail hosts (McManus et al., 2018). There are two main genera of snail hosts responsible for the transmission of *Schistosoma* spp. in Africa, *Bulinus* and *Biomphalaria*, transmitting species of the *S. haematobium* and *S. mansoni* groups, respectively (Brown, 1994; Rollinson et al., 2001). The *S. haematobium* species group is diverse with nine closely related species capable of utilizing a range of snail and definitive hosts for transmission, with host-parasite species/strain specificities observed (Webster et al., 2006).

In Senegal, *S. haematobium* and *S. mansoni* coexist in the Senegal River Basin with permanent transmission and prevalence above 95% (Webster et al., 2013a), while in central Senegal, including the Niakhar study area, only *S. haematobium* transmission occurs seasonally in temporal ponds with prevalence varying from 10 to 57% (GAHI, 2012; Senghor et al., 2014). There are also two other *Schistosoma* spp. endemic in Senegal,

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Schistosoma bovis and *Schistosoma curassoni*, infecting livestock. *Schistosoma curassoni* has a more restricted distribution to the south-east and central regions of Senegal whereas *S. bovis* is widespread throughout the country, and elsewhere in Africa (Diaw & Vassiliades, 1987; Diaw et al., 1989).

Five species of *Bulinus* (*Bulinus forskalii*, *Bulinus globosus*, *Bulinus umbilicatus*, *Bulinus senegalensis* and *Bulinus truncatus*) are endemic to Senegal each reported to transmit one or more of the three *S. haematobium* group species (Table 1). Early identifications of which species of snail were transmitting which species of *Schistosoma* were primarily based on morphology, differences in the chronobiology of cercarial shedding (Frandsen & Christensen, 1984; Vassiliades et al., 1985; Diaw & Vassiliades, 1987; Mouahid et al., 1991) or by cercarial chaetotaxy characteristics (Bayssade-Dufour, 1982; Albaret et al., 1984, 1993; Vassiliades et al., 1985), which all carry a degree of uncertainty. In other cases, *Schistosoma* species identifications were inferred after experimental infections in laboratory animals followed by egg morphology analysis (Diaw & Vassiliades, 1987). Reliable and specific molecular identification of snail hosts and their emerging trematodes are now routinely used to better inform on the epidemiology of human and animal schistosomiasis in endemic countries (Webster et al., 2015; Tian-Bi et al., 2019). Specifically, this helps identify human schistosomiasis transmission foci so that targeted interventions can be implemented in the correct areas. Additionally, precise species identifications are needed for the monitoring of transmission as human schistosomiasis elimination or eradication is reached. Multi-locus molecular analyses of *Schistosoma* cercariae are also vital for precise species identification and to identify *S. haematobium* group hybrids (namely, *S. haematobium-S. bovis*, *S. haematobium-S. curassoni* and *S. bovis-S. curassoni* hybrids in Senegal) which may have an effect on disease epidemiology (Huyse et al., 2009; Webster et al., 2013b; Leger & Webster, 2017).

The present study used DNA sequencing to confirm the transmission of species of the *S. haematobium* group and their respective hybrid forms in Niakhar, central Senegal, together with molecular characterisation of the *Bulinus* species involved in transmission.

2. Materials and methods

2.1. Study area

The study was carried out in Niakhar, a rural area located in the Fatick region, 135 km east of Dakar, the capital of Senegal. Niakhar has two distinct seasons: a dry season (November-May/June) and a rainy season (June/July-October). Many temporary ponds, which constitute the only transmission sites for urogenital schistosomiasis, during the rainy season. For a detailed description of the study area, see Senghor et al. (2015).

2.2. Bulinus spp. collection and testing for Schistosoma spp. infections

Bulinus spp. were collected in November 2015 from the main pond of the village of Ngangarlam (14.54269, -16.49194) when the water level was low, but before it became completely dry in December. Snails were collected using a scoop for 30 min from vegetation and from the mud and floating debris, placed in plastic containers with water and transferred to the laboratory. All snails were identified to the species level based on shell morphology, and placed individually into a 24-well ELISA plate containing 2 ml of filtered water per well. The snails were exposed to artificial light for 1 h at 10:00 h and re-exposed at 13:00 h in order to induce cercariae shedding, taking into account differences in chronobiological rhythms of different *Schistosoma* spp. (Théron, 2015). Snails were induced to shed cercariae over two consecutive days and any *Schistosoma* spp. cercariae released from the snails were morphologically identified using a binocular microscope and a taxonomic key (Frandsen & Christensen, 1984).

