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Molecular Identification of an Invasive *Sarotherodon* Species from the Atchakpa Freshwater Reservoir (Ouémé River Basin, Benin) and Comparison within *S. melanotheron* Using COI Markers

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Abstract: The Atchakpa freshwater reservoir (Ouémé Basin, Benin) was found to harbour an unexpected population of a cichlid species that was presumed to be *Sarotherodon melanotheron*. This species became dominant in the reservoir and became the main fisheries target species. We applied DNA barcoding to identify this population. Besides specimens from the reservoir, we also sequenced *S. melanotheron* from its native range in Benin at the lower Ouémé and Sô Rivers, and from Lake Nokoué, and Porto-Novo Lagoon. High sequence similarity indicated that all specimens were conspecific. Hence, we cannot exclude that a natural range extension led to the presence of the species in the reservoir. A comparison with sequences from NCBI GenBank confirmed that all samples belonged to the subspecies *S. m. melanotheron*, which is native to Benin. This comparison also showed that this subspecies was previously introduced in the Philippines. We call for further studies to investigate the socioeconomic, ecological and environmental impacts of the species in the Atchakpa reservoir.

Keywords: cytochrome c oxidase subunit I; black-chin tilapia; DNA barcoding; Savè; species introductions

1. Introduction

The black-chin tilapia, *Sarotherodon melanotheron* (Rüppell, 1852), is an estuarine species endemic to West and Central Africa [1–3] that is well known from the brackish waters of southern Benin. In the Ouémé River basin, its limit of frequent occurrence is the village of Agonlin-Lowé (Commune of Adjohoun, department of Ouémé) [4]. This locality is considered the boundary between the continental and the coastal domains in Benin [4] (Figure 1). However, rare specimens of *S. melanotheron* have been found upstream of Agonlin-Lowé in southern Benin (rare occurrences at the Ouémé River at Toué, Commune of Covè, department of Zou [4], at the Hlan River at Lokoli, Commune of Zogbodomey, department of Zou [5] and in the Lake Hlan at Kpomè, Commune of Toffo, department of Atlantic [6]) (Figure 1).

A large population of presumed *S. melanotheron* was discovered prior to 2001 in the Atchakpa freshwater reservoir (SUCOBE dam built in 1982), which is located in central Benin, about 230 km north of Agonlin-Lowé [4,7]. This population, which was only morphologically identified, thrived in the landlocked reservoir. Despite its proximity to



Figure 2. Satellite image highlighting the absence of direct communication between the main course of the Ouémé River and the Atchakpa freshwater reservoir (source: Google maps).

The fisheries in the Atchakpa reservoir ensure the supply of fish for the city of Savè. Currently, the population morphologically identified as *S. melanotheron* dominates the catches of local fishermen in terms of abundance. According to [8], *S. melanotheron* represents 72% (numerical abundance) of the fish caught at the Atchakpa reservoir and sold at the fish market in Savè. It thus surpasses the Nile tilapia *Oreochromis niloticus* (Linnaeus, 1758), which was introduced into the reservoir through aquaculture (direct restocking in 1985 and cage culture in 2014). Nile tilapia is currently rare whereas it was very common in the 1990s.

Sarotherodon melanotheron is known to be invasive, i.e., it has the potential to spread widely, reproduce rapidly and persist in a new environment with a large range of impacts [9,10]. Knowing the correct identity of an invasive species is an important prerequisite for its efficient management. The identification of invasive cichlids using only metric and meristic parameters requires caution due to their mega-diversity and the widespread introduction of alien species [11,12]. Therefore, molecular evidence is often required to provide additional verification. Hence, we chose to, for the first time in Beninese fisheries, apply a DNA barcoding approach. DNA barcoding is a molecular technique for fast and accurate determination of species identity [13]. In animals, DNA barcoding is usually performed by sequencing a fragment of the mitochondrial cytochrome c oxidase subunit I gene, COI (barcode), of the specimen under investigation and its comparison with a reference library of barcode sequences [14,15].

Sarotherodon melanotheron contains substantial intra-specific geographic structure, with three subspecies: *S. melanotheron melanotheron* Ruppell, 1852, *S. m. heudelotii* (Dumeril, 1859), *S. m. leonensis* (Thys van den Audenaerde, 1971) currently recognised [16]. The latter subspecies contains the synonym *S. m. paludinosus* (Trewavas, 1983) [16,17]. Additionally, *S. melanotheron* is highly similar to its sister species *S. nigripinnis* (Guichenot, 1861). Although the subspecies are difficult to distinguish on morphological grounds, they can be delineated with mitochondrial markers [16,17]. A molecular approach could hence allow us to confirm the subspecific nature of the *Sarotherodon* population, along with the implied geographic affinities. According to the management committee of the Atchakpa fisheries, no species of *Sarotherodon* has been stocked in the reservoir. However, it remains to be

verified whether the population in the reservoir belongs to the nearest strain from the Ouémé, or whether it has been introduced from elsewhere.

