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Characterization of *Bulinus* Snails in Sô-Ava and Azowlissè, Two Localities in Southern Benin, Using PCR-RFLP

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Abstract

Schistosomiasis is a public health concern in Benin. Freshwater snails of the genus *Bulinus* serve as intermediate hosts for schistosomes, trematode parasites responsible for bilharzia. The urinary form, caused by *Schistosoma haematobium*, is the most widespread and is transmitted to humans by these mollusks, with *Bulinus truncatus* and *Bulinus globosus* being the most important species. Effective strategies to combat the transmission of these parasites require a prior understanding of the molecular characterization of *Bulinus* snails. For this purpose, 293 *Bulinus* snails were collected and morphologically identified from two localities in southern Benin, Sô-Ava and Azowlissè. The snails were preserved in absolute alcohol. To achieve the set objectives, DNA was extracted from the collected biological material, and SSU gene fragments were amplified. Using PCR-RFLP, the amplified fragments were digested with the restriction endonucleases HaeIII, HinfI, and DdeI to perform molecular characterization. In this study, 80 individuals of *B. globosus* and 10 of *B. truncatus* were subjected to molecular analysis. The PCR-RFLP profiles showed bands of different sizes for the *Bulinus* species when analyzed with the three endonucleases using the SSU molecular marker. PCR-RFLP analysis revealed that the snails belonged to the freshwater genus *Bulinus*, including *Bulinus globosus* and *B. truncatus*, based on reference profiles from studies conducted in Nigeria, which enabled precise identification of these gastropods. This study provided initial insights, although still incomplete, into the molecular diversity of these species.

Keywords

Bulinus globosus, *Truncatus*, Molecular Diversity, PCR-RFLP, Benin

1. Introduction

This schistosomiasis is a parasitic infection caused by flatworms known as schistosomes or blood flukes, which are hematophagous trematodes with separate sexes that inhabit the circulatory system. This disease is a major parasitic endemic, and its intermediate host is the freshwater snail *Bulinus*, which transmits the trematode. Intestinal human schistosomiasis is a debilitating disease found in Africa, West Asia, and some parts of South America [1]. It is characterized by a range of symptoms including abdominal pain, diarrhea, and in severe cases, hepatosplenomegaly and esophageal varices [2]. The urinary form, caused by *S. haematobium*, is the most widespread and is primarily transmitted to humans by these mollusks, with *Bulinus truncatus* and *Bulinus globosus* being the most significant intermediate hosts [3]. In 2006, the World Health Organization (WHO) reported that more than 200 million people were infected with either the hepato-intestinal or urogenital forms of schistosomiasis in 76 developing countries, with approximately 80% of cases occurring in Africa (WHO, 2006). Following the spread of this disease, transmission has been confirmed in 78 countries (WHO, 2021). In 2021, the global coronavirus pandemic and the increasing efforts to mitigate its effects led to a decrease in interventions against neglected tropical diseases, further exacerbating the risk. This is particularly concerning in Africa, where underdevelopment, poor hygiene, and the persistence of fecal and urinary contamination facilitate the spread of the disease. The highest infection rates and morbidity are often found among school-aged children, especially in environments with inadequate hygiene and sanitation facilities [4]. Infected human hosts suffer from acute hyperemia, abnormal growth, internal bleeding, fibrosis, and tissue thickening [5].

Human activities such as creating water reservoirs for development purposes have caused ecological disruptions, leading to the proliferation of certain biological species, including disease vectors like the intermediate host snails of schistosomes [6]. *Bulinus* species are found in many African countries, including neighboring regions [7]. However, identifying *Bulinus* species based on morphology is unreliable due to the limited morphological differences within species groups. Several studies have identified and classified *Bulinus* species into groups based on malacological physical parameters, such as shell appearance and internal structures [8].

However, these classifications are not always reliable when used for higher-resolution classification and can be extremely confusing. The development of new molecular biology techniques, such as PCR-RFLP markers [9], has improved the study of certain aspects of the biology and population diversity of *Bulinus* species. Therefore, the use of more advanced tools to distinguish *Bulinus* species is essential to better understand the identification and characterization of *Bulinus* species through PCR-RFLP using the Small Subunit rRNA (SSU) marker in two localities, Sô-Ava and Azowlissè in southern Benin, where urogenital schistosomiasis is endemic, but no similar study has been conducted to identify *Bulinus* species in Benin.

