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Novel sequences of schistosomes and their snail intermediate host species in the Democratic Republic of Congo: first phylogenetic analyses

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Research Paper

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Abstract

Malacological surveys were conducted in 2021 in the Kimpese region of Central Kongo Province, west of the Democratic Republic of Congo (DRC). Snail specimens were collected following a standardised protocol, identified using morphological and molecular methods, and tested for schistosome infection using a diagnostic PCR assay. Positive snail samples were sequenced to characterise the infecting schistosome species. Partial mitochondrial cytochrome c oxidase subunit 1 (COX1) gene sequences were used in phylogenetic analyses to explore the evolutionary position of these snail species within the broader African context. At least four intermediate snail hosts were identified: *Bulinus truncatus*, *Bulinus forskalii*, *Biomphalaria pfeifferi*, and a *Biomphalaria* species belonging to the Nilotic species complex (tentatively named *Biomphalaria* cf *sudanica)*, of which the species identity needs to be confirmed. A total of 37 out of 1,196 snails (3.1%) tested positive for schistosome infection, with an infection prevalence of 7.4% for *B. truncatus* with *Schistosoma haematobium* and 1.5% for *Biomphalaria* spp. with *Schistosoma mansoni*. The *S. mansoni* sequence retrieved from these samples formed a basal clade relative to Zambian isolates, whereas *S. haematobium* grouped with the most frequently characterised haplotype cluster previously identified across mainland Africa. It is important to note that no animal schistosome species were identified in this study. Both the sequences from the snail hosts and the parasites represent novel contributions from the DRC. Additionally, the findings update the current knowledge of schistosomiasis transmission in the Kimpese region by providing insight into the phylogenetic placement, species diversity, and infection status of local snail populations.

Introduction

Schistosomiasis, also known as bilharzia, is one of the major tropical infectious diseases and is caused by trematodes of the genus *Schistosoma*. Worldwide, it is second only to malaria in prevalence and has a significant socio-economic impact in Sub-Saharan Africa (Gryseels *et al.* 2006). It especially affects poor communities lacking adequate sanitation infrastructure and access to safe water, thereby relying on natural and artificial waterbodies that can be contaminated. In Africa, *Schistosoma haematobium* is responsible for causing urogenital schistosomiasis and relies on snails of the genus *Bulinus* as a mandatory intermediate host, whereas *Schistosoma mansoni* causes intestinal schistosomiasis and exploits *Biomphalaria* spp. snails as intermediate host (Dankoni and Tchuenté 2014; Tchuem Tchuenté *et al.* 2017). Chronic infection leads to anaemia, learning disorders, and stunted growth in children, while complications in adults include liver fibrosis and hepatocarcinoma, bladder fibrosis, cancer, and infertility (Gryseels *et al.* 2006). Schistosomiasis is highly endemic in the Democratic Republic of Congo (DRC), with about 11.19 million school-aged children being exposed to schistosomiasis (WHO 2023). Schistosomiasis is present in several health zones, including Kimpese in Kongo Central province (Atila *et al.* 2021; Mbuyi-Kalonji *et al.* 2020). As outlined by the World Health Organization in their 2021–2030 roadmap for eliminating neglected tropical diseases, reducing schistosomiasis transmission necessitates a comprehensive approach, including snail control measures (WHO 2023).

In order to design such effective strategies, a thorough knowledge of the diversity, spatiotemporal distribution, and ecology of snail intermediate hosts is needed. However, a recent systematic literature search revealed a lack of malacological data from the DRC (Kapour *et al.* 2024a). Moreover, in most of the existing studies, snail identification relied solely on morphological

characteristics like the shell shape, which is not sufficient to reliably identify some of the closely related species or juvenile stages (Brown 1994; Jarne *et al.* 2011; Raahauge and Kristensen 2000). There is one study conducted approximately 20 years ago, which employed molecular markers, including internal transcribed spacers 1 and 2 and 16S ribosomal DNA to identify the neotropical *Biomphalaria tenagophila* that was introduced in the Kinshasa area (Pointier *et al.* 2005). The second study identified *Bulinus truncatus* from the Albertine Rift and included *B. truncatus* samples from lakes in the Kivu region in its phylogenetic analysis (Nalugwa *et al.* 2011).

The snail's infectious status is typically determined using the cercarial shedding test or snail dissection, followed by microscopic observation of free-swimming cercariae or sporocysts in the snails' tissues, respectively. Since the morphological diagnostic features are limited, schistosome cercariae cannot be identified up to species level (Atila *et al.* 2021; Bennike *et al.* 1976; Colaert *et al.* 1977; Frandsen 1978), favouring more sensitive molecular assays (Schols *et al.* 2019).

Despite the public health significance of schistosomiasis, only limited molecular data are available from the DRC concerning its primary snail hosts and their associated *Schistosoma* parasites. To date, just 14 relevant intermediate host sequences have been submitted to GenBank (Table 1). The vast majority of these sequences (11 out of 14) originate from eastern DRC, with only three from Kinshasa and none from the Kongo Central region, where the present study was conducted. This highlights a significant spatial bias in sampling and underscores the need for broader molecular surveillance across the country. Regarding *Schistosoma* spp. samples, no publicly available sequences for *S. mansoni* or *S. haematobium* were found at the time of this publication. *Schistosoma intercalatum* was the only species with available sequence data, likely reflecting its endemicity in the DRC, which may have drawn the attention and resources of earlier studies (Sealey *et al.* 2023; Webster *et al.* 2012). The first reported outbreak of *S. intercalatum* in the DRC occurred over 90 years ago in Lualaba Province, specifically in the Yakusu-Kisangani, Ubundu, and Lokandu-Kindu areas in the eastern part of the country (Fischer 1934). In western DRC, an outbreak was also documented in Kinshasa (Tchuem Tchuenté *et al.* 1997).

The absence of reliable information on the distribution and diversity of intermediate host species within DRC's ecosystems presents a significant challenge in the fight against schistosomiasis, particularly for snail control. Therefore, there is an urgent need for new molecular and ecological research to update the species status and their role in schistosomiasis transmission (Kapour *et al.* 2024a). This study is the first to provide genetic sequences of *S. mansoni* and *S. haematobium* from the DRC, along with the associated snail species. Furthermore, it offers an updated overview of the distribution of schistosomiasis intermediate hosts in the Kimpese region, which is known to be endemic to urinary and intestinal schistosomiasis (Mbuyi-Kalonji *et al.* 2020).