Tilapia species, including *S. melanotheron*, have been extensively introduced outside of their native range and often established feral populations. This also holds for *S. melanotheron*, which is, among others, found in the Philippines [9,18], Florida [19] and Hawaii [20]. As most tilapia introductions have been poorly documented, the correct identity of feral populations often remains unknown. DNA barcoding can aid in discovering the origin of introduced populations. However, as African freshwater fishes are underrepresented in barcoding libraries [21], there is a need to barcode specimens from the species' native range.

The aims of this study are twofold: (i) the molecular identification of the invasive cichlid species from the Atchakpa reservoir and (ii) constructing a databank of DNA barcodes of *S. melanotheron* from its range in the Ouémé River in Benin, and comparing these sequences with previously published sequences of the same species in GenBank.

2. Materials and Methods

Sampling took place at five sites in the Ouémé River in Benin (Figure 1). Besides the Atchakpa reservoir, we sampled in two brackish waters: Lake Nokoué and Porto-Novo Lagoon, and two freshwaters: Agonlin-Lowé (Ouémé River) and Sô-Ava (Sô River), where *S. melanotheron* is native. Sampling took place in January 2020 and specimens were identified using the identification key of [22]. At each site, twenty-two specimens of *S. melanotheron* were collected with the help of local fishermen. We also sampled at the localities of rare occurrence (Toué, Lokoli and Kpomè, see Figure 1), but failed to collect any specimens of *S. melanotheron* there.

Caudal fin clips were sampled and put into 1.5 mL sterile Eppendorf tubes containing pure ethanol as a preservation medium. Four specimens per site were fixed in formalin and deposited at the Royal Belgian Institute for Natural Sciences (RBINS) under collection numbers: Atchakpa water reservoir (25907, 25908, 25909, and 25910), Sô-Ava (Sô River) (25923, 25924, 25925, and 25926), Agonlin-Lowé (Ouémé River) (25911, 25912, 25913, and 25914), Porto-Novo Lagoon (25915, 25916, 25917, and 25918) and Lake Nokoué (25919, 25920, 25921, and 25922). Fin clips were stored at -20°C at the Molecular Systematics Laboratory of the RBINS.

Total genomic DNA was extracted using NucleoSpin[®] Tissue Kit following the instructions of the manufacturer (Macherey-Nagel, Düren, Germany). The mitochondrial COI gene region for each of five specimens per locality, except only four for Lake Nokoué, was amplified by polymerase chain reaction (PCR) using the 'Fish Cocktail': an M13 tailed primer combination of VF2_t1, FishF2_t1, FishR2_t1 and VR1d_t1 [23]. A standard 25.2 μL PCR mix consisted of 2.5 μL PCR buffer (10 \times); 2.5 μL dNTP (2 mM); 1.25 μL 'Fish Cocktail' (2 μM); 0.2 μL Taq DNA Polymerase (5 units per μL); 16.75 μL mQ-H₂O and 2.0 μL of the extracted DNA sample. The PCR profile was 3 min at 94°C , followed by 35 cycles of 40 s at 94°C , 40 s at 52°C , and 1 min at 72°C , plus a final extension of 10 min at 72°C . Amplicons were examined with 1% agarose gel electrophoresis. Products were purified with ExoSAP-IT[™] PCR Product Cleanup Reagent and bidirectionally sequenced using BigDye Terminator v.3.1. Fragments were analysed on an ABI 3130 capillary sequencer. Forward and reverse sequences were edited, used to build consensus sequences, visually checked in CodonCode Aligner 5.1.4 (CodonCode Corporation) and aligned in MEGA 5.2 using Muscle Alignment [24]. Sequences were compared to the database of NCBI GenBank using nucleotide BLAST (Basic Local Alignment Search Tool). Based on earlier studies, the sequence similarity of at least 98% was used to separate conspecific from heterospecifics [25,26].

We downloaded all 39 COI sequences of *S. melanotheron* available on GenBank (Table 1) and analysed them together with our 17 newly generated sequences (Dataset S1). We constructed a minimum spanning network using PopART [27]. Additionally, a neighbour-joining (NJ) tree [28] based on Kimura-2-Parameter (K2P) distances and using *S. galileus*

(KY84676) as an outgroup was constructed using MEGA 5.2. Nodal support was assessed with bootstrapping, using 1000 replicates. Kimura-2-Parameter (K2P) distances were calculated using unique haplotypes, obtained using FaBox 1.5.