2. Methodology

2.1. The Study Area

This study was conducted in early 2024 in two localities across two departments in southern Benin: Azowlissè in Adjohoun (Ouémé) and Sô-Ava in the Nokoué Lake region (Atlantique). These areas are situated in southern Benin and are characterized by a sub-equatorial climate with high humidity [10]. The region experiences two dry seasons (December to March and mid-July to mid-September), alternating with two rainy seasons (April to mid-July and mid-September to November). The temperatures are typically around 30˚C or higher, with frequent rainfall averaging 1200 mm annually. Human activities in these areas include heavy reliance on water sources for agriculture (market gardening, seasonal and annual crops, and perennial plantations), fisheries, and aquaculture (fish farming ponds).

Bulinus species were collected from natural water retention areas. These sites were selected based on visual observation and preliminary sociological surveys. The survey aimed to gather information from local residents about the aquatic environments they frequently use for their daily needs. In each identified water point, *Bulinus* snails were collected. The georeferencing of the sampling sites was conducted using a Global Positioning System (GPS). At each site, random sampling was carried out over approximately two hours in various parts of the water, covering areas of varying sizes. The collected *Bulinus* snails were placed in perforated jars and transported to the Unit of Research in Phylogeography, Genetics, and Evolution (UR-PGE) at the Faculty of Sciences and Technology of the University of Abomey-Calavi (FAST-UAC) for breeding and comprehensive identification, which included additional ecological and morphological studies (shell morphology and determination of parasitic infestation rates for each identified species). The snails were then preserved in 70% alcohol for subsequent molecular analyses.

2.2. Molecular Characterization of Collected Samples

DNA extraction from *Bulinus* out of the 293 *Bulinus* snails collected and morphologically identified, a random selection was made from each species previously confirmed to be infected, to form a sample for molecular analysis. In total, 80 individuals of *Bulinus globosus* and 10 of *Bulinus truncatus* were analyzed. To better assess the genetic diversity at each site, two to five snails were randomly selected per locality [11]. The preserved *Bulinus* snails were transported to the Laboratory of Biology and Molecular Typing in Microbiology at the University of Abomey-Calavi for DNA extraction. The snails were immersed in absolute ethanol for two days, and the procedure was repeated, replacing the ethanol if it became cloudy or yellowish due to mucus secretion. Approximately 20 mg of tissue (excluding the shell) from the whole snail was immersed in 1 mL of absolute ethanol for two days to eliminate any mucus residue. The tissue was then crushed and lightly ground with a pestle in a porcelain mortar before being transferred to a 2 mL Eppendorf

tube containing 1 mL of ethanol. After two days, 750 µL of TE buffer were added to the Eppendorf tube to eliminate the remaining ethanol, and the mixture was incubated for 1 hour at room temperature (30˚C) to soften the tissue. Afterwards, the TE buffer was discarded, and distilled water was added to each tube, followed by another 1-hour incubation. The water was then discarded, and 600 µL of pre-heated Urea-SDS buffer (60˚C) and 10 µL of Proteinase K were added.

The mixture was subjected to mechanical disruption using a silica bead in a grinder for 45 seconds and left in a 60˚C water bath overnight (10 - 14 hours) to lyse the cells. Then, 750 µL of phenol-chloroform-isoamyl alcohol (PCIA) solution (24:1) were added and mixed by inversion for 5 minutes. The mixture was centrifuged at 10,000 rpm at 4˚C for 15 minutes, and the supernatant was carefully transferred to a new 1.5 mL Eppendorf tube, ensuring the white intermediate layer remained intact. The aqueous phase was precipitated with 750 µL of cold absolute ethanol (−20˚C) and 40 µL of 3M sodium acetate. The mixture was gently stirred for a few minutes and incubated at −20˚C for 1 hour. The pellet was recovered by centrifugation at 10,000 rpm at 4˚C for 10 minutes, then washed with 600 µL of 70% ethanol. This step was repeated two or three times, depending on the appearance of the pellet. The tubes were left to dry at room temperature on blotting paper. Finally, the pellet was resuspended in 100 µL of ultrapure water. After the complete dissolution of the pellets, the DNA extracts were quantified using a NanoDrop spectrophotometer after 4 hours to assess the quality and quantity of native DNA. The purity index (I) averaged between 1.8 and 2, confirming satisfactory conditions for DNA extraction.