2.3. Schistosoma spp. cercariae collection and preservation

Individual *Schistosoma* cercariae from each shedding snail were placed in a volume of 3 µl of water using a micropipette, under a binocular microscope and then directly placed on Whatman FTA® cards (Webster, 2009). All samples were transferred to the Natural History Museum, London, UK, for molecular analyses.

2.4. Molecular identification via mitochondrial cox1 analyses of Schistosoma-infected Bulinus spp.

Infected *Bulinus* spp. snails were photographed for morphological referencing (Brown, 1994) and DNA was extracted from the soft tissue, using a modified Qiagen DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) protocol (Pennance et al., 2018). For molecular identification, a partial mitochondrial *cox*1 DNA region (the 'Folmer' region, 643–658 bp), was amplified for each sample using forward/reverse primers BulCox1/HCO2198 and/or LC1490/HCO2198 (Folmer et al., 1994; Kane et al., 2008). Additionally, the nuclear ITS rDNA region was amplified from selected samples using the primers ETTS1 and ETTS10 (Kane et al., 2008), for further species clarification. Amplicons were visualised on a Gel Red agarose gel before being Sanger sequenced. The sequences were manually edited and trimmed using Sequencher v5.4.6 (Genecodes Corp., Michigan, USA) and then compared to reference sequence data using BLASTn (NCBI). The *cox*1 sequences were aligned with available reference sequences (Kane et al., 2008; Zein-Eddine et al., 2014; Pennance et al., 2020) using MAFFT v7.450 (Katoh & Standley, 2013) in Geneious Prime v11.1.4. The alignment was trimmed to the length of the shortest sequence; this resulted in a 591 bp region available for analysis.

Table 1
Previously reported role of *Bulinus* species in the transmission of species and hybrid forms of the *Schistosoma haematobium* group in Senegal based on non-DNA sequence identification methods such as identification based on morphology/behaviour (M/B) and those confirmed by mitochondrial and nuclear DNA sequencing (DNA), including those identified in the present study (DNA)[a] which also include snail identification.

Schistosoma spp./*Bulinus* spp.	*S. haematobium*	*S. bovis*	*S. curassoni*	*S. haematobium-S. bovis* hybrids	Reference
B. truncatus	M/B	M/B		M/B	Ernould (1996)
	DNA	DNA		DNA	Sène et al. (2004); Huyse et al. (2009); Léger et al. (2020)
B. globosus	M/B	M/B	M/B	M/B	Albaret et al. (1984); Vercruysse et al. (1985); Diaw & Vassiliades (1987); Picquet et al. (1996)
	DNA	DNA		DNA	Huyse et al. (2009); Léger et al. (2020)
B. senegalensis	M/B				Albaret et al. (1984); Diaw & Vassiliades (1987); Senghor et al. (2015)
	DNA[a]	DNA[a]		DNA[a]	Present study
B. umbilicatus	M/B		M/B		Albaret et al. (1984); Albaret et al. (1985); Diaw & Vassiliades (1987); Senghor et al. (2015)
	DNA[a]				Léger et al. (2020); present study
B. forskalii		M/B			Albaret et al. (1984); Diaw& Vassiliades (1987)

[a] Identification of the present material.

A haplotype network analysis was performed for the *cox*1 data using PopART v.1.7 (Leigh & Bryant, 2015), to investigate the relationships and diversity of infected *B. senegalensis* and *B. umbilicatus* to other *Bulinus* species and haplotypes (Kane et al., 2008; Zein-Eddine et al., 2014; Zhang et al., 2018; Pennance et al., 2020).

2.5. Molecular species identification of Schistosoma spp. cercariae

DNA was prepared from individual cercariae stored on the Whatman FTA cards, using the alkaline elution method, as previously described (Webster et al., 2015). Six cercariae were analysed from each snail. Individual cercariae were characterised using three molecular markers: a partial region of the mitochondrial *cox*1 gene, the nuclear rDNA (ITS1-5.8S-ITS2) region and a partial region of the 18S rRNA gene, to determine the species of *Schistosoma* or hybrid status of each cercaria as described in Pennance et al. (2020). Sequence identity from trimmed and manually edited data were confirmed by comparison to reference data (Webster et al., 2010). Following successful sequencing and analysis, the mitochondrial (*cox*1) and nuclear (ITS + 18S rDNA) profiles were asigned to each cercaria to determine species or hybrid status.

