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MOLECULAR CHARACTERISATION OF *BULINUS* SNAILS – INTERMEDIATE HOSTS OF SCHISTOSOMES IN OGUN STATE, SOUTH-WESTERN NIGERIA

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ABSTRACT: Freshwater snails of the genus *Bulinus* O. F. Müller, 1781 are intermediate hosts for schistosomes, trematode parasites which cause schistosomiasis. The genus includes closely related species complexes with restricted gene flow between populations of each taxon. Despite their importance as intermediate hosts, unambiguous identification of these snails remains challenging. We applied molecular approach to their identification to achieve a better understanding of the epidemiology of schistosomiasis in an endemic region, south-western Nigeria. A total of 149 snails were collected and their genomic DNA was screened for schistosome infection using PCR amplification of schistosome *Dra*I repeat sequence. The snails were identified by PCR-RFLP and/or sequencing of an amplicon of their entire ITS region including the 5.8S ribosomal RNA (rRNA) gene. Four *Bulinus* species, namely *B. globosus* (Morelet, 1866), *B. forskalii* (Ehrenberg, 1831), *B. camerunensis* Mandahl-Barth, 1957 and *B. senegalensis* O. F. Müller, 1781 were identified, and 34.9% (n = 52) of the 149 snails were infected: *B. globosus* 25.5% (n = 38), *B. forskalii* 5.4% (n = 8), *B. camerunensis* 2.7% (n = 4) and *B. senegalensis* 1.3% (n = 2). Restriction fragment profiles of the ribosomal ITS region for *B. globosus* closely matched those obtained in our previous study thus confirming the view that ribosomal ITS region of these snails could be well suited for taxonomic studies. The use of sequencing for species identification was costly and time-consuming, but it was effective in resolving true identities of snails whose restriction profiles were similar and inconclusive.

KEY WORDS: species identification, *Bulinus*, ITS, *rRNA* gene, schistosome, schistosomiasis, Nigeria

INTRODUCTION

Schistosomiasis, an infection caused by blood flukes of the genus *Schistosoma* Weinland, 1858, is endemic in Nigeria (AKOGUN & AKOGUN 1996, MAFE et al. 2000). Both *Schistosoma haematobium* (Bilharz, 1852) and *S. mansoni* Sambon, 1907 infections occur in all the states of the federation (EJEZIE et al. 1989, MAFE et al. 2000, OFOEZIE 2002, OGBE 2002), and an estimated 33.5 million people, mainly children aged 5–19 years, are at risk (HOTEZ & KAMATH 2009). Freshwater pulmonates of the genera *Bulinus* O. F. Müller, 1781 and *Biomphalaria* Preston, 1910 act as intermediate hosts for the parasites; the snails

occur commonly throughout much of Africa and its adjacent regions. Their focal distribution has been reported in many parts of Nigeria (OKWUOSA & UKOLI 1980, IMEVBORE et al. 1988, NDIFON & UKOLI 1989, OFOEZIE 1999, OWOJORI et al. 2006) where *Biomphalaria pfeifferi* (Krauss, 1848), *Bulinus globosus* (Morelet, 1866) and *B. truncatus* (Audouin, 1827) were recorded from diverse freshwater habitats. Urinary schistosomiasis is caused by *S. haematobium*, with some snails of the genus *Bulinus* acting as the intermediate hosts. Among the intermediate hosts of *S. haematobium, B. globosus* was reported to be widely distributed and common while *B. truncatus* was rare. IMEVBORE et al. (1988), NDIFON & UKOLI (1989), OFOEZIE (1999), NALE et al. (2003) and OWOJORI et al. (2006) reported the presence of *B. globosus* and *B. truncatus* in the River Kibanni, at Zaria, north-western Nigeria. BETTERTON et al. (1988) investigated 165 freshwater habitats throughout Kano state, north-western Nigeria, and recorded a number of potential intermediate host species: *Bulinus senegalensis* O. F. Müller, 1781, *B. forskalii* (Ehrenberg, 1831), *B. globosus*, *B. truncatus* and *Biomphalaria pfeifferi*; the most widespread was *B. senegalensis*. NTONIFOR & AJAYI (2007) recorded five medically important snail species comprising *B. globosus*, *B. truncatus*, *B. forskalii*, *B. pfeifferi*, and *Radix natalensis* (Krauss, 1848) in two Local Government Areas of Bauchi State, north-eastern Nigeria. It is noteworthy that in all these studies the snail identification was based on morphological characters.