Amplification and Enzymatic Digestion of the SSU Gene Using Restriction Endonucleases (HaeIII, DdeI, HinfI)

PCR (Polymerase Chain Reaction) was used to obtain numerous copies of a specific DNA segment, called the target DNA. A fragment of approximately 1020 bp from the SSU gene located on the nuclear genome was amplified using primers with the following sequences: 18S Baso3F: 5'-GTGCTCTTCNCTGAGGGTCC-3' and 18S Baso9R: 5'-TACGGAAGCCTTGTTACGA-3' (Jorgensen *et al*. 2011). The PCR program included an initial denaturation at 95˚C for 4 minutes, followed by 35 cycles of denaturation (30 seconds at 94˚C), annealing at 68˚C, and elongation (60 seconds at 72˚C), ending with a final elongation phase at 72˚C for 5 minutes. The reaction mixture consisted of 5 µL of PCR buffer (6X green buffer), 1 µL of 200 µM dNTP, 2.5 µL of each primer at 0.2 µM, 1 µL of 25 mM $MgCl_2$, 0.1% BSA (Bovine Serum Albumin), 0.2 U of OneTaq polymerase, 3 µL of 10 ng/µL DNA, and sufficient water for a final volume of 25 µL. The amplified products were stored at 4˚C before being digested with the restriction endonucleases HaeIII, DdeI, and HinfI. The digestion mixture was prepared with 5 µg of PCR products, 5 µL of digestion buffer, 1.5 µL of each enzyme, and ultrapure water to a final volume of 25 µL. The digestion mixtures were incubated at 37˚C overnight (approximately 10 hours) for optimal activation, and then inactivated at 65˚C (for DdeI) or 80˚C (for HaeIII and HinfI) for 20 minutes, depending on the character-

istics of each restriction enzyme. The resulting fragments, hydrolyzed according to specific cutting sites, were separated by electrophoresis on a 2% agarose gel using 10 µL of digestion products mixed with loading buffer. The migration was carried out under 80V for 45 minutes to 1 hour in 0.5X TBE buffer. A 1.5 µL volume of ethidium bromide (BET) was added to each gel well. After migration, the gel was visualized under UV light using a transilluminator. A 100 bp size marker was used during migration to estimate the approximate size of the DNA fragments generated by digestion and to assess their polymorphism.

3. Results

3.1. Identification and Distribution of *Bulinus* Species in the Localities

A total of 283 *Bulinus* snails were collected from the two sampling sites during the 2024 recession period (Table 1). Two different species of *Bulinus* were identified based on previously reported morphological criteria: *Bulinus globosus* and *Bulinus truncatus*.

Table 1. Population size of *Bulinus* species in different localities.

Locality	*Bulinus globosus*	*Bulinus truncatus*	Total
Sô-Ava	170	20	190
Azowlissè	90	3	93
Total	260	23	283

3.2. Infestation Rate of *Bulinus* Species

The Kruskal-Wallis non-parametric test indicated a significant difference (P = 0.01) in the number of infested *Bulinus* between the two localities. Out of the 283 *Bulinus* snails tested for cercarial shedding, 265 excreted cercariae, leading to an overall infestation rate of 69.19% ± 6.80%. However, this infestation rate varied according to the locality: Sô-Ava: 64.12% ± 4.6% Azowlissè: 76% ± 4.90%. For *Bulinus truncatus*, 17 out of 23 samples released schistosome cercariae, with an infestation rate of 73.91% ± 10.65%. The infestation rate of *B. truncatus* also showed variability between localities: Azowlissè: 66.67% ± 33.33%; Sô-Ava: 75.00% ± 11.18%. Furthermore, out of 260 *Bulinus globosus* samples, 248 shed schistosome cercariae, resulting in an infestation rate of 73.37% ± 2.80%. These infestation rates also showed variability between the localities: for *B. truncatus*, 66.67% ± 33.33% in Azowlissè and 75.00% ± 11.18% in Sô-Ava; for *B. globosus*, 76.00% ± 4.90% in Azowlissè and 64.12% ± 4.06% in Sô-Ava. Consequently, the infestation rate of *B. globosus* is higher in Azowlissè than in Sô-Ava, while *B. truncatus* shows a higher rate in Sô-Ava compared to Azowlissè. The variability in the infestation rate of *B. truncatus* suggests a less consistent infection pattern in Sô-Ava than in Azowlissè. These observed differences could be attributed to environmental and ecological factors affecting the infection dynamics of *Bulinus* species in the two localities. (Table 2)

Table 2. Infestation rate of *Bulinus* species by schistosomes in different localities.