3. Results and discussion

From the 98 *Bulinus* snails collected from the study area, 62 were morphologically identified as *B. senegalensis* and 36 as *B. umbilicatus*. Of these a single snail of each species shed *Schistosoma* spp. cercariae (Fig. 1).

The morphologically identified *B. senegalensis* (Fig. 1D and E) was molecularly confirmed as *B. senegalensis* (GenBank: OP779241), with 99.32% mitochondrial *cox*1 similarity to published sequences from *B. senegalensis* sampled in Senegal (GenBank: MW167056) (Fig. 2). The

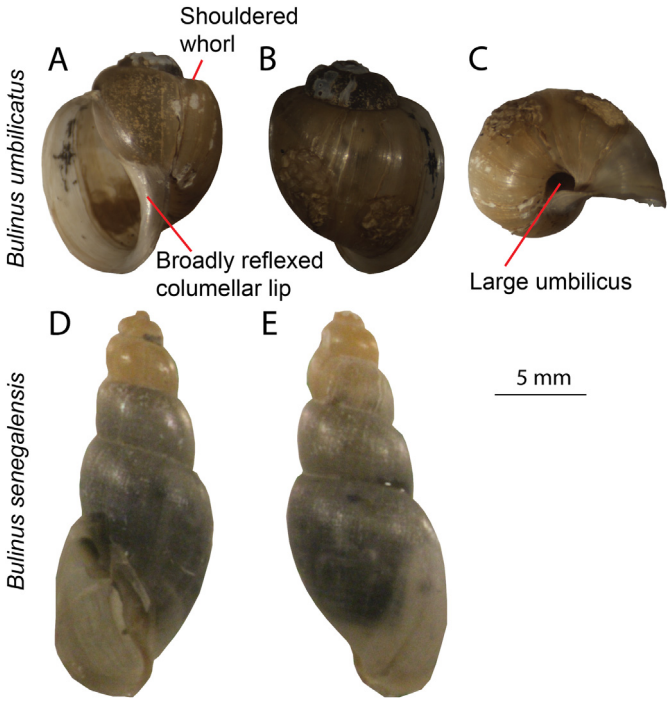

Fig. 1. Snail shell photos, taken under a dissecting microscope, showing the key morphological features of *Schistosoma*-infected *Bulinus umbilicatus* (**A**, **B** and **C**) and *Bulinus senegalensis* (**D** and **E**) from Niakhar, Senegal. For the *Bulinus umbilicatus* the front (**A**), back (**C**) and apex (**B**) of the shell are shown. The angular shouldered whorl, broadly reflexed columellar lip and well-defined large umbilicus are all key morphological features for *B. umbilicatus*. The front (**D**) and back (**E**) views of the *Bulinus senegalensis* show the curved and non-carinate whorls, which are more rounded, without shoulder angle.