Methods

Study site

The Kimpese region is one of the better-documented schistosomiasis-endemic areas in Kongo Central province, located in the western part of the DRC (Lengeler *et al.* 2000). From an administrative standpoint, this region includes villages within the Kimpese health zone, as well as those in the neighbouring health zones of Kwilu-Ngongo (to the east) and Nsona-Mpangu (to the west) (Lengeler *et al.* 2000; Madinga *et al.*

Table 1. Information on publicly available sequences from the Democratic Republic of the Congo (retrieved from GenBank)

Snail or parasites species	No. sequences	Collection site	Genbank reference	Genetic sequences	Source
Bulinus truncatus	1	Lake Kivu	PP510656.1	Partial COX1	Dusabe *et al.* (2024, unpublished)
Biomphalaria tenagophila	3	Mangungu River (Kinshasa)	AY631867.1; AY631861.1; AY631864.1	Partial 18s, Partial 16s, Complete ITS1 and complete ITS2	Pointier *et al.* (2005)
Biomphalaria choanomphala	3	Lake Kivu and Lake Edward	PP510663.1; PP510664.1; PP510672.1	Partial COX1	Dusabe *et al.* (2024, unpublished)
Biomphalaria cf smithii	1	Semliki River	PP510671.1	Partial COX1	Dusabe *et al.* (2024, unpublished)
Bulinus truncatus	1	Lake Kivu	HQ121561.1	Partial COX1	Nalugwa *et al.* (2011)
Bulinus truncatus	1	Unknown	EU076726.1	Partial 16s	Jørgensen *et al.* (2007)
Bulinus sp.	4	Unknown	ON117873.1; ON117850.1; ON112343.1; ON181249.1	Partial COX1, complete ITS2 and partial 5.8s	Tumwebaze *et al.* (2022)
Schistosoma intercalatum	8	Kinshasa	AJ519515.1; AJ519519.1; AJ419779.1; AJ419781.1; AJ519527.1; AJ519529.1; AJ416894.1; AJ416899.1	Partial COX1, partial 12s, partial 28s and partial nd6 gene	Kane *et al.* (2003)
Schistosoma intercalatum	3	Kinshasa	DQ354362.1; DQ354363.1; DQ354364.1	Partial 28s, partial 18s and Partial COX1	Webster *et al.* (2006)
Schistosoma intercalatum	2	Kinshasa	JX844184.1; JX844185.1	Tetraspanin 23 (TSP–23) gene, TSP–23–1 allele, partial cds	Sealey *et al.* (2023)

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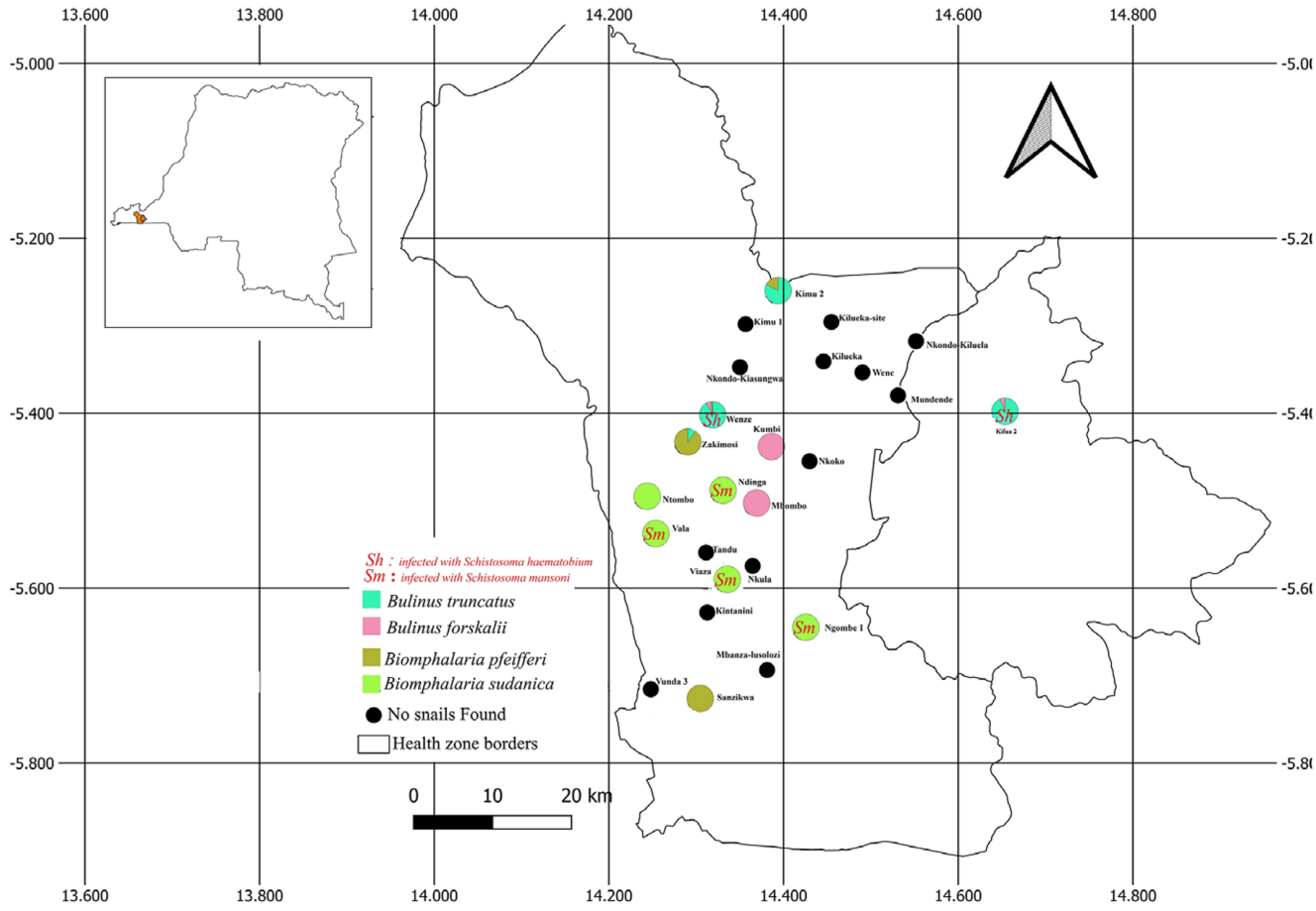

Figure 1. Map of the Kimpese and Kwilu-Ngongo health zones in DRC, showing the locations of villages where the malacological survey was conducted. The locations of specific snail intermediate host species are indicated, along with the respective schistosome infections based on diagnostic PCR assays.

2015). The present study was conducted in 25 villages, comprising 24 in the Kimpese health zone and one in the Kwilu-Ngongo health zone (Figure 1).

Village selection was guided by weekly epidemiological surveillance data (cases and deaths) of schistosomiasis reported by the health workers in the health zone. The health zone is the operational unit of care in the DRC and is subdivided into health areas. Kimpese health zone consists of 20 health areas. The schistosomiasis prevalence in each health area was calculated by dividing the number of reported cases between 2017 and 2019 by the average population of the health area during the same period. Prevalence ranged from 2.47 to 863.91 cases per 10,000 inhabitants. An equidistant discretization method was applied to classify the data into three 'prevalence categories': [2.47-289.62]; [289.63-576.77], and [576.78-863.91] (Kapour 2019). Health areas were then stratified into three endemicity levels: high, medium, and low. Two health areas were randomly selected from each category, resulting in a total of six health areas. Among the 250 villages located within these selected areas, 49 villages were chosen at random. An additional 50th village from the neighbouring Kwilu-Ngongo health zone was included due to its particular characteristics observed by field surveyors, including the presence of snails, its geographical proximity to Kimpese city, and the presence of schistosomiasis as reported in previous studies (De Clercq 1987; Mbuyi-Kalonji *et al.* 2020).

A pilot visit was made to each of these 50 villages to select 25 villages, based on several criteria, including the presence of snails in the water bodies used by the community and accessibility. In total, the 72 most frequently used water points in the 25 villages were included as sampling sites.

Snail collection

Snails were collected monthly from August 2020 to December 2021. At each site, sampling was conducted within a 5-meter radius upstream and downstream (De Clercq 1987). These sites were either rivers, streams, or ponds, but no lakes. The collection was performed manually using circular scoops fitted with a 2-millimetre mesh net attached to a 1-meter handle. Each site was sampled for 15 minutes by two consistent collectors throughout the study period (Bagalwa *et al.* 1996). The manual collection involved actively searching for snails on various surfaces, including submerged aquatic vegetation, dead leaves, discarded fabric, and plastic debris.