Locality	*Bulinus globosus*	*Bulinus truncatus*	Average Infestation Rate (%)
Sô-Ava	64.12% ± 4.60%	75% ± 11.18%	68.6% ± 4.07%
Azowlissè	76% ± 4.90%	66.67% ± 33.33%	69.78% ± 5.01%

3.3. Physico-Chemical Characteristics of Surveyed Localities

The recorded water temperature in Sô-Ava is 28˚C, slightly higher than the 27.9˚C observed in Azowlissè. The pH values are 7.31 in Sô-Ava and 7.01 in Azowlissè, indicating neutral conditions in both locations. Dissolved oxygen levels are higher in Sô-Ava (6.71 mg/L) compared to Azowlissè (5.06 mg/L). Conductivity is 45 S/m in Sô-Ava but significantly higher at 93 S/m in Azowlissè. Lastly, salinity is 0 ppt in Sô-Ava and 0.01 ppt in Azowlissè. (Table 3)

Table 3. Effects of physico-chemical parameters on the number of *Bulinus* individuals collected and those releasing cercariae.

Locality	Latitude and Longitude	Density water hyacinth	Temperature (˚C)	pH	Dissolved oxygen (mg/l)	Conductivity (s/m)	Salinity unit (ppt)
Sô-Ava	6˚28'35"N, 2˚24'86"E	highest	28	7.31	6.71	45	0
Azowlissè	6˚40'51"N, 2˚29'11"E	high	27.9	7.01	5.06	93	0.01

3.4. Effects of Physico-Chemical Parameters on the Number of *Bulinus* Individuals Collected and Those Releasing Cercariae

The generalized linear regression model of the fish family shows that all the parameters evaluated have a significant effect at the 0.1% threshold on the number of *Bulinus* snails collected and those releasing cercaria (Table 4). The analysis reveals that conductivity and pH are negatively correlated with the number of *Bulinus snails* collected, while there is a positive relationship between the other parameters and the number of *Bulinus* snails. Analysis of the same tables shows that conductivity and pH are negatively correlated with the number of *Bulinus* snails infested, while there is a positive relationship between the other parameters and the number of *Bulinus* snails infested.

Table 4. Effect of physico-chemical parameters on the number of *Bulinus* snails collected.

	Bulinus collected				*Bulinus* snails releasing cercariae			
Settings	Estimate	Standard error	Z value	Pr (>|z|)	Estimate	Standard error	Z value	Pr (>|z|)
Intercepte	130.76	163.54	0.4	0.21	322.07	144.58	1.11	0.013*
Conductivity	−0.22	0.037	−2.94	0.91e−09***	−0.18	0.035	−2.69	3.81e−08***
Dissolved oxygen	83.49	6.22	6.71	<1e−16***	83.13	5.51	5.27	<1e−16***
pH	−186.88	26.15	−3.57	4.51e−13***	−154.29	23.87	−3.23	0.51e−06***
Salinity	567.36	118.67	2.39	0.87e−06***	510.29	106.61	2.395	0.85e−06***
Temperature	28.02	2.33	6.01	<1e−16***	17.46	2.010	4.26	<1e−16***

*Significant at the 5% level; ** Significant at the 1% level; *** Significant at the 0.1% level.