Table 1. Information on newly generated COI sequences of *S. melanotheron* and those retrieved from GenBank.

Aquatic Ecosystems	Number of COI Sequences	Accessions Numbers GenBank	Codes for Our Samples	Country	Status	Reference
Atchakpa reservoir	05	MT180099 (1) MT180100 (1) MT180101 (1) MT180102 (2)	ATCH2 ATCH3 ATCH4 ATCH5 and ATCHF-H01	Benin		This study
Agonlin-Lowé (Ouémé River)	04	MT180103 (1) MT180106 (3)	FOUE3 FOUE2, FOUE4F-H03 and FOUE5F-G03	Benin	Native	This study
Sô-Ava (Sô River)	01	MT180104 (1)	RISO1	Benin	Native	This study
Porto-Novo Lagoon	04	MT180102 (4)	LAPO2F-E03, LAPO1F-F03, LAPO3F-D03 and LAPO4F-C03	Benin	Native	This study
Lake Nokoué	03	MT180107 (1) MT180108 (2)	LANO2 LANO3 and LANO1F-A03	Benin	Native	This study
Manila Bay Lake	04	KM212014- KM212016; KM212018	-	Philippines	Feral	[9]
Taal Lake	05	HQ654753- HQ654757	-	Philippines	Feral	[18]
Laguna de Bay Lake	05	HQ682721- HQ682725	-	Philippines	Feral	[29]
<i>Not specified</i>	01	<i>MT666031</i>	-	Hawaii	Feral	Unpublished
Odooba River	02	KX231781- KX231782	-	Nigeria	Native	[30]
Banc d'Arguin National Park (PNBA)	16	KJ938183- KJ938191 ; KJ938208- KJ938214	-	Mauritania	Native	[31]
Guiers Lake	04	KJ938215-KJ938218	-	Senegal	Native	[31]
State Zhongji Tilapia Farm, Hebei Province	02	NC_015611 JF894132	-	China	Farmed	[32]

In the column "Accessions numbers GenBank", the accession number in bold was mislabelled as *S. melanotheron* but instead belongs to *Coptodon guineensis*. Hence, it was not included in the phylogenetic analysis; the accession number in italics was shorter (313 bp vs. 443 bp) and was only directly compared to other sequences; numbers in brackets denote how many specimens are contained in a single sequence.

3. Results

3.1. Molecular Identification of the Specimens

A total of 22 partial mitochondrial COI sequences (five for each of the following ecosystems: Atchakpa water reservoir, Ouémé River and Porto-Novo Lagoon, and three and four for Lake Nokoué and Sô River, respectively) were successfully obtained from 24 samples. For 17 of these, a complete length of 443 bp was obtained (Table 1). The dataset

consisted of three unique haplotypes that differed by only one or two nucleotides. The first contained 14 samples from all locations. The other haplotypes contained two (one from the Atchakpa Reservoir, one from Porto Novo Lagoon), and one sample (from Agonlin-Lowé). BLAST revealed a similarity of 99.8–100% with sequences from *Sarotherodon melanotheron* deposited by [18] (HQ654754 and HQ654753) and [29] (HQ682724 and HQ682721). This confirmed the identification of our samples as *S. melanotheron*.

3.2. Comparison with Available Sequences of *S. melanotheron*

Thirty-nine COI sequences identified as *S. melanotheron* were retrieved from NCBI GenBank (Table 1). One of these (KJ938191) was considered mislabelled and was removed from further analyses. This sequence was coded as *Coptodon guineensis* in [31]. The COI sequence of this specimen matched that of *C. guineensis* and differed by at least 15% from all *S. melanotheron* sequences. We constructed a minimum spanning network (Figure 3) and an NJ tree (Figure 4) with all available sequences of length 443 bp. This revealed three clusters in *S. melanotheron*. The first cluster included all sequences from Benin (present study), as well as sequences from Nigeria, and some sequences from the Philippines (Taal Lake and Laguna de Bay). The sequence from Hawaii was, although shorter, identical to those of the main haplotype of cluster 1 (not shown). The second, central, cluster contained sequences from Mauritania and Senegal. The third cluster contained the remaining sequences from the Philippines as well as those from China. Maximum within-cluster divergence ranged from 0.23 to 0.45%, minimum between cluster divergence ranged from 0.68% (1 vs. 2), 0.91% (2 vs. 3) and 1.14% (1 vs. 3).