Collected snails were sorted, counted, and morphologically identified based on conchological characteristics, such as shell shape, size, coiling, and aperture, using the identification keys developed by Mandahl-Barth and Brown (Brown 1980: Mandahl-Barth 1962). Shell length and width were measured using graph paper. The geographical coordinates of each site were taken and recorded directly using the Kobo Collect application installed on an Android tablet (www.kobotoolbox.org). Specimens were transported in plastic tubes filled with water from the collection site and labelled with the village initial, watercourse, identified species or genus name, contact point number, and the sampling month. Only snails morphologically identified as potential intermediate

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hosts of *Schistosoma* species, those belonging to the *Bulinus* and *Biomphalaria* genera, were retained for further analysis. Non-target snail species were returned to their aquatic environment. Retained specimens were grouped by species, collection date (month and year), and site, and preserved in 70% ethanol in 50-ml tubes. Samples were stored in a refrigeration at the medical parasitology laboratory, Department of Tropical Medicine, University of Kinshasa, and later transferred to the Biology Department of the Royal Museum for Central Africa in Tervuren, Belgium, for molecular analysis.

Snail DNA extraction

DNA extraction was performed on 1,196 snail specimens collected from the field and preserved in ethanol. Before extraction, the workbench was sterilized using a DNA/RNase decontamination solution, and the tools were sterilized using the solution and then flamed over a Bunsen burner flame. The latter was repeated between the extraction of the individual snail specimens. The soft tissues of the snails were carefully separated from the shells, and residual ethanol was removed using absorbent paper. Tissues were then homogenized with a scalpel that was previously sterilized under a Bunsen burner flame to maintain a sterile environment.

Genomic DNA was extracted from the homogenized tissues using the E.Z.N.A.® Mollusc DNA Kit (OMEGA Bio-Tek, Norcross, GA, USA), specifically designed for extracting DNA from mucopolysaccharide-rich tissues. The extraction protocol consisted of two elution steps, each of 75 µl, resulting in a total of 150 µl of DNA extract per sample. DNA extracts were stored at -20°C. Before PCR amplification, DNA samples were diluted 1:10 in Milli-Q water (Merck, Darmstadt, Germany) (Schols *et al.* 2019).

Snail infection status

Infection detection was performed using a two-step approach involving two multiplex polymerase chain reaction (PCR) assays, as previously described by Schols *et al.* (2019). The first assay employed primers targeting 1) snail 18S rDNA as an internal positive control, 2) trematode 18S rDNA to detect general trematode infections, and 3) internal transcribed spacer 2 (ITS2) rDNA specific to *Schistosoma* spp. All samples that tested positive for *Schistosoma* spp. (Figure 2) were further analysed using a second PCR assay to identify medically and veterinary important *Schistosoma* species at the species level. This assay targets the Asmit region of the mitochondrial cytochrome c oxidase subunit I (COX1) gene, using a general forward primer and species-specific reverse primers that generate species-specific amplicon lengths that can differentiate among *S. haematobium*, *S. mansoni*, *S. mattheei*, and the *S. bovis*, *S. curassoni*, *S. guineensis* group using gel electrophoresis. PCR assays were run in a 20-µL reaction volume using the Qiagen™ Taq DNA polymerase kit comprising 0.6 mM dNTP mix, 2 µL of 10x PCR buffer, 1.5 mM MgCl2, 0.12 µL of Taq Polymerase at 5 units/µL, 0.8 µM of each primer, and 2 µL DNA extract (1:10 diluted). The PCR cycling parameters were adapted to the respective primers used following Schols *et al.* (2019); PCR amplifications were performed in a Biometra® Tprofessional Thermal Cycler. The resulting PCR products were separated and visualised using agarose gel electrophoresis with Midori Green Direct® staining using a UV transilluminator (Schols *et al.* 2019).

Sequencing and species identification

Schistosome sequences from four positive snail samples (three snail specimens infected with *S. haematobium* and one with *S. mansoni*) were successfully obtained for the partial COX1 mitochondrial gene using the species-specific primers of the RD-PCR described above (Schols *et al.* 2019). For 26 specimens of the intermediate snail hosts, the HCO2198 LCO1490 primers, which amplify about 650 bp of the COX1 gene, were used to generate the sequences. All sequencing PCRs were conducted using the same cycling conditions as the initial PCR, but in simplex format. The samples were then run on a 1.5% agarose gel for electrophoresis. Amplicons visible on the gel were purified using the ExoSAP (Fermentas™) PCR purification protocol before being sent for Sanger sequencing using BigDye® chemistry, outsourced to Macrogen™ Europe BV.

The obtained sequences were validated, cleaned, and assembled using Geneious software (version R10.0.9). The sequences were subsequently analysed by querying the GenBank database using the Basic Local Alignment Search Tool (BLAST). Publicly available sequences of intermediate host species, as well as *S. mansoni* and *S. haematobium* from other countries, were downloaded and aligned with our sequences to confirm species identity (their accession number can be found in the Results and the respective phylogenetic trees below). Alignments were performed using AliView software (version 1.26) and the MUSCLE alignment algorithm (Edgar 2004). Alignments were trimmed to 337 bp for the *S. mansoni* alignment and 427 bp for the *S. haematobium* alignment. Additionally, a third alignment of 450 bp for the snail dataset was created by including our sequences along with sequences from species from the *Bulinus* and *Biomphalaria* genera, representing the species diversity present in Eastern and Central Africa.

Each alignment was scanned using the online sRNAtoolbox to identify identical haplotypes that were subsequently removed. For the intermediate host, a phylogenetic tree was constructed in

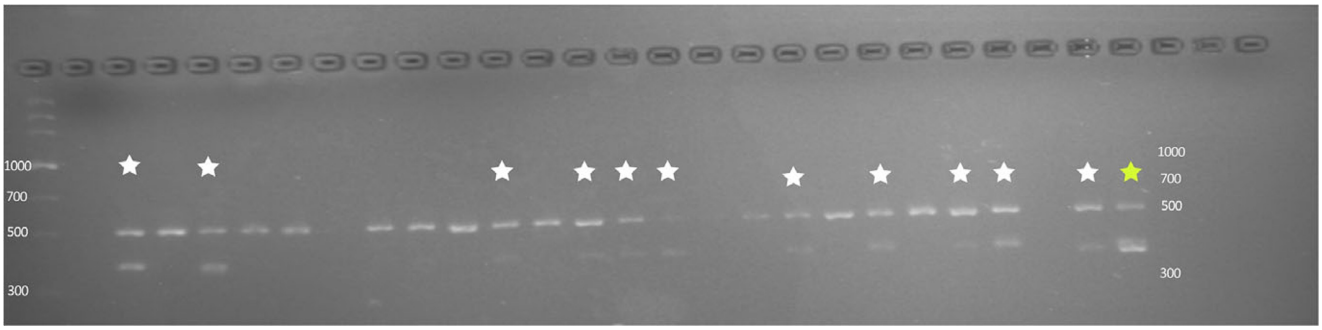

Figure 2. Gel electrophoresis image, showing specimens positive for general trematode infection (white star, amplicon of 392 bp) and for *Schistosoma* spp. infection (yellow star, additional band of 369bp). The first band is the internal control (snail amplicon).