3.5. Molecular Identification of *Bulinus* Species

3.5.1. *Bulinus globosus*

1) Case of the HinfI Endonuclease

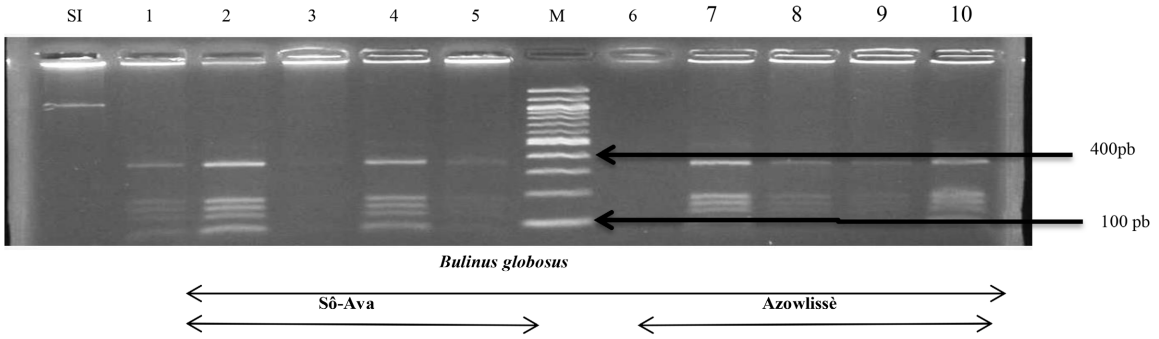

Figure 1. Enzymatic digestion of the SSU gene of *Bulinus globosus* in the localities of Sô-Ava and Azowlissè. Legend: M: Marker (100 bp), SI: Sequence of interest, 1, 2, 3, 4, 5 = *Bulinus globosus* (Sô-Ava), 6, 7, 8, 9, 10 = *Bulinus globosus* (Azowlissè).

2) Case of the HaeIII Endonuclease

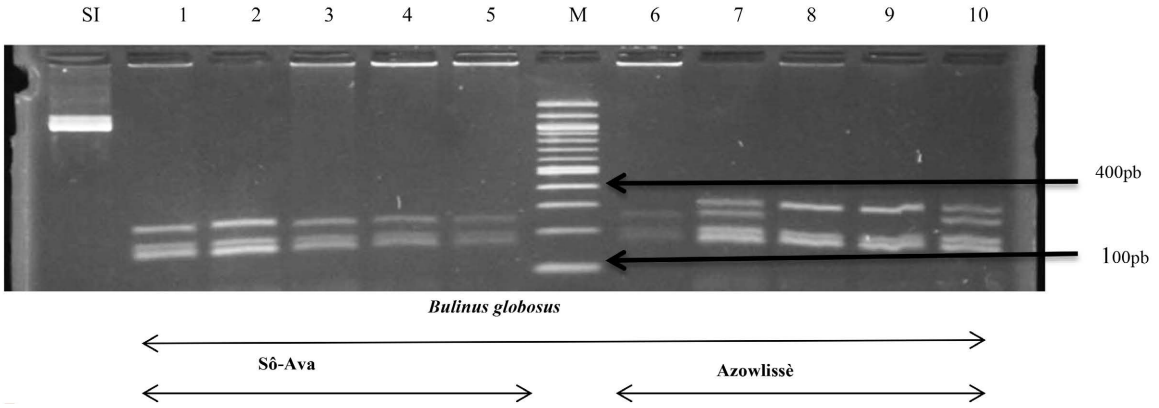

Figure 2. Enzymatic digestion of the SSU gene of *Bulinus globosus* in the localities of Sô-Ava and Azowlissè. Legend: M: Marker (100 bp), SI: Sequence of interest, 1, 2, 3, 4, 5 = *Bulinus globosus* (Sô-Ava), 6, 7, 8, 9, 10 = *Bulinus globosus* (Azowlissè).