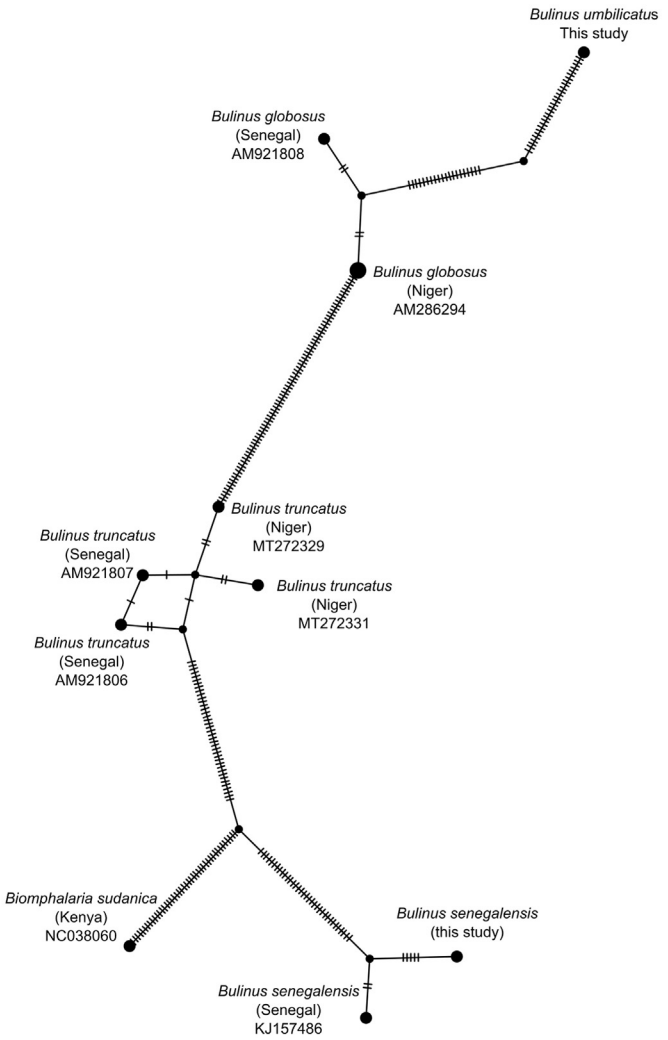

Fig. 2. Median-joining *cox*1 haplotype network constructed with PopART v.1.7 including the data from the infected *Bulinus senegalensis* (GenBank: OP779241) and *B. umbilicatus* (GenBank: OP779242) of the present study together with previously published sequences for *Bulinus* spp. (GenBank: AM286294, AM921806, AM921807, AM921808, MT272329, MT272331, NC038060 and KJ157486) (Kane et al., 2008; Zein-Eddine et al., 2014; Zhang et al., 2018; Pennance et al., 2020). The haplotype network shows clear species divergence for *B. globosus*, *B. truncatus*, *B. senegalensis* and *B. umbilicatus*. The unlabelled circles represent unsampled haplotypes; the hatch marks on the connecting lines indicate the number of mutational steps between haplotypes.

morphologically identified *B. umbilicatus* snail showed clear morphological features matching this species as detailed in Brown (1994), with a broadly reflexed columella lip (Fig. 1A) and large umbilicus (Fig. 1B). However, a comparison of our *B. umbilicatus cox*1 sequence and those published by Zein-Eddine et al. (2014) (GenBank: KJ157490 and KJ157491) revealed that sequences were extremely divergent. Further analysis of the data from Zein-Eddine et al. (2014) (GenBank: KJ157490 and KJ157491) suggested that the published sequences were not from a species of *Bulinus* but from an annelid, and therefore, should not be used as reference data for *B. umbilicatus*. We further confirmed the validity of our *B. umbilicatus cox*1 sequence data through comparison with independently collected *Bulinus* specimens from Bandafassi, Senegal (Ruben Schols and Tine Huyse, personal communication). The partial ITS sequence of our *B. umbilicatus* (720 bp) was therefore also analysed further confirming the sequence identity as a species of the *Bulinus africanus* species group (Brown, 1994), and so we provide novel molecular data for *B. umbilicatus* (GenBank: OP779242 (*cox*1) and OP778350 (ITS)).

The cercariae shed from the *B. senegalensis*, were molecularly confirmed as *S. haematobium* (GenBank: OP779237), *S. bovis* (GenBank: OP779238) and *S. haematobium-S. bovis* hybrids (GenBank: OP779239), with the hybrids presenting a *S. bovis* mitochondrial/*S. haematobium* nuclear DNA profile. For the infected *B. umbilicatus*, all of the cercariae were identified as *S. haematobium* (GenBank: OP779240) with all of the *S. haematobium* mitotypes being identical to each other and also to the common H1 haplotype found across mainland Africa (Webster et al., 2012). All of the *S. bovis* mitotypes from *S. bovis* cercariae were also identical to each other and showed a 100% match to *S. bovis* isolated from cattle and rodents in Senegal (Catalano et al., 2018; Leger et al., 2020). The two cercariae that presented the hybrid profile (*S. bovis* mitotype/*S. haematobium* nuclear profile) presented a *S. bovis* mitotype that was 100% identical to previously described *S. haematobium-S. bovis* hybrids identified from humans in Senegal (Huyse et al., 2009; Webster et al., 2013b). All ITS and 18S rDNA profiles were identical to the reference data for the respective species, *S. haematobium* or *S. bovis* (Pennance et al., 2020, 2021).

Interactions between schistosomes and their snail hosts is specific and complex. Snails and cercariae are not easily morphologically identifiable to the species level, therefore the use of molecular tools for identification has proven to be essential to clarify which snail species is transmitting which *Schistosoma* species in different geographical areas, enabling mapping of human and animal schistosomiasis transmission and risk (Pennance et al., 2020).