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IQ-TREE based on the alignment of 52 unique COX1 sequences, trimmed to 450 bp (10 of our snail samples and 41 already publicly available sequences), employing the Maximum Likelihood method with 10,000 bootstrap replicates. The GTR+G model was selected as the best-fit evolutionary model based on the Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) values derived from model selection analysis in MEGA X software (Tamura *et al.* 2021). The *S. mansoni* alignment consisted of 120 unique sequences that were aligned and trimmed to a length of 337 bp. This alignment included 118 publicly available sequences from GenBank and our own *S. mansoni* sequence. The second tree focused on *S. haematobium*, based on an alignment of 427 bp, which included 48 unique haplotypes from GenBank, supplemented with our haplotype (the three obtained sequences were identical). Both trees were constructed using the Maximum Likelihood method with 10,000 bootstrap replicates. The HKY+G model was selected for both the *S. mansoni* and *S. haematobium* alignment based on the AIC and BIC values resulting from the MEGA X model selection tool.

Results

Snail species identification

Across the 72 sampling sites in the Kimpese region, a total of 172,491 snails were collected, of which 4,899 (2.8%) were morphologically identified as possible intermediate hosts of *Schistosoma* spp. These included 3,812 (77.8%) belonging to the *Bulinus* genus, and 1,087 (22.2%) to *Biomphalaria*. A total of 2,000 specimens were shipped to Belgium for further analysis, and DNA was successfully extracted from 1,519 specimens, of which 1,196 were confirmed as intermediate hosts: 403 *Bulinus* and 793 *Biomphalaria* specimens. Other species, such as *Gyraulus* and *Amerianna* spp., were also found.

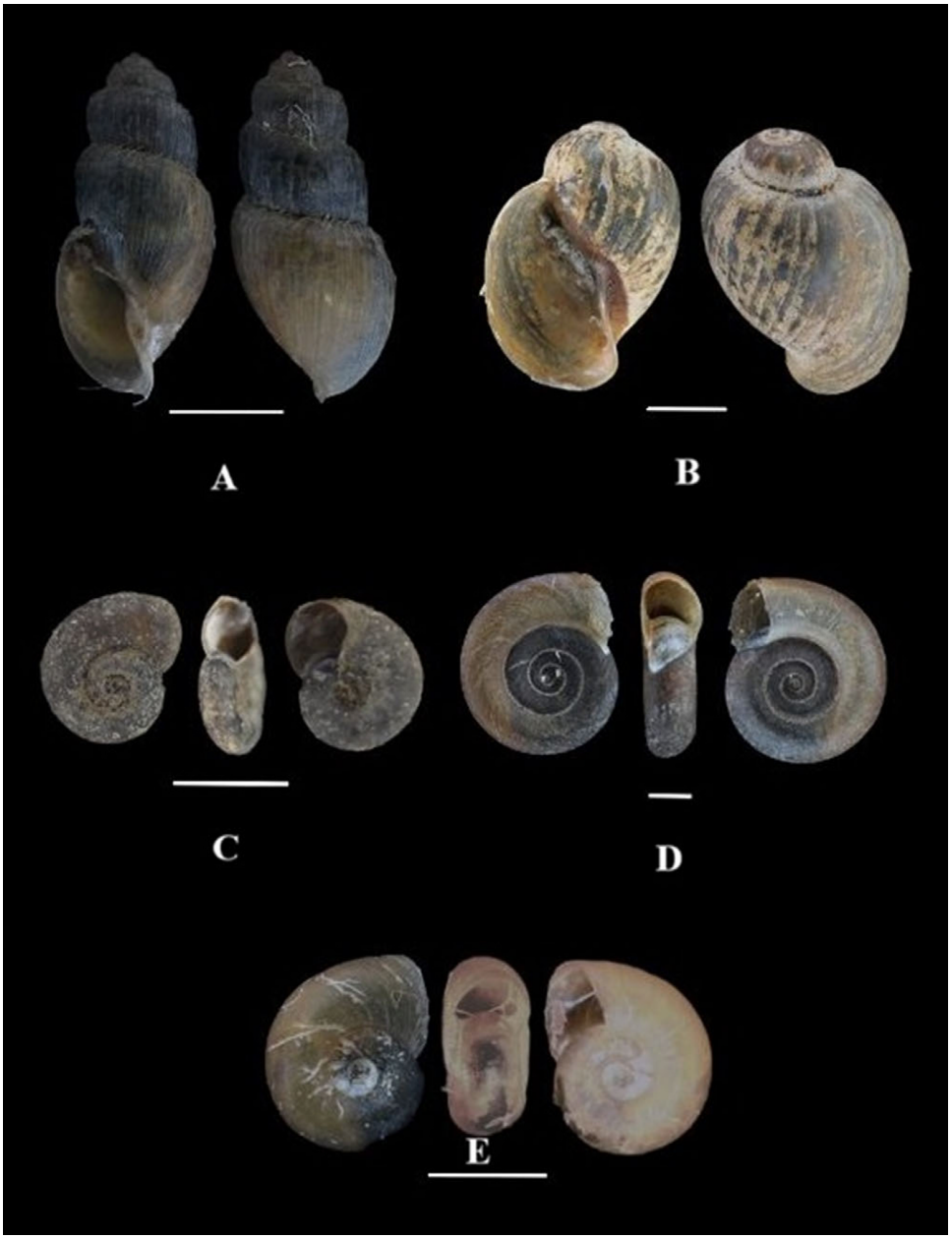

Figure 3. Freshwater snail species found in the Kimpese region. A: *Bulinus forskalii*; B: *Bulinus truncatus*; C: *Gyraulus* sp.; D: *Biomphalaria* cf *sudanica*; and E. *Biomphalaria pfeifferi.*

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We added a high-resolution picture of the *Gyraulus* specimen in Figure 3. The sequence (accession number PV839866) showed a closest hit with *Gyraulus costulatus* from Gabon (96.62% identity), followed by *Gyraulus connollyi* from South Africa (95.82% identity). The exotic *Amerianna* species is described elsewhere (Kapour *et al.* 2024b).

A subsample of 26 individual snail specimens was selected based on morphological identification and processed for COX1 sequencing. This led to 10 unique sequences and the identification of four potential intermediate host species of *Schistosoma* spp., as confirmed by the BLAST queries (Table 2; Figure 3), and the phylogenetic tree (Figure 4): *B. truncatus* (3 sequences), *B. forskalii* (3 sequences), *B. pfeifferi* (2 sequences), and *Biomphalaria* cf *sudanica* (2 sequences; all accession numbers can be found in Table 2). The two *B. pfeifferi* sequences were very similar to each other (p-distance of 0.12%) and clustered with other *B. pfeifferi* species (Figure 3), matching most closely with accession numbers MG431962.1, MG431962.1, and PP510667.1 collected from Kenya and Rwanda. The two *B.* cf *sudanica* sequences (the p-distance between them was 0.37%) clustered in the Nilotic species complex but could not confidently be determined up to species level, given the poorly resolved phylogenetic tree (Figure 4) and the limited power of shell diagnostics. However, given the uniform brown color and the flatter shell and the absence of angulated whorls (Figure 3) (Brown 2005), we tentatively name it *B.* 'cf' *sudanica*. Recent studies suggest that the taxonomy of the Nilotic species complex may require revision; we will go deeper into this in the Discussion. Based on the BLAST query and uncorrected pairwise distances, our two *Biomphalaria* cf *sudanica* sequences are 99.3% similar to each other, and both sequences (Figure 4) were most closely related to *B. choanomphala* from Kenya, *B.* cf *sudanica* from Uganda, and *B. sp.* from Lake Victoria, showing 98.52%, 98.52%, and 99.22% identity, respectively, in the BLAST results, and the lowest pairwise distances 1.26% and 1.01%, respectively—with the Ugandan *B. choanomphala* sample in the phylogenetic tree. Additionally, the pairwise distances among samples within the Nilotic species complex are all below 5%, typically around 1.5%. Values of 1.6% and 1.26% were observed when comparing *B. sudanica* from Uganda to our *B.* cf *sudanica* samples, further highlighting the close genetic relationships within this complex. When compared to *B. choanomphala* previously identified from Lake Kivu in the DRC (Dusabe *et al.* 2024), we obtained pairwise distances of 1.5 and 1.1%, respectively, with our two samples.