3) Case of the DdeI Endonuclease

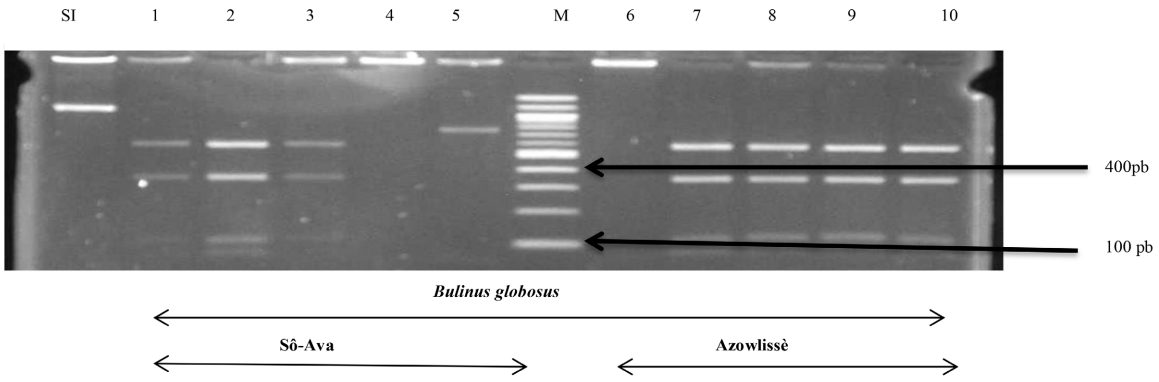

Figure 3. Enzymatic digestion of the SSU gene of *Bulinus globosus* in the localities of Sô-Ava and Azowlissè. Legend: M: Marker (100 bp), SI: Sequence of interest, 1, 2, 3, 4, 5 = *Bulinus globosus* (Sô-Ava), 6, 7, 8, 9, 10 = *Bulinus globosus* (Azowlissè).

3.5.2. *Bulinus truncatus*

1) Cases of the endonucleases HinfI, HaeIII, and DdeI

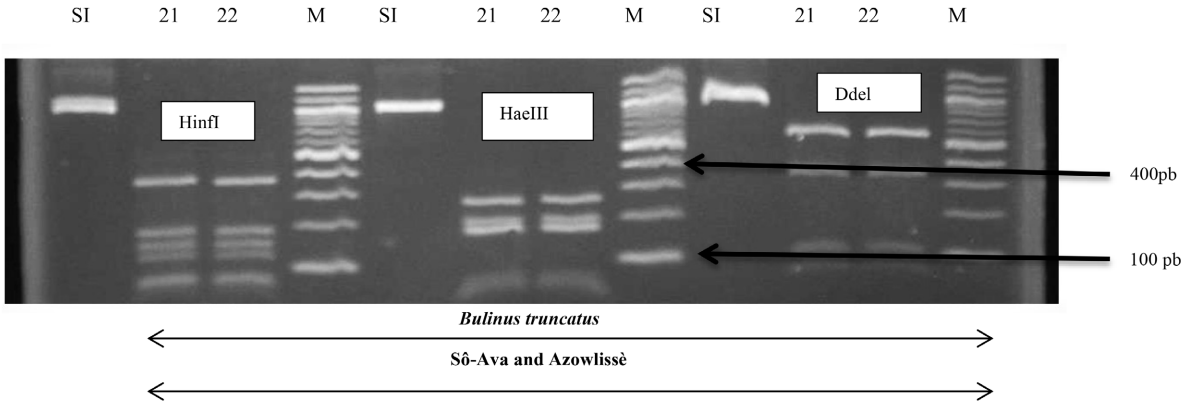

Figure 4. Enzymatic digestion of the SSU gene of *Bulinus truncatus* in the localities of Sô-Ava and Azowlissè. Legend: M: Marker (100 bp), SI: Sequence of interest, 21 = *Bulinus truncatus* (Sô-Ava), 22 = *Bulinus truncatus* (Azowlissè).

PCR amplification performed on the species of *Bulinus*, with highly intact indices on agarose gels, was subsequently subjected to RFLP analysis (**Figures 1-4**). The species *Bulinus globosus* produced the same profiles in Sô-Ava and Azowlissè, regardless of the endonuclease used. Similarly, the species *Bulinus truncatus* also produced the same profiles in Sô-Ava and Azowlissè, irrespective of the endonuclease employed. Typical bands of the *Bulinus* species were obtained from 13 snails in Nigeria [12]. Consistent with these published RFLP reference profiles from Nigeria, which clearly identified *B. truncatus* and *B. globosus*, the profiles obtained in Sô-Ava and Azowlissè can be identified as *Bulinus globosus* and *Bulinus truncatus* using the aforementioned RFLP reference profiles.

4. Discussions

4.1. Distribution of *Bulinus* Species and Effect of Physico-Chemical Parameters on the Number of *Bulinus* Snails Collected

The results of this study demonstrated a heterogeneous distribution of the species *Bulinus globosus* and *Bulinus truncatus*, as well as infestation rates in the two localities in Benin studied. The species *Bulinus truncatus* and *Bulinus globosus* each exhibit distinct ecological structures. These species are unevenly distributed across the collection sites. The causes of this dissimilar distribution can be traced to both abiotic and biotic factors. This difference may be explained by the density of water hyacinths and the physicochemical parameters of the water (temperature, salinity, turbidity, dissolved oxygen) [13]. The pH and conductivity correlated negatively with the number of collected bulins and the number of infested bulins. The slightly basic pH obtained respectively in Sô-Ava and Azowlissè (7.01 and 7.31) confirms the work carried out by [14] in the waters of the Senegal River which showed that the pH values measured in the waters of the Senegal River are not significant and have no influence on the life of these gastropods. The data