Correct identification of *Bulinus* species based on morphology alone may be difficult (RAAHAUGE et al. 1984), since the morphological distinctiveness within these species groups is limited. There are 37 recognised species of the genus *Bulinus* divided into four major species groups, namely *B. forskalii*, *B. africanus*, *B. truncatus/tropicus* complex and *B. reticulatus* (BROWN 1994). Within each of these groups, there are species that act as intermediate hosts for schistosomes in part or all of their geographical range, while the specificity of the snail-parasite interaction is such that only certain species are involved in transmission of the parasite (ROLLINSON et al. 1998). Since the interaction between schistosomes and snails is very specific, and compatibility may differ over fairly small geographical ranges, a reliable taxonomy of the genus *Bulinus* is a prerequisite for understanding the epi-

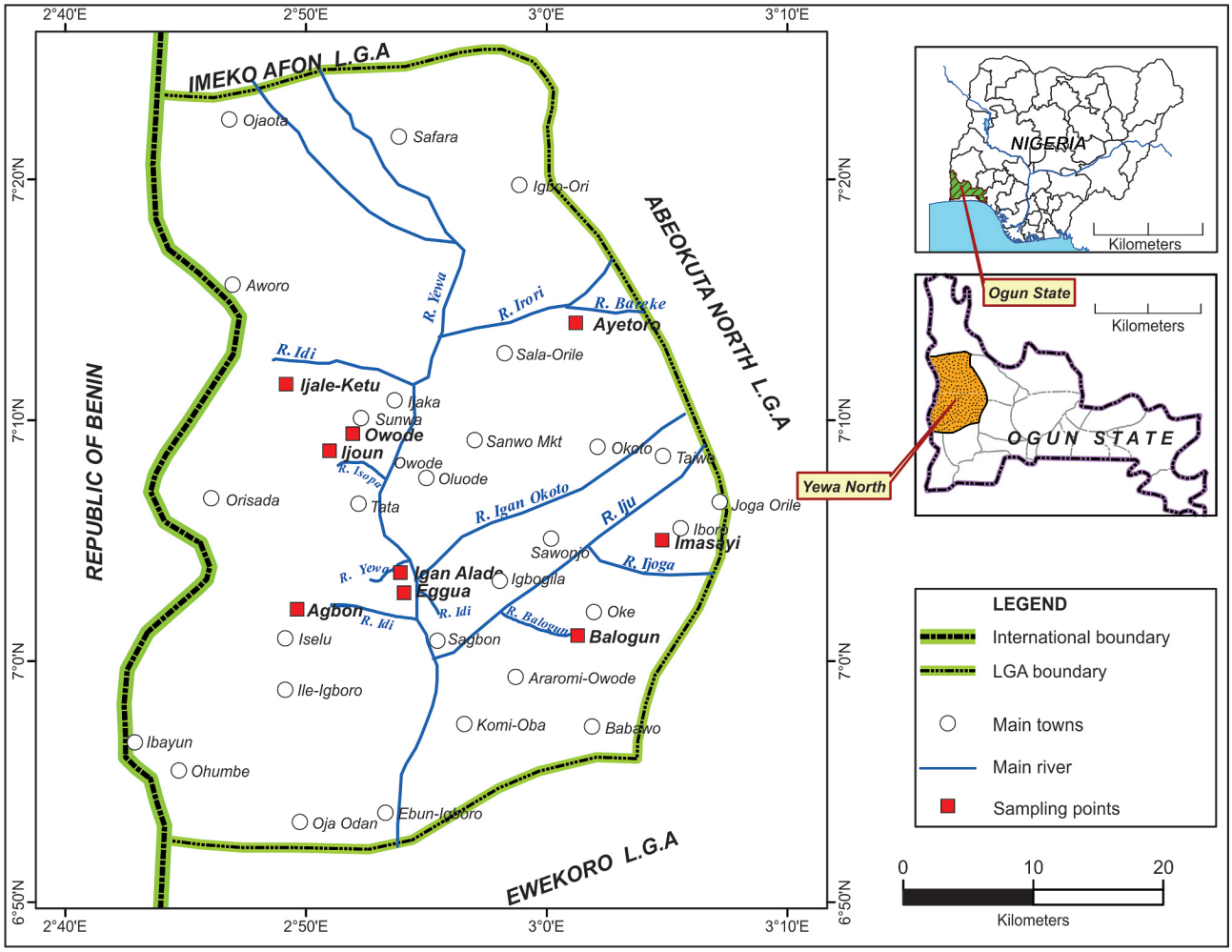


Fig. 1. Map of Yewa North LGA, Ogun state, Nigeria, showing the study areas

demiology of schistosome infection. Consequently, considerable efforts have been focused on providing reliable methods for their differentiation and identification in order to specify the species and strains playing a major role in the disease transmission. The studies of ploidy (GOLDMAN et al. 1983) and allozymes (ROLLINSON & SOUTHGATE 1979, NASCETTI & BULLINI 1980, MIMPFOUNDI & GREER 1989) have played an increasing role in *Bulinus* species identification in addition to enzyme electrophoresis (BIOCCA et al. 1979, JELNES 1979, 1986, ROLLINSON & SOUTHGATE 1979, NJIOKOU et al. 1993). Various molecular methods such as PCR amplification of the snails' ribosomal ITS region and restriction fragment length polymorphism (RFLP), the use of randomly amplified polymorphic DNA (RAPDs), ITS2 secondary structure and sequence analysis of mitochondrial genes (SIMPSON et al. 1984, ROLLINSON & KANE 1991, JOHNSTON et al. 1993, KANE & ROLLINSON 1994, STOTHARD et al. 1996, 1997, 2001, STOTHARD & ROLLINSON 1997, RAAHAUGE & KRISTENSEN 2000, JONES et al. 2001, JØRGENSEN et al. 2007, 2011, 2013, KANE et al. 2008, AKINWALE et al. 2011, NALUGWA et al. 2011, NYAKAANA et al. 2013) have recently been applied to *Bulinus* species identification. This study therefore aimed to characterise *Bulinus* species involved in schistosome transmission in an endemic region, south-western Nigeria through PCR-RFLP and/or sequencing of amplicons of the snails' entire ribosomal ITS region.