We obtained three unique *B. truncatus* sequences that differed from each other with uncorrected pairwise distances of about 1% (data not shown). The sequences clustered within other *B. truncatus* sequences, strongly supporting their species identity. We also found three unique *B. forskalii* sequences. One of them strongly clustered with other *B. forskalii* species of Tanzania, Kenya, and Uganda, with 98.89% and 98.44% identity, the second shared 93.32% identity with the first of our *B. forskalii* sequence, and the third sequence was even more differentiated, with 90.22% and 93.11% shared identity with the other two sequences.

Infection status

Of the 1,519 successfully extracted snails, 1,196 specimens belonging to *Bulinus* or *Biomphalaria* were processed for infection PCR. A total of 37 snails (3.1%) tested positive for *Schistosoma* spp. infection (Table 3), and 15.3% of them were positive for other trematodes. The highest infection rate was found in *B. truncatus* with *S. haematobium* (7.4%), while 1.5% of all *Biomphalaria* species were infected with *S. mansoni*. No infections were found in *B. forskalii* (n=67).

Table 2. Information on the obtained sequences (accession number and BLAST query results: only the closest match is reported)

Own sequences (450bp)				BLAST results				
Accession number	Latitude	Longitude	Species identified	Accession number	Query cover	Percent identity	Scientific name	Origin
PV530231	–5,4236313	14,6431994	*Bulinus truncatus*	MG407342.1	100%	97.33	*Bulinus truncatus*	France (Corsica)
PV530232	–5,4228912	14,3168843	*Bulinus truncatus*	MG407342.1	100%	99.11	*Bulinus truncatus*	France (Corsica)
PV530233	–5,4236313	14,6431994	*Bulinus truncatus*	MG407342.1	100%	99.56	*Bulinus truncatus*	France (Corsica)
PV530237	–5,4091794	14,3529937	*Biomphalaria pfeifferi*	MG431962.1	100%	99.11	*Biomphalaria pfeifferi*	Kenya
PV530240	–5,4228912	14,3168843	*Biomphalaria pfeifferi*	MG431962.1	100%	99.11	*Biomphalaria pfeifferi*	Kenya
PV530241	–5,4991559	14,2449849	*Biomphalaria* cf *sudanica*	MG431964.1	100%	99.22	*Biomphalaria choanomphala*	Kenya
				HM768903.1			*Biomphalaria cf sudanica*	Uganda
				HM769230.1			*Biomphalaria sp.*	Lake Victoria (East Africa)
PV530253	–5,6491800	14,3166382	*Biomphalaria* cf *sudanica*	MG431964.1	100%	98.52	*Biomphalaria choanomphala*	Kenya
				HM768903.1			*Biomphalaria cf sudanica*	Uganda
				HM769230.1			*Biomphalaria sp.*	Lake Victoria (East Africa)
PV530254	–5,4973448	14,2434177	*Bulinus* cf *forskalii*	LT671967.1	100%	97.33	*Bulinus forskalii*	Angola
PV530255	–5,4221592	14,6445125	*Bulinus forskalii*	OP233108.1	100%	98.89	*Bulinus forskalii*	Tanzania
PV530256	–5,5254885	14,3782185	*Bulinus forskalii*	MT272322.1	100%	100	*Bulinus forskalii*	Niger

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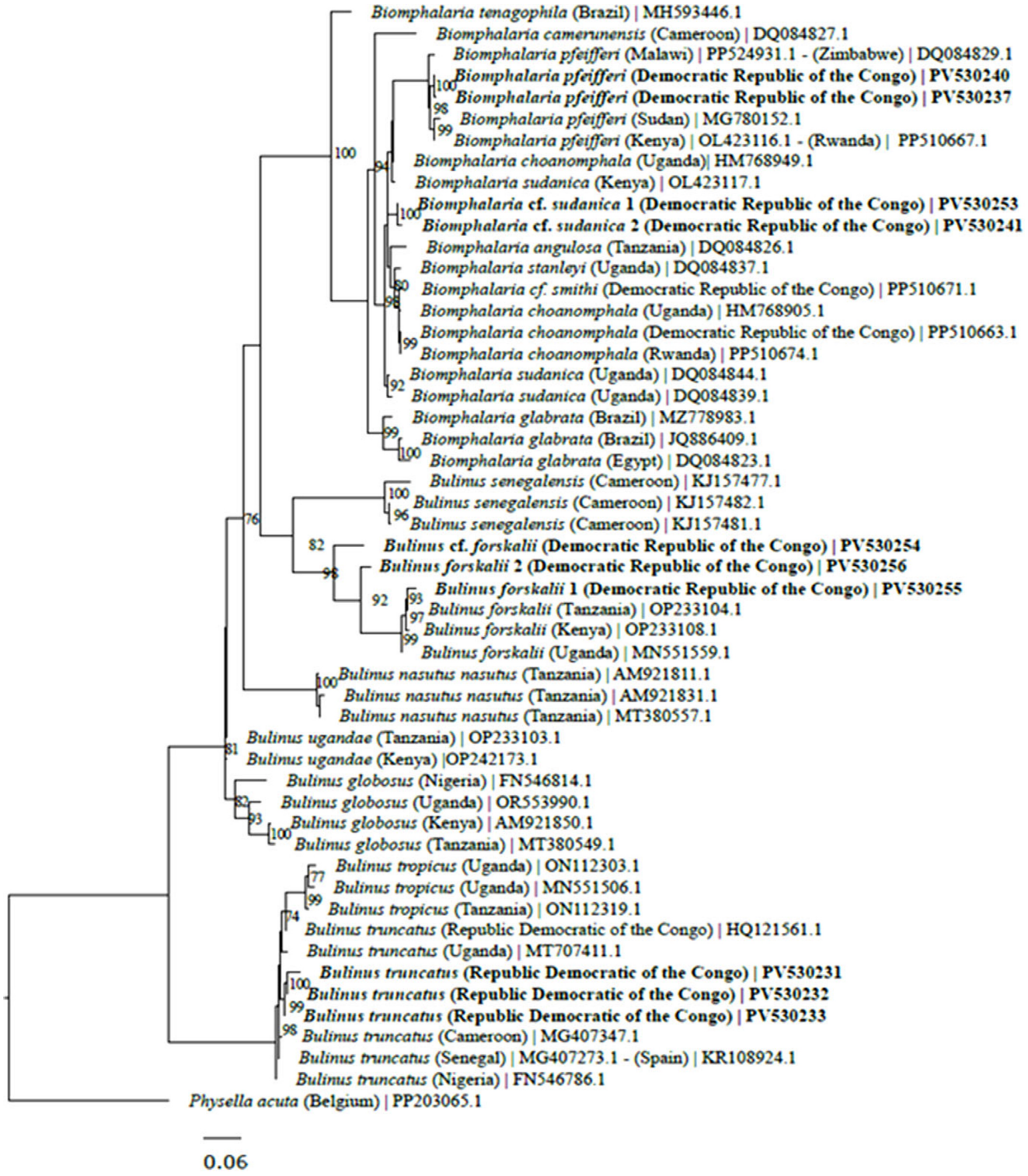