Previous malacological studies across Senegal and laboratory challenge experiments have shown the complexities of the *Bulinus* species involved in the transmission of different *Schistosoma* species and the need for molecular clarification to determine human and/or animal schistosomiasis transmission. Here, we have molecularly confirmed that *B. senegalensis* naturally transmits *S. bovis*, *S. haematobium* and *S. haematobium-S. bovis* hybrids and that *B. umbilicatus* transmits *S. haematobium* whereas previously *B. senegalensis* was only speculated to transmit *S. haematobium* (based on morphological data). We further confirm the involvement of *B. umbilicatus* in the transmission of *S. haematobium* corroborating previous morphological data summarized in Table 1.

Bulinus senegalensis is the most widespread intermediate host species existing in almost all of the water bodies (especially temporary pools) in all the regions of Senegal, where urogenital schistosomiasis is endemic (Diaw et al., 1989; Ndir, 2000). This species is also widespread in the neighbouring country Gambia, where it is reported to transmit *S. bovis* and *S. haematobium* (Smithers, 1956), although this needs to be confirmed by molecular methods. In the Niakhar area, Gaye et al. (2021) reported the presence of a mixed DNA profile (*S. haematobium* and *S. bovis*) from a single B. *senegalensis* sample, suggesting co-infection with both species and/or hybrid forms (Gaye et al., 2021). In the present study, we provide conclusive evidence of the transmission of *S. bovis* and *S. haematobium-S. bovis* hybrid forms in Niakhar by *B. senegalensis.*

Bulinus umbilicatus has a limited distribution only being found in temporary ponds, but it also plays a significant role in seasonal urogenital schistosomiasis transmission. Indeed, in the Niakhar area, previous malacological surveys in 2012–2013 found morphologically identified *B. umbilicatus* to be infected with *Schistosoma* spp. at a high prevalence of 13.79% and 11.90%, in 2012 and 2013, respectively (Senghor et al., 2015).

Schistosoma haematobium group hybrids (*S. haematobium-S. curassoni* and *S. haematobium-S. bovis*) appear common in Senegal, with human infections being observed in sympatric zones (Webster et al., 2013b). It is thought that the genetic exchange *via* these hybridisation events enables an increase in compatible intermediate snail host species (i.e. being able to infect both primary snail hosts of the parental species) leading to enhanced transmission (Webster & Southgate, 2003; Huyse et al., 2009; Rey et al., 2021). Four out of the five *Bulinus* species endemic in Senegal are now known to transmit *S. haematobium*, with three of these also known to be able to transmit the *S. haematobium* group hybrids. Similarly, four of *Bulinus* species are now known to transmit *S. bovis* (Table 1). This snail host range and compatibility with different *Schistosoma* species and hybrid forms permit human and animal schistosomiasis transmission in all ecological zones of Senegal, where different snail habitats exist (Webster et al., 2013b; Leger et al., 2020). This could possibly explain the high endemicity of disease across the country with a high force of transmission. However, this study is small and involves very few samples and a single sampling point. Further sampling is imperative to fully confirm the involvement of *Bulinus* species in the transmission of the different *Schistosoma* species and hybrid forms throughout Senegal, and beyond. Additionally, a more in-depth molecular analysis, at the genome level, of the *Schistosoma* cercariae would provide valuable insights into the levels of genetic inter-species introgression (Rey et al., 2021), which could be supporting enhanced snail host-schistosome compatibilities leading to dynamic transmission of both human and animal schistosomiasis.

4. Conclusions

Our results confirm that both *B. senegalensis* and *B. umbilicatus* are responsible for the transmission of *S. haematobium* in Niakhar, central Senegal. Moreover, we provide the first conclusive evidence of the transmission of *S. bovis*, and *S. haematobium-S. bovis* hybrids by *B. senegalensis* in Niakhar. This study demonstrates the importance of molecular identification of snails and cercariae during malacological monitoring and raises not only concern for human and livestock health but also for the control and elimination of urogenital schistosomiasis in the several seasonal transmission foci in Senegal.

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Ethical approval

Not applicable.

CRediT authorship statement

Bruno Senghor: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing. Bonnie Webster: Conceptualization, Methodology, Data curation, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. Tom Pennance: Investigation, Validation, Formal analysis, Writing – review & editing. Mariama Sène: Writing – review & editing. Souleymane Doucouré: Writing – review & editing. Doudou Sow: Writing – review & editing. Cheikh Sokhna: Conceptualization, Methodology, Supervision, Writing – review & editing.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All the data supporting the conclusions of this article are included within the article. The newly generated sequences are submitted to the GenBank database under the accession numbers OP779237-OP779242 and OP778350.

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