AREAS STUDIED

Five rivers running through nine rural communities in Yewa North Local Government Area (LGA) of Ogun state, south-western Nigeria (Fig. 1), were surveyed for freshwater snails of the genus *Bulinus*. The main occupations of the people in the area (Table 1) are fishing and farming. They suffer from endemic urinary schistosomiasis. Yewa North LGA, located at 7°15'N and 3°03'E in a deciduous/derived savannah zone, has the largest land area in the state (ONAKOMAYA et al. 1992).

Table 1. Geographical coordinates, sample origins, number of snails collected and prevalence of schistosome infection

	Location	River	GPS coordinates	No. of snails screened	No. infected	Prevalence of infection (%)
1	Imasayi	Iju	7°05'N, 3°05'E	34	7	20.6
2	Eggua	Idi	7°00'N, 2°54'E	3	3	100.0
3	Ijale Ketu	Idi	7°11'N, 2°49'E	10	8	80.0
4	Balogun	Balogun	7°02'N, 3°01'E	5	2	40.0
5	Agbon	Idi	7°02'N, 2°50'E	5	3	60.0
6	Ijoun	Yewa	7°08'N, 2°51'E	10	10	100.0
7	Ayetoro	Bareke	7°12'N, 3°12'E	34	8	23.5
8	Owode	Yewa	7°07'N, 2°53'E	42	8	19.0
9	Igan Alade	Yewa	7°03'N, 2°54'E	6	3	50.0
				149	52	

MATERIAL AND METHODS

A total of 149 snails morphologically conforming to the genus *Bulinus* were collected between March and May 2013 from five rivers. Human-water contact sites in the rivers were located with the aid of GPS (Magellan Explorist 310, MiTAC Digital corporation, CA 95050 USA) and were searched for *Bulinus*. Sampling was carried out along the shoreline of each contact site using a standard snail scoop net by the same collector: 30 passes of the net were supplemented with manual search for 30 minutes according to the method of OLOFINTOYE & ODAIBO (1999). Snails attached to macrophytes were collected manually. The snails were transported to the laboratory in pre-labelled plastic containers, rinsed, sorted and counted. Each snail was identified to species based on its morphological characters according to BROWN (1994), and then preserved in 70% ethanol.

Genomic DNA was extracted from each snail following a modified method of STOTHARD et al. (1996). Each snail was removed from the 70% ethanol and soaked in TE buffer, pH 7.4 (10 mM Tris HCl and 1 mM EDTA) overnight so as to remove residual ethanol. Tissue from each snail was placed in a sterile 1.5-ml microcentrifuge tube, with 500 μl of CTAB solution (0.2% 2-mercaptoethanol, 2% hexadecyltrimethyl-ammonium bromide (CTAB), 100 mM Tris (hydroxymethyl) amino-methane, 16 mM EDTA, 1.4 M sodium chloride) and the tissue homogenised. Proteinase K solution (10 μl at 20 mg/ml) was added and the digests were incubated at 55°C for 1 hour,

with intermittent gentle mixing. Genomic DNA was extracted from the digests by adding an equal volume of chloroform : isoamyl alcohol (24 : 1) to each tube. The organic and aqueous layers were gently mixed for 5 minutes and centrifuged at 13,000 g for 20 minutes. From each sample, the supernatant was removed into another sterile micro centrifuge tube and an equal volume of 100% ethanol was added, mixed, and the sample was incubated at –20°C overnight so as to enhance DNA precipitation. The precipitate was centrifuged at 13,000 g for 20 minutes and the pellet washed with 70% ethanol and centrifuged for another 20 minutes. The supernatant was removed and the pellet was dried at room temperature. When completely dry, the pellet was re-suspended in 50 μl of double distilled water.

All 149 snails were screened for schistosome infection by PCR amplification of the *Dra*I repeat using the forward primer, Sh1, 5'-GATCTCACCTATCAGACGAAAC-3' and reverse primer, Sh2, 5'-TCACAACGATACGACCAAC-3' following the method of HAMBURGER et al. (2001). The amplified products were visualised on 1.5% agarose gel and then photographed. PCR amplification of the entire ribosomal ITS region including the 5.8S rRNA gene was performed on all 149 snails following the methods of KANE & ROLLINSON (1994). The sequences for the primers used for the amplification were based on conserved regions of the 3' end of the 18S rRNA gene (ETTS1) and the 5' end of the 28S rRNA gene (ETTS2). The primers were ETTS1 (5'-TGCTTAAGTTCAGCGGGT-3') and ETTS2 (5'-TAACAAGGTTTCCGTAGGTGAA-3'). Cycling parameters were 95°C for 4 minutes, followed by four cycles of 95°C for 15 seconds, 48°C for 15 seconds and 72°C for 1 minute and then a further 41 cycles of 95°C for 15 seconds, 53°C for 45 seconds and 72°C for 1 minute, with the final extension period of 72°C for 3 minutes. The amplification was confirmed through visualisation on 1.5% agarose gel.