Figure 4. Phylogenetic tree of intermediate host species based on COX1 sequences (450 bp), constructed using maximum likelihood and the General Time Reversible + gamma (gamma = 0.286) model. Nodal support is indicated as bootstrap percentages when it equals or exceeds 70 (10,000 bootstrap replicates). Sequences retrieved from GenBank are shown with their accession numbers, sampling location, and species names. Sequences from this study are written in bold; their accession numbers can be found in Table 1. *Physella acuta* was used as an outgroup.

Genetic profile of *Schistosoma* specimens

The profile of our two *Schistosoma* haplotype sequences was analysed through phylogenetic analysis to determine their genetic position within the haplotype network of African specimens. The Congolese *S. mansoni* sequence (accession number PV528764) clustered within the East African haplotype group (designated Group 3 *sensu* Webster *et al.* (2013); Figure 5). The *S. haematobium* haplotype (all three sequences were identical; accession number PV528767) clustered within the overall African haplotype group, which was characterised by Webster and colleagues as the H1 cluster in their study (Webster *et al.* 2012) (Figure 6).

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Table 3. Prevalence of schistosome infection by snail species as identified with the Rapid Diagnostic PCR

Genus	Snail species	*Schistosoma* spp.	Total tested	Positive	% of infection
Bulinus	*Bu. truncatus*	*S. haematobium*	336	25*	7.4
	Bu. forskalii		67	0	0.0
Biomphalaria	*Biomphalaria* cf *sudanica*	*S. mansoni*	793	12**	1.5
Total	**All**		**1,196**	**37**	**3.1**

*24 samples from Wenze village and 1 from Kifua II village.
**6 samples from Ngombe village, 2 from Ndinga, 1 from Valla, and 3 from Viaza

Distribution of infection in schistosome intermediate hosts

The distribution of snail intermediate hosts of schistosomes is heterogeneous in the study area, as shown in Figure 1. *Biomphalaria* cf *sudanica* was most widespread (present in five sites, where no other snail intermediate hosts were found), while the other species were restricted to three sites, sometimes overlapping in distribution.

Six sites harboured schistosome-infected snails, including two sites with *B. truncatus* infected with *S. haematobium*, with the majority of specimens (24/25) found in one site, the Fwamaza River in the village of Wenze. Four of the five sites with *B.* cf *sudanica* harboured snails that were infected with *S. mansoni*, as shown in Table 4.

Discussion

This study aimed to identify the *Schistosoma* and snail species involved in the transmission of schistosomiasis in the Kimpese region. A total of at least four intermediate snail host species were identified: *Bulinus forskalii*, *Bulinus truncatus*, *Biomphalaria pfeifferi*, and *Biomphalaria* cf *sudanica*. Among these, *B. forskalii*, *B. truncatus*, and *B. pfeifferi* have already been documented in the Kimpese region (Atila *et al.* 2021) and other localities in the same province of Kongo Central (De Clercq *et al.* 1985; Frandsen 1979), but all these studies were based solely on morphology. In contrast, *B.* cf *sudanica* or any other species of the Nilotic species complex (including *Biomphalaria choanomphala*, *Biomphalaria sudanica*, *Biomphalaria alexandrina*, and *Biomphalaria smithi*) has not yet been reported from any schistosomiasis-endemic area in the DRC. Consequently, our study is, to our knowledge, the first report of the presence of species belonging to the *Biomphalaria* Nilotic species' complex in the Kongo central region of the DRC. This finding is thus very unexpected for two reasons. Firstly, early malacological surveys (De Clercq 1987) reported only *B. pfeifferi* and *B. camerunensis* in the western Congo Basin, with the latter being completely replaced by the former by the 1980s. Next to that, the distribution of the Nilotic species complex was expected to closely follow the Nile drainage system (Brown 2005). However, *B. choanomphala* was recently reported in the northeastern region of the DRC by means of genetic analyses (Dusabe *et al.* 2024), confirming an earlier morphological study by Chartier *et al.* (1993), extending the known species range. Dusabe *et al.* (2024) raise the possibility of either an overlooked broader range or a recent anthropogenic introduction.

Despite the wide geographic separation, our two *B.* cf *sudanica* specimens differed from *B. choanomphala* from Lake Kivu by only 1–1.5% p-distance, with just 9 and 12 mutations across a 450 bp fragment. This low divergence mirrors previous studies showing that genetic data do not reliably separate members of the Nilotic complex—including *B. sudanica*, *B. choanomphala*, and *B. smithi*—into discrete taxa. Indeed, there is a longstanding debate over whether *B. sudanica* and *B. choanomphala* should be considered distinct species or ecophenotypes. Traditional malacological literature treated them as separate based on habitat and shell morphology: *B. sudanica* inhabiting shoreline marshes with flatter, larger shells, and *B. choanomphala* occupying deeper lake zones with smaller, more conical shells and angular whorls (Brown 1994; Mandahl-Barth 1958). However, mitochondrial genome and rDNA sequencing found 98.3% nucleotide identity between *B. sudanica* and *B. choanomphala*, greater than observed between different *B. glabrata* strains, suggesting they may represent eco-phenotypic variants of a single species (Zhang *et al.* 2018). Standley *et al.* (2011) proposed that the two taxa should be treated as subspecies, based on mitochondrial COX1 and ITS2 data and the presence of intermediates, reinforcing the ecotype hypothesis. Pennance *et al.* (2024) further supported this by showing that both shoreline and deep-water forms from Lake Victoria shared ancestry and immune-associated loci that strongly influence schistosome susceptibility regardless of shell phenotype or microhabitat.

As such, we cannot confidentially identify our specimens to species level, which is also reflected in our unresolved phylogeny (Figure 4). But based on its shell morphology (Figure 3), especially the flat shell, the overall brown colour, and the absence of angulated whorls (Brown 2005), we tentatively name it *B.* cf *sudanica*. Integrating detailed morphometric and morphological analysis (including the internal organs) with multilocus sequencing of all members of the Nilotic complex will help to clarify this matter.

With respect to the *Bulinus* species, our Congolese *B. forskalii* sequences appear basal to the East African lineages (Figure 4), while the Congolese *B. truncatus* sequences cluster firmly together but within the larger monophyletic *B. truncatus* clade. It should be noted that the genetic diversity within *B. forskalii* is rather high, with the highest p-distance exceeding 5%. Such a high figure can be indicative of the existence of another closely related species, but also here, future morphological and molecular research is needed to address this question.