obtained during this study comply with the standard recommended by the WHO (6.5 < pH < 8.5). The electrical conductivity (EC) which is a measure of the capacity of an aqueous solution to conduct electric current shows less significant variations for the localities Sô-Ava and Azowlissè (45 and 93 mS/cm). These low contents obtained in Sô-Ava (45 mS/cm) and Azowlissè (93 mS/cm) would be due to the low inputs of organic matter in these waters, leading to very low mineralization. Our results are consistent with those of [15] who showed that conductivity contents (74 and 77.4 mS/cm) obtained in the Ivory Coast on the dams of Lake Taabo did not achieve a significant correlation with the populations of these gastropods because of its low values. The temperatures recorded in Sô-Ava and Azowlissè could be explained by the fact that the freshwater ecosystem found in these environments is less penetrated by light due to its highly developed eutrophication which would have an impact on the multiplication of the number of bulins, hence an increase in the number of bulins and the infestation rate of these bulins. The measured salinity correlated positively with the harvested molluscs and the number of infested molluscs; This could be due to its very low content. The very low salt contents indicate that the watercourses of the two localities Sô-Ava and Azowlissè are freshwater ecosystems. This absence of salinity would favor the proliferation of these aquatic species, more specifically water hyacinths (Eichhornia crassipes) which is very high in Sô-Ava and Azowlissè which, by developing, would prevent the penetration of solar rays into the water and contribute to the depletion of dissolved oxygen (O_2) [16]. These bulins are often stuck to water hyacinths which are their host plant species. The positive influence of oxygen measurements on molluscs shows that the latter record optimal values favoring life in the habitat of these gastropods. Environmental factors influencing the distribution of snails are often overlooked, even though they can vary significantly from one site to another and across ecological zones, even over short distances [17]. It should be noted that the environments of these two localities better meet the ecological requirements of these two species of *Bulinus*, and that there is a nearly constant communication established between them, facilitating a continuous exchange of organisms and physicochemical elements. The predominance of *Bulinus globosus* and *Bulinus truncatus* in Sô-Ava (Lake Nokoué) compared to Azowlissè (Ouémé River) can be attributed to the low marine current and the shallow gradient of the lake, although dissolved oxygen levels and salinity are not negligible factors. The results of the infestation rates in this study clearly indicate that Sô-Ava and Azowlissè are two localities that exhibit significant infestation rates.

4.2. Molecular Identification of *Bulinus* Species

The molecular characterization study of *Bulinus* using endonucleases (HaeIII, HinfI, and DdeI) with the molecular marker SSU through PCR-RFLP amplification showed specific cuts for each locality and each species of *Bulinus*, releasing nearly the same fragment size per enzyme for all individuals of the *Bulinus* popu-

lations. Regardless of the endonucleases used, Sô-Ava and Azowlissè revealed the same profiles for each species of *Bulinus*. Our results demonstrating the different profiles of *Bulinus globosus* and *Bulinus truncatus* obtained in this study at Sô-Ava and Azowlissè with the endonucleases (HaeIII, HinfI, and DdeI) on the SSU gene in southern Benin are consistent with the work conducted in Nigeria [18]-[20] in Kenya, who confirmed that these *Bulinus* species obtained were indeed *Bulinus globosus* and *Bulinus truncatus*, among other species. The observed data indicate that stable genetic markers are present for *B. globosus* and *B. truncatus* in Benin. The evolution of the SSU molecular marker is rapid enough to assist in species identification. Accurate identification of the host and parasite would not only clarify species diversity but also facilitate the development of strategies to significantly reduce transmission and find means to combat this pathology.

5. Conclusion

The results obtained from the molecular characterization study of *Bulinus* represent the initial conclusions of the analysis on the length of restriction fragments, particularly with endonucleases using the molecular marker SSU in southern Benin. This study confirmed the identification of *Bulinus* species in Sô-Ava and Azowlissè in southern Benin using the PCR-RFLP method, which is a cost-effective means of identifying *Bulinus* species. Further research should be pursued to identify the species that has long been established in the Benin territory, which could clarify the species that serves as the main intermediate host of *S. haematobium* in Benin.

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Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this article.

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