RFLP analysis was performed according to the method of STOTHARD et al. (1996). Briefly, the PCR amplified products were digested with *Rsa*I, a 6-base cutting restriction enzyme, in a digestion reaction consisting of 10 μl PCR product, 1.75 μl 10 × buffer, 0.75 μl enzyme, 0.5 μl bovine serum albumin (BSA) and 2 μl H_2O in a final volume of 15 μl. The digestion mixture was incubated for 30 minutes at 37°C. Digestion products were separated by electrophoresis on 1.5% agarose gel stained with ethidium bromide.

Thirty representative samples, including those species whose restriction fragment profiles were not sufficiently different to enable identification by RFLP alone, were selected from the nine study sites for sequencing. Gel slices containing the PCR products were excised from agarose gels using sterile scalpel blades and purified with Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol, and were selected for subsequent sequence analysis. Each sample was quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies Inc., Wilmington, Delaware, USA). Both the forward and reverse strands of the purified PCR products were sequenced using a dilution of the original PCR primers ETTS1 and 2. Sequencing was performed on Applied Biosystems ABI 3500 XL Genetic Analyzer platform using the Sanger sequencing approach. Following basic editing and compilation, the ITS sequences were used to undertake BLAST searches (ALTSCHUL et al. 1990). These were necessary to ensure that contaminating sequences, such as those from internal and/or external molluscan parasites and other organisms, were absent, and also to give a reasonable first estimate of the species identity, as it would be expected that close matches with *Bulinus* species ITS sequences submitted to the European Molecular Biology Laboratory (EMBL) database on previous occasions (AKINWALE et al. 2011) should be returned.

Phylogenetic relationships were inferred using genetic distance analysis by Neighbor-Joining method (SAITOU & NEI 1987). Prior to phylogenetic analysis, sequences of ITS1 and ITS2 were aligned using ClustalW (THOMPSON et al. 1994). NJ trees were generated from evolutionary distances computed using the Maximum Composite Likelihood method (TAMURA et al. 2004) and were in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were conducted in MEGA4 (TAMURA et al. 2007). The tree branches were supported by bootstrap analysis with 1,000 replicates (FELSENSTEIN 1985).

RESULTS

PCR amplification of the schistosome *Dra*I repeat from the DNA of the snails showed that 52 out of the 149 snails – *B. globosus* (38), *B. forskalii* (8), *B. camerunensis* (4) and *B. senegalensis* (2) from the nine study sites (Table 1) were positive for schistosome infection. Some PCR amplifications using the *Dra*I primers resulted in partial, uncertain profiles (Fig. 2, lane 11), where it was not clear whether the snail was only lightly infected or subject to miracidial penetration not followed by actual development of the parasite.

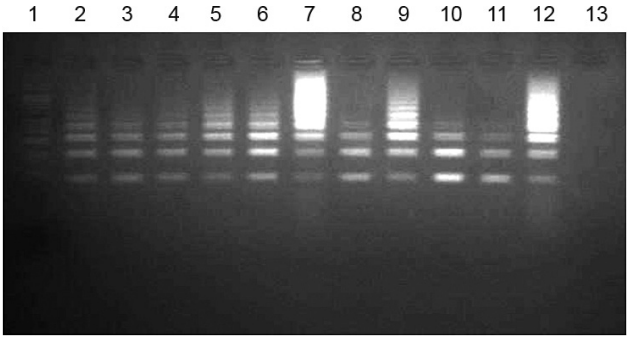
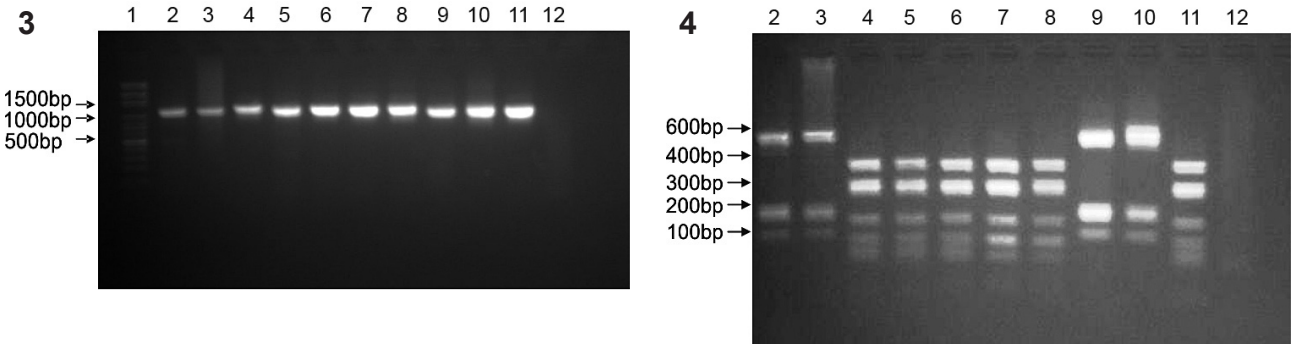