Another interesting result is the absence of *Bulinus globosus*, a species previously described in the Kimpese region by two earlier studies (Atila *et al.* 2021). Regarding identification methods, the aforementioned studies primarily used morphological identification, whereas our study employed both morphological and molecular identification. In environments characterised by high species diversity, relying solely on morphological identification can lead to misclassification of certain species (Jarne *et al.* 2011; Maes *et al.* 2021; Raahauge and Kristensen 2000). A more comprehensive understanding of the malacological fauna in this region requires covering a variety of ecological zones, collecting data at different times of the year, and utilising molecular techniques to confirm or refute the presence of *B. globosus* and possibly other potential

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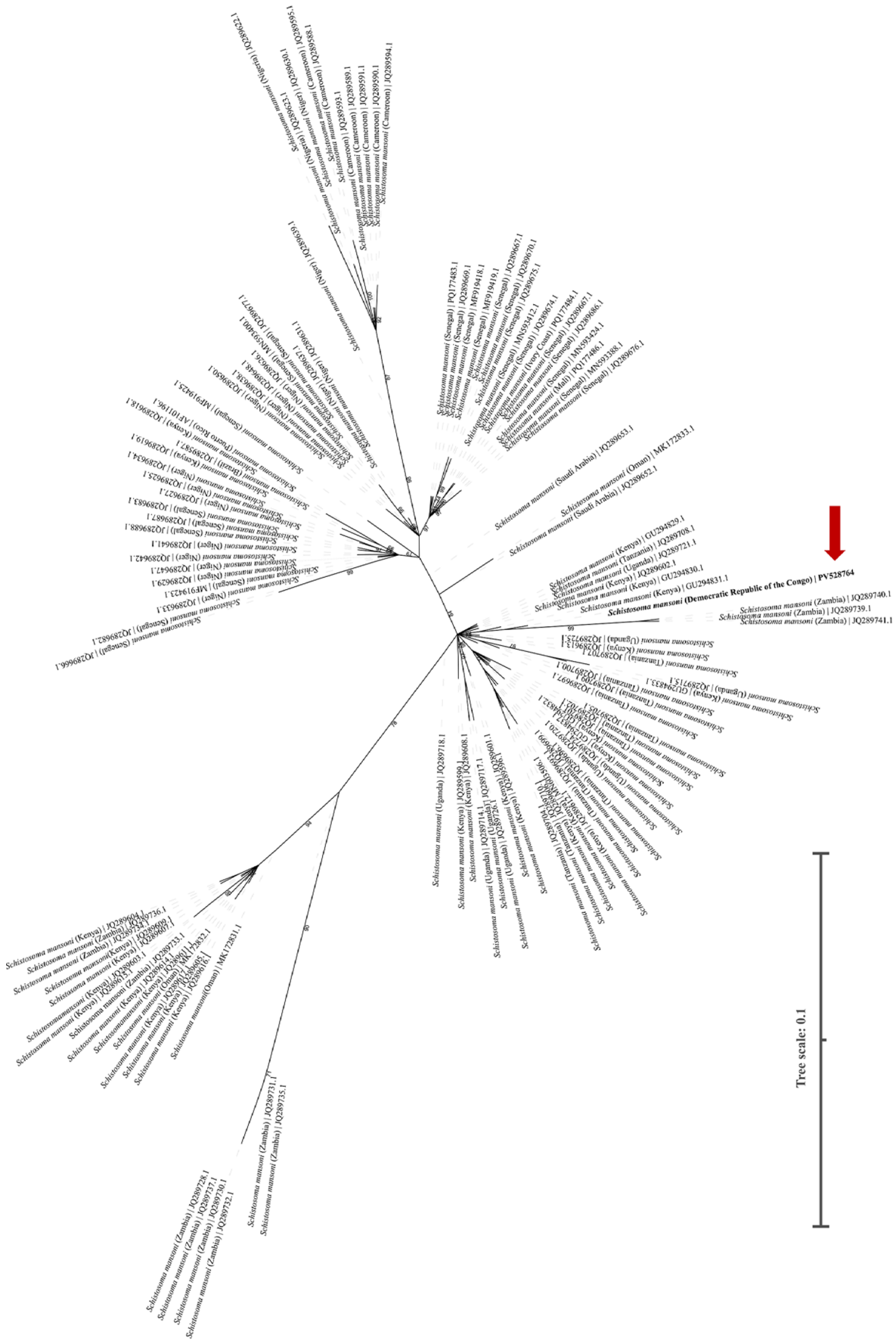

Figure 5. Phylogenetic tree of *Schistosoma mansoni* sequences based on COX1 sequences (337 bp), constructed using the maximum likelihood method and the Hasegawa-Kishino-Yano + gamma (gamma = 0.210) model. Nodal support is indicated as bootstrap percentages when it equals or exceeds 70 (10,000 bootstrap replicates). The sequence from this study is indicated with a red arrow.

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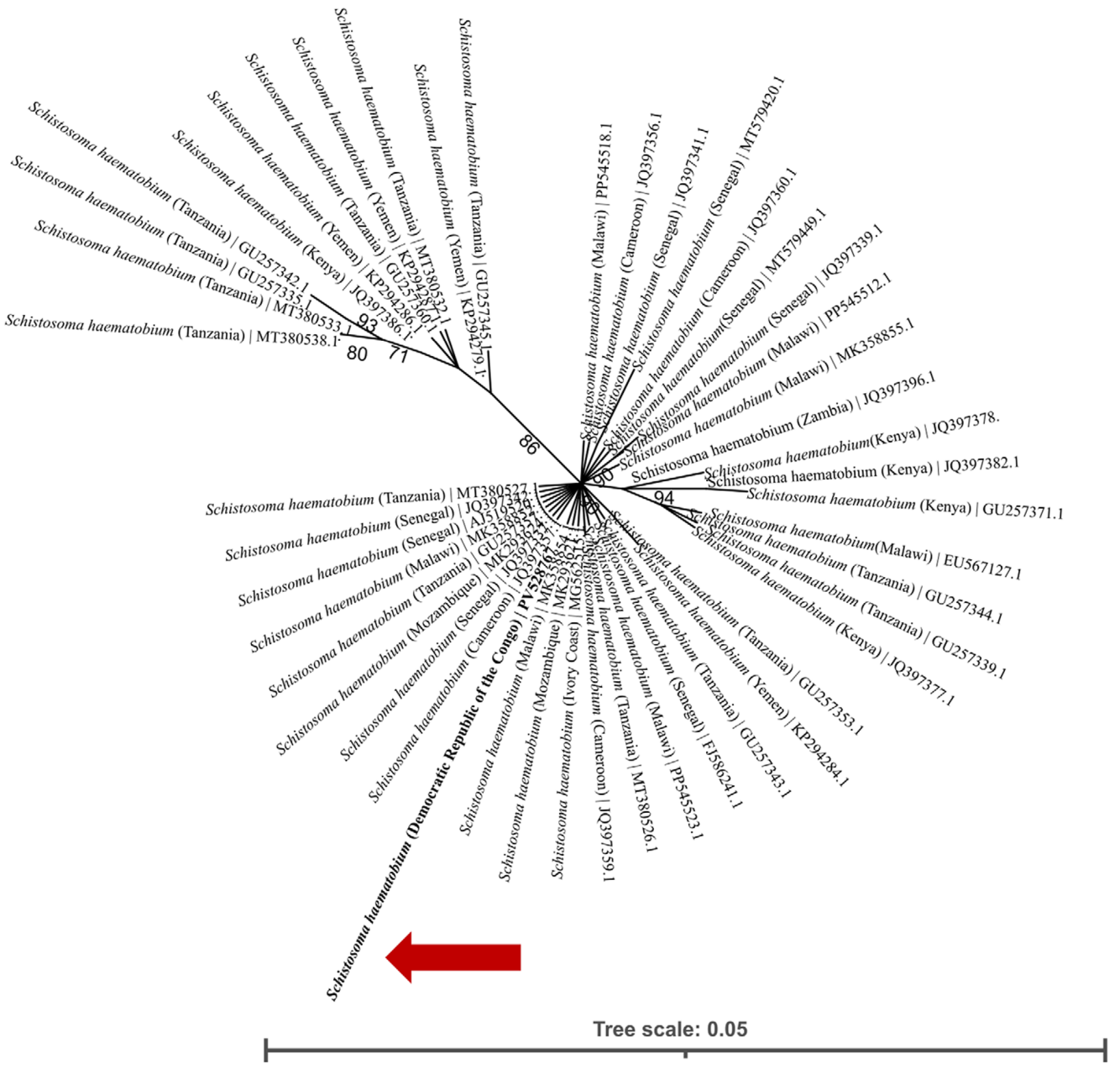