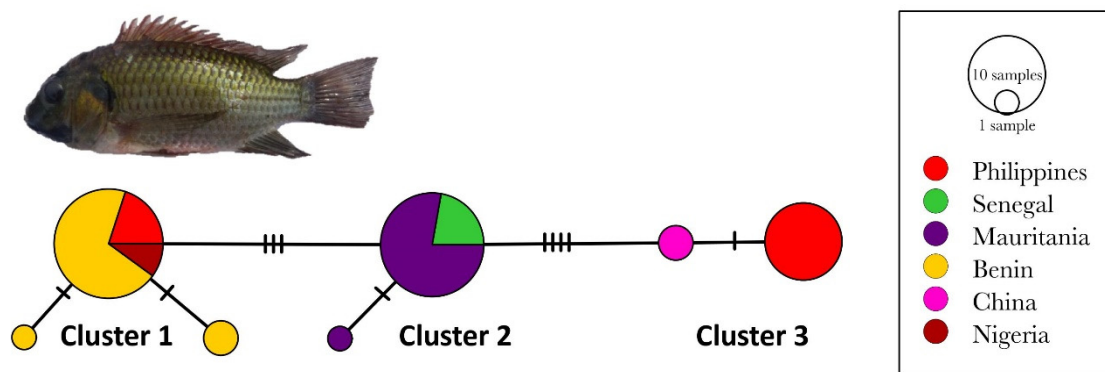


Figure 3. Minimum spanning network constructed from COI sequences of *S. melanotheron* obtained in our study and from GenBank. Colours denote countries where specimens were sampled, node size the number of sequences in a haplotype. The photograph represents a specimen of *S. melanotheron* from the Atchakpa reservoir (Photo credit: R.O.E. Pèlèbè).



Figure 4. NJ tree constructed from the COI sequences of *S. melanotheron* obtained in our study and from GenBank.

4. Discussion

In this study, the use of DNA barcoding allowed us to identify the cichlid population that became landlocked in the Atchakpa freshwater reservoir as *Sarotherodon melanotheron*. Although we could not unambiguously determine the origin of the population, we found

no indication that the population in the Atchakpa reservoir belongs to a different strain than the rest of the specimens from Benin. Hence, it could have arrived in the reservoir naturally, or via pumping of river water into the reservoir. The former explanation raises doubt on whether the reservoir has always remained physically separated from the river. The mechanisms by which *S. melanotheron* replaced *O. niloticus* remain unknown. As *S. melanotheron* has a broad ecological tolerance, its success could be linked to environmental parameters in the reservoir. This, however, remains to be investigated.

The three clusters in *S. melanotheron* found using COI correspond to those found by [33] using the mitochondrial control region, which corresponds to three of the four subspecies. These are *S. melanotheron heudelotti* found from the coasts of Mauritania to Guinea (cluster 2), *S. m. leonensis* occurring from Sierra Leone to western Liberia (most likely cluster 3), and *S. m. melanotheron* known from Ivory Coast to Nigeria (cluster 1) [16,17,30,31,33]. Although we lack sequences from native populations in cluster 3, it can be assumed that this cluster corresponds to *S. m. leonensis*. All sequences from Benin fell in cluster 1, which corresponds to *S. melanotheron melanotheron*. The comparison of COI sequences revealed interesting patterns about the introduced populations of *S. melanotheron*. The presence of a cluster 1 haplotype in the specimen from Hawaii is in accordance with the recorded introduction of the species on the islands in 1962 [20]. This strain derived via Europe and New York [34] from the area of the subspecies *S. m. melanotheron* in Africa [35]. As the farmed population in the State Zhongji Tilapia Farm in China [36] has a cluster 3 haplotype, it most likely stems from the range of *S. m. leonensis*. Finally, the populations present in the Philippines contain haplotypes of both clusters 1 and 3, which suggests that at least two introductions took place on the islands.

Although we were able to identify the invasive population in the Atchakpa freshwater reservoir, further studies on its socioeconomic, ecological and environmental impacts should be done. This study represents the first successful usage of DNA barcoding in Beninese fisheries research. Its success highlights that investigations must be undertaken to assemble a national fish DNA barcodes reference library in Benin, useful for reliable monitoring and effective management and conservation of fish biodiversity.

Supplementary Materials: The following is available online at <https://www.mdpi.com/article/10.3390/d13070297/s1>, Dataset S1: COI sequences used for constructing the NJ phylogenetic tree.

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Data Availability Statement: The data used in this study are openly available in NCBI GenBank (<https://www.ncbi.nlm.nih.gov/nuccore/?term=PELEBE>) and the dataset on COI sequences is in the Supplementary Materials.

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