Fig. 2. Ethidium bromide stained agarose gel showing infection status of 10 snails using the *Dra*I repeat. Lanes: 1 – size marker (Solis BioDyne 100bp DNA Ladder); 2–11 – infected snails; 12 – DNA from adult *S. haematobium* as positive control; 13 – negative control (no DNA). Sequencing confirmed five of the infected snails as *B. globosus* (lanes 2, 4–7), one as *B. forskalii* (lane 3), two as *B. camerunensis* (lanes 8 and 11) and two as *B. senegalensis* (lanes 9 and 10)

Following PCR amplification of the 1.3 kbp fragment (Fig. 3), PCR-RFLP analysis of the ITS region from the snails showed that the dominant species was *B. globosus*. However, the restriction fragment profiles were not sufficiently different between *B. senegalensis*, *B. forskalii* and *B. camerunensis* (Fig. 4) to enable easy identification by RFLP alone, hence thirty representative samples including these species had their identity confirmed through sequencing. BLAST searching and phylogenetic analysis confirmed that 17 out of the 30 snails were *B. globosus* while the others were *B. camerunensis* (7), *B. forskalii* (4) and *B. senegalensis* (2) (Table 2). The sequences were submitted to the GenBank database (accession numbers: KF989347 to KF989359, KJ184520, KJ361799 to KJ361814). In addition, the majority of the 149 *Bulinus* snails characterised in this study using a combination of sequencing and RFLP profiles were *B. globosus* (73.2%; n = 109) with the others identified as *B. forskalii* (14.7%; n = 22), *B. senegalensis* (7.4%; n = 11), and *B. camerunensis* (4.7%; n = 7).

Table 2. Sample origin, species and GenBank accession number

Sample origin	Sample Id	Snail species identification		Accession number
		Morphological	Molecular	
Owode	Oy8Bg	*Bulinus globosus*	*Bulinus globosus*	KF989347
Eggua	E1086Bg	*Bulinus globosus*	*Bulinus globosus*	KF989348
Imasayi	Im21Bg	*Bulinus globosus*	*Bulinus globosus*	KF989349
Ijale Ketu	Ik4Bg	*Bulinus globosus*	*Bulinus globosus*	KF989350
Ijale Ketu	Ik11Bj	*Bulinus jousseaumei*	*Bulinus globosus*	KF989351
Agbon	RA4Bg	*Bulinus globosus*	*Bulinus globosus*	KF989352
Igan Alade	Ia2	*Bulinus globosus*	*Bulinus globosus*	KF989353
Owode	Oy43Bs	*Bulinus senegalensis*	*Bulinus camerunensis*	KF989354
Ayetoro	A37Bs	*Bulinus senegalensis*	*Bulinus camerunensis*	KF989355
Ayetoro	A42Bs	*Bulinus senegalensis*	*Bulinus camerunensis*	KF989356
Owode	Oy42Bc	*Bulinus camerunensis*	*Bulinus forskalii*	KF989357
Ijale Ketu	Ik1Bc	*Bulinus camerunensis*	*Bulinus forskalii*	KF989358
Owode	Oy41Bc	*Bulinus camerunensis*	*Bulinus forskalii*	KF989359
Ayetoro	A24Bs	*Bulinus senegalensis*	*Bulinus forskalii*	KJ184520
Owode	Oy9Bg	*Bulinus globosus*	*Bulinus globosus*	KJ361799
Owode	Oy38Bc	*Bulinus camerunensis*	*Bulinus camerunensis*	KJ361800
Ayetoro	A4Bj	*Bulinus jousseaumei*	*Bulinus camerunensis*	KJ361801
Ayetoro	A6Bj	*Bulinus jousseaumei*	*Bulinus cmerunensis*	KJ361802
Ayetoro	A19Bs	*Bulinus senegalensis*	*Bulinus senegalensis*	KJ361803
Ayetoro	A26Bs	*Bulinus senegalensis*	*Bulinus senegalensis*	KJ361804
Ayetoro	A28Bs	*Bulinus senegalensis*	*Bulinus camerunensis*	KJ361805
Ijale Ketu	Ik9Bj	*Bulinus jousseaumei*	*Bulinus globosus*	KJ361806
Eggua	E1992Bg	*Bulinus globosus*	*Bulinus globosus*	KJ361807
Eggua	E2	*Bulinus globosus*	*Bulinus globosus*	KJ361808
Ijoun	I10Bj	*Bulinus jousseaumei*	*Bulinus globosus*	KJ361809
Igan Alade	Ia3Bg	*Bulinus globosus*	*Bulinus globosus*	KJ361810
Igan Alade	Ia4Bg	*Bulinus globosus*	*Bulinus globosus*	KJ361811
Balogun	B5Bg	*Bulinus globosus*	*Bulinus globosus*	KJ361812
Imashayi	Im12Bg	*Bulinus globosus*	*Bulinus globosus*	KJ361813
Imashayi	Im13Bg	*Bulinus globosus*	*Bulinus globosus*	KJ361814