Figure 6. Phylogenetic tree of *Schistosoma haematobium* sequences based on COX1 sequences (427 bp), constructed using the maximum likelihood method and the Hasegawa-Kishino-Yano + gamma (gamma = 0.1524) model. Nodal support is indicated as bootstrap percentages when it equals or exceeds 70 (10,000 bootstrap replicates). The sequence from this study is indicated with a red arrow.

intermediate host species. Also, Jarne *et al.* (2011) plea for an integrative approach combining molecular, morphological, ecological, and reproductive data to clarify species limits, especially within the *Biomphalaria* genus. Nevertheless, this study can serve as a baseline for future malacological surveys.

A total of 1,196 snails were tested for trematode infection using a rapid diagnostic PCR assay. Out of these snails, 37 were infected with schistosomes, resulting in an overall infection rate of 3.1%, and 15.3% of them were positive for other trematodes. That means a high prevalence of trematodes compared to schistosomes in snails may slow the transmission of schistosomiasis through competition and antagonism mechanisms (Laidemitt *et al.* 2019). This infection rate appears to be lower than those reported in previous studies in the DRC, with 11% found in Kimpese by Atila *et al.* (2021) and 7.27% in creeks and 3.79% in ponds in Lwiro (Bagalwa 1998). It is important to note that these studies used the cercarial emission test, complicating direct comparisons with our study. Also, our sampling design intentionally included endemic and non-endemic areas for comparative analysis, diluting the infection signal, whereas the cited studies probably only focused on the endemic areas. Nevertheless, our results confirm the active circulation of *Schistosoma* parasites at the studied sites despite the ongoing mass drug administration (MDA) program. This can be explained by the lack of adequate sanitary facilities in the study area (Madinga *et al.* 2017), which contributes to water contamination by untreated, infected individuals and leads to reinfection of children after MDA campaigns. However, given that infected snails remain infectious throughout their life span, perpetuating transmission, vector

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Table 4. Distribution of infected snail specimens in Kimpese DRC by collection site

Village	River	LAT	LONG	Bu. truncatus	Bi. cf sudanica
Kifwa 2	Tiki	–5.4221592	14.6445125	0	0
Kifwa 2	Ngongo	–5.4237012	14.6431456	1	0
Kifwa 2	Ngongo	–5.4236313	14.6431994	0	0
Kimu 2	Nduka	–5.374791	14.3091988	0	0
Kumbi	Bobo	–5.49734483	14.24341773	0	0
Kumbi	Kyungulu	–5.49469669	14.23936702	0	0
Kumbi	Nkaku	–5.49915591	14.2449849	0	0
Mbombo	Mbombo	–5.5254885	14.3782185	0	0
Ndinga	Muzala	–5.461279	14.3618196	0	2
Ngombe 1	Lusolozi	–5.6468814	14.45789897	0	6
Ntombo	Ntombo	–5.5374546	14.2317194	0	0
Sanzikwa	Zakiamundele	–5.7064579	14.2372675	0	0
Sanzikwa	Sanzikwa	–5.7105965	14.2358921	0	0
Vala	Nkazu	–5.5485979	14.2543855	0	1
Viaza	Mpanganzi	–5.64918	14.3166382	0	0
Viaza	Nkama	–5.657692	14.3118003	0	3
Wenze	Fwamaza	–5.42289123	14.31688426	24	0
Zakimosi	Zakimosi	–5.40917942	14.35299371	0	0
Zakimosi	Lubi	–5.41398557	14.34712291	0	0
TOTAL				**25**	**12**

control, and health education should be implemented alongside MDA in the study area.

It is also crucial to note that the absence of a schistosome intermediate host does not necessarily indicate the absence of schistosomiasis. For instance, no *Biomphalaria* specimens were found in the village of Kifua 2, despite it being known to be endemic for *S. mansoni* (Mbuyi-Kalonji *et al.* 2020). This underscores the need to enhance and extend malacological surveys to include all habitat types and possible human water contact sites to improve the resolution of schistosomiasis risk maps.

The distribution of infected snails was very heterogeneous, with almost all infected *B. truncatus* found in a single site in Wenze village, which was intensely used by the community for washing and bathing. The infected *Biompharia* specimens were found in four sites, spread over four villages, in slow-flowing sections of the river. The community uses the river for bathing, washing up, and laundry. Domestic animals are prevented from accessing it by wooden fences on the path leading to the area. This might be one of the reasons why no animal schistosome species were identified in this study. This may also be linked to the absence of intensive cattle farming in the area, although goats and sheep are present. Livestock watering points are typically located farther from the villages, and some key human water contact sites are physically shielded from animal access, which might be an efficient strategy.

The four infected sites contained snails of the *Biomphalaria* Nilotic complex. Those sites have been characterised by more or less fast-flowing rivers, with adjacent pockets of stagnant water that could shelter snails, with a substrate of organic matter and turbid-looking water. These characteristics are similar to the habitat description for this species (Diaw *et al.* 1999).

Finally, the results of this study provide the first molecular data on *S. mansoni* and *S. haematobium*, the two main species responsible for schistosomiasis in the DRC. Also, *S. intercalatum* is endemic in the DRC, but it is much less prevalent and was not found in this study. Our Congolese *S. mansoni* haplotype clustered together with East African lineages, and not with those from Senegal, Niger, and Cameroon. Regarding *S. haematobium*, our haplotype appeared to belong to the predominant H1 group (Webster *et al.* 2013), which lacks any geographic structuring. This is consistent with the low genetic diversity observed across the African mainland for *S. haematobium*, which Webster and colleagues hypothesised to result from an evolutionary bottleneck event. Our Congolese sample seems to support this hypothesis, although more samples from different geographic regions in the DRC should be included.

Conclusion

This study explores the diversity of intermediate host snail species in the Kimpese region and presents the first genetic sequences of both *S. haematobium* and *S. mansoni* in this area, along with the first determination of their evolutionary relationships within the African diversity of the respective species. Additionally, the identification of a *Biomphalaria* species of the Nilotic species complex in Kimpese marks a first record for the Kongo Central region. However, there is a clear need for integrative taxonomy to resolve cryptic diversity and ecophenotypic plasticity within this group of freshwater snails. More studies are needed to fully capture the genetic diversity and exact species status of some of these species by

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increasing sampling effort in space and time, and combining morphological, ecological, and molecular analyses.

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