Figs 3–4. Agarose gels stained with ethidium bromide showing: 3 – PCR products following amplification of ITS region of 10 snails; 4 – species-specific banding patterns obtained from digestion of PCR product containing ITS region using *Rsa*I enzyme. Lanes: 1 – size marker (Solis BioDyne 100bp DNA Ladder); 2 – *B. senegalensis*; 3 – *B. forskalii*; 4 to 8 & 11 – *B. globosus*; 9 – *B. forskalii*; 10 – *B. camerunensis*; 12 – negative control. All 10 snails were further confirmed by sequencing of their ITS regions

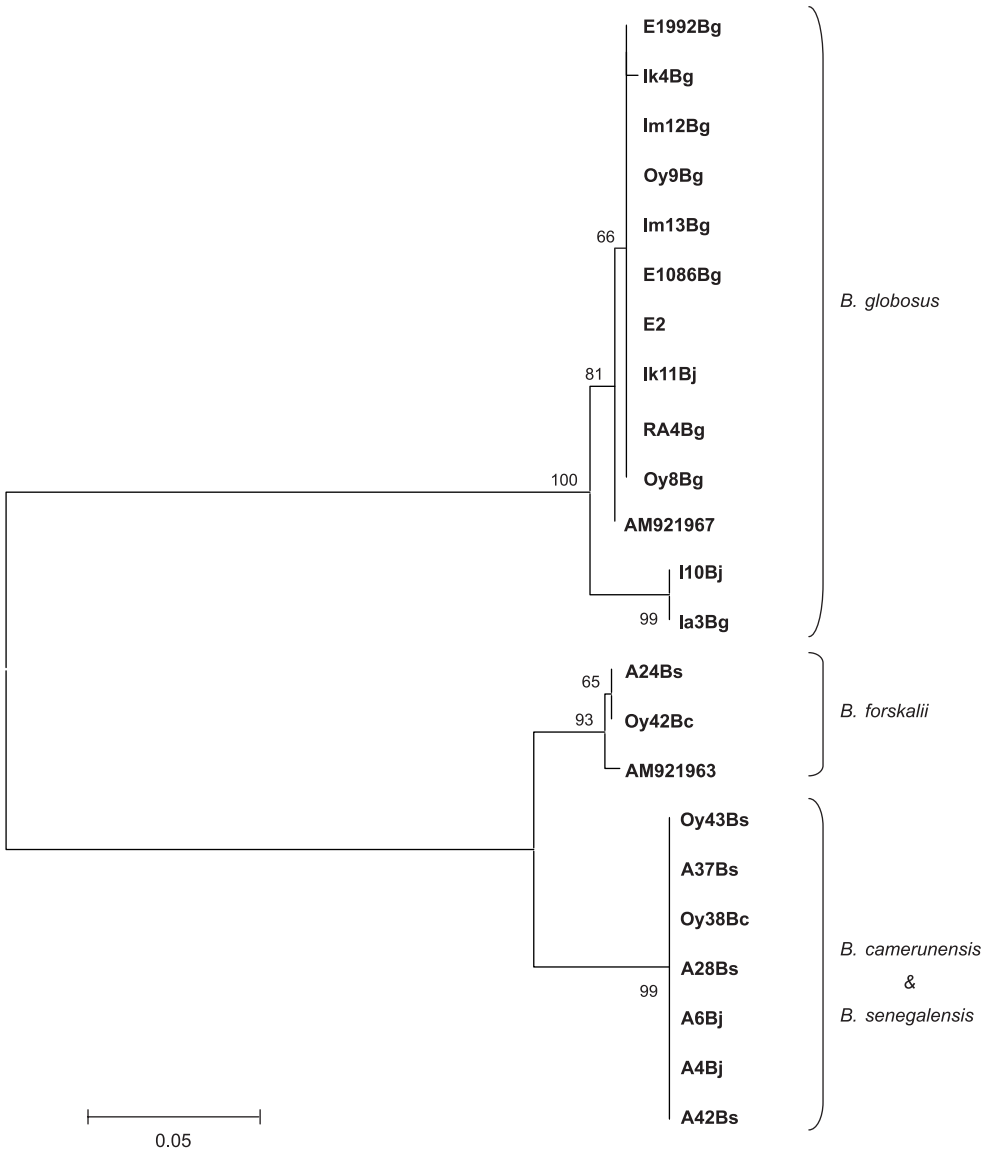


Fig. 5. Neighbour-Joining tree based on ITS2 sequences of 21 selected *Bulinus* specimens and two sequences, AM921963 and AM921967 for *B. forskalii* and *B. globosus*, respectively, taken as references from GenBank (KANE et al. 2008). Numbers next to branches represent percentage of 1,000 bootstrap replicates (bootstrap values below 50 not shown). Scale bars show the number of base substitutions per sequence position

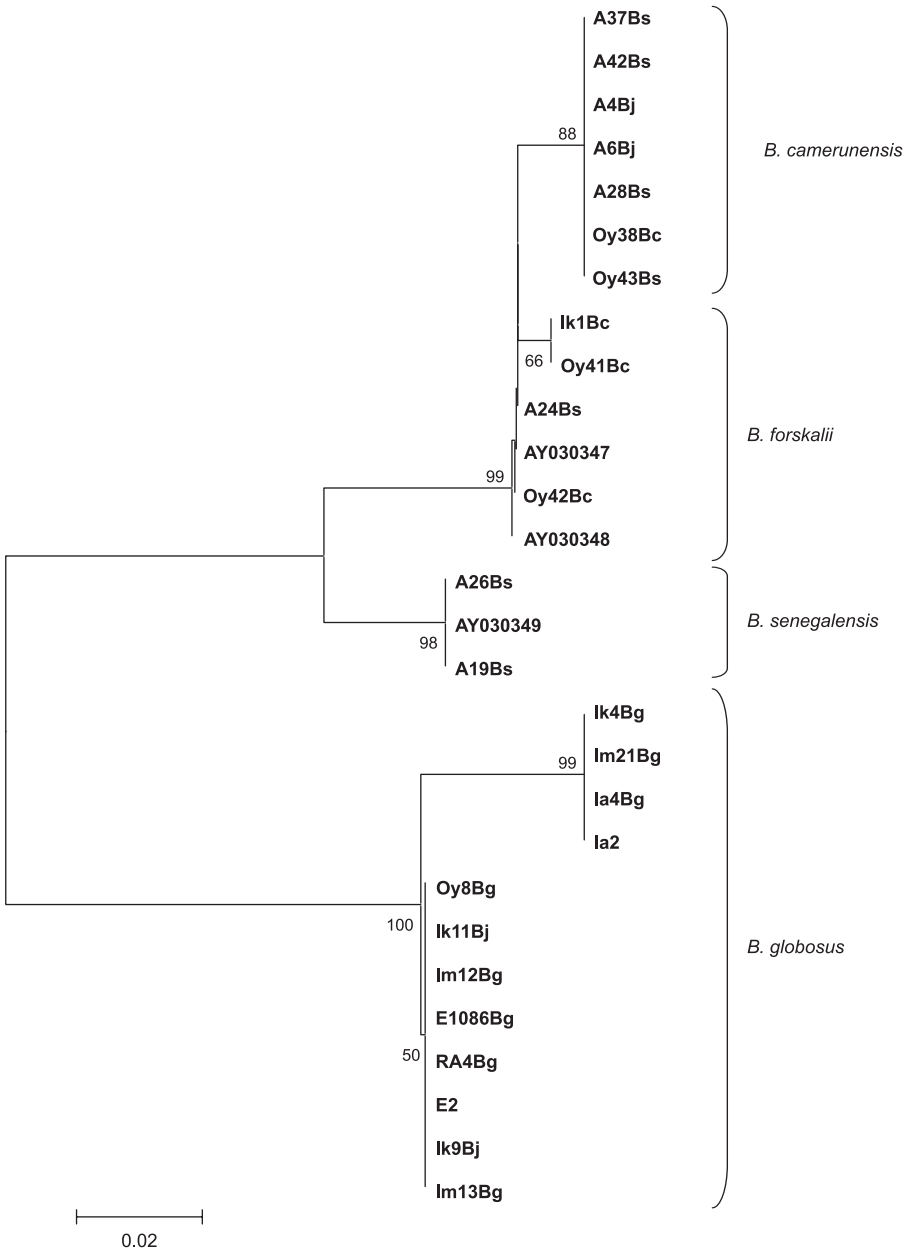


Fig. 6. Neighbour-Joining tree based on ITS1 sequences of 25 selected *Bulinus* specimens and three sequences AY030347, AY030348 for *B. forskalii*, AY030349 for *B. senegalensis*, obtained from GenBank (JONES et al. 2001). For other explanations see: Fig. 5

The obtained 30 sequences of ITS region differed in length. To compare them with GenBank data, two phylogenetic trees were constructed, separately for ITS2 and ITS1 fragments (Figs 5 and 6). Thirteen of our sequences of ITS2 fragment (Fig. 5) were grouped together with the sequence AM921967 deposited in GenBank for *B. globosus* (KANE et al. 2008) which allowed us to identify our specimens as *B. globosus*. Similarly, the sequence AM921963 deposited in GenBank for *B. forskalii* (KANE et al. 2008) made it possible to identify two specimens (Oy42Bc and A24Bs) as *B. forskalii* because these three sequences were in one tree branch. Unfortunately seven further ITS2 sequences grouped together (Fig. 5) and therefore they represented both *B. camerunensis* and *B. senegalensis*. The phylogenetic tree constructed on the basis of ITS1 fragment sequences (Fig. 6) allowed a better identification of species. One group of sequences represented *B. globosus* because they included specimens identified as this species in the ITS2 analysis. The sequence AY030349 from GenBank (JONES et al. 2001) made it possible to recognise two specimens (A26Bs and A19Bs) as *B. senegalensis*, two other sequences AY030347 and AY030348 from GenBank (JONES et al. 2001) allowed identification of four specimens (Ik1Bc, Oy41Bc, A24Bs, Oy42Bc) as *B. forskalii*. We assumed that the last group of seven ITS1 sequences represented *B. camerunensis*.

DISCUSSION

The 149 snails used in this study were field-caught specimens; 52 (34.9%) of them carried schistosome infection. All four species identified were naturally infected, or at least penetrated by some miracidia of the *S. haematobium* group species as shown by *Dra*I amplification. Upstream and downstream sites did not differ significantly in their snail species composition or infection prevalence (Tables 1 and 2). The *Dra*I repeat is a useful tool to determine whether or not a snail has been exposed to and infected with schistosomes. In transmission loci, snail species are invariably always exposed to schistosome and the snail may be susceptible or refractory; however, it will not reveal which species of the parasite is responsible for infection. All snails collected from Agbon, Balogun, Igan Alade, Ijoun, Eggua and Imasayi were identified as *B. globosus*, a few through sequencing and others through their restriction profiles and morphological characteristics. There were mixed populations of *B. globosus*, *B. camerunensis* and *B. forskalii* in Owode; *B. globosus* and *B. forskalii* in Ijale Ketu; *B. forskalii*, *B. camerunensis* and *B. senegalensis* in Ayetoro. *B. camerunensis*, which was previously known only from western Cameroon, where it was recorded from two crater lakes; Barombi Koto and Debundsha (BROWN 1994), was also reported for the first time in Nigeria by SALAWU & ODAIBO (2014). They found the snails in two rivers: Bareke and Isopa, located in Yewa North LGA, Ogun State, the area where the present study was carried out. Sampling in our study was limited to a few endemic areas in Yewa North LGA and, in consequence, the absence of other species groups such as *B. truncatus/tropicus* complex may have been the effect of the sampling strategy.

Restriction fragment profiles for *B. globosus* obtained in this study closely matched those provided by AKINWALE et al. (2011) for the same species. This is in agreement with observations of ROLLINSON et al. (1998) that genomic ribosomal RNA gene complex could be well suited for taxonomic studies as it contains regions which evolve at different rates. In addition, it revealed *B. globosus* as the dominant intermediate host species in these regions. According to STOTHARD et al. (1996) using restriction fragment analysis of the ribosomal ITS region for identification of snails in the genus *Bulinus* could be cheaper and faster than sequencing. Thus the method was employed in this study in addition to sequencing and morphology of the snails. Phylogenetic inferences clustered the samples into two species groups: *B. forskalii* (*B. forskalii, B. camerunensis* and *B. senegalensis*) and *B. africanus* (*B. globosus*). This supports BROWN's (1994) grouping of *B. forskalii*, *B. camerunensis* and *B. senegalensis* in the *B. forskalii* complex. However, the ITS1 and ITS2 fragments failed to make a clear distinction between the three species within the *B. forskalii* group, as *B. senegalensis, B. camerunensis* and *B. forskalii* were all grouped together (Figs 5 and 6). ZEIN-EDDINE et al. (2014) also suggested that ITS region was unable to separate clearly between *B. globosus* and *B. umbilicatus* in the the *B. africanus* group as well as between the three species within the *B. forskalii* group. KANE et al. (2008) and ZEIN-EDDINE et al. (2014) suggested that analyses of mitochondrial genes were more suitable for identification of *Bulinus* species.

The inferred phylogenetic trees (Figs 5 and 6) also showed that some snails were misidentified morphologically before sequencing was carried out. The snails identified molecularly as *B. forskalii* were initially identified morphologically as *B. senegalensis* (A24Bs) or *B. camerunensis* (Oy42Bc, Ik1Bc, Oy41Bc). Others proved to be *B. globosus* misidentified morphologically as *B. jousseaumei* (Ik9Bj and I10Bj), and *B. camerunensis* misidentified as *B. senegalensis* (A37Bs, A42Bs, Oy43Bs) or *B. jousseaumei* (A4Bj and A6Bj). This study therefore shows that in the case of *Bulinus*, species identification based on shell characters alone, is doubtful. Though preliminary identification based on such characters may be possible, unambiguous identification at the species level needs other more reliable methods such as the use of DNA analysis.

CONCLUSION

Understanding the systematics of freshwater snails which play the crucial role in the transmission of schistosomiasis is very important for monitoring the present and future infection levels as well as the distribution of schistosomes in Nigeria. This study contributes to development of methods of identifying the snail species which are schistosomiasis vectors in the study areas. Although the use of sequencing for species identification is very expensive and time-consuming, it helps to resolve the true identities of some of the snails whose restriction profiles are very similar and inconclusive.

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