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DNA Sequence Characteristics and Phylogenetics of Three Oligonucleotides Markers on Clariid Species

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Authors' contributions

This work was carried out in collaboration between all authors. Author OTA designed and carried out the experiment and wrote the first draft of the manuscript. Authors OOO and OAA supplied the software and interpreted the data. Author IA managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The aim of this study is to express the profiles of three oligonucleotide markers corresponded to reproductive genes that may be different between the two Clariid species (*Clarias gariepinus* and *Heterobranchus bidorsalis*) and their phylogenetics.
Methodology: Total DNA isolation was carried out on the whole blood of the two strains of Clariid species – 100 species *Clarias gariepinus* male and female (1.2 – 1.5 kg, 34 – 52 cm) separately; 100 species *Heterobranchus bidorsalis* (1.7 – 2.2 kg, 38 – 60 cm) respectively using the Quick-gDNA Zymo research kit. Having ascertained the DNA stability on 0.8% agarose gel, NCBI database and Clustal analyses were employed to design primers to reproductive genes that may be different between the two catfishes and may participate in their differential reproducibility. We have used quantitative real-time PCR to investigate the expression of three selected oligonucleotides markers on the catfish. CLC Sequence viewer 7 software was used to analyze the nucleotide alignment percentage and develop the phylogenetics tree.

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Place and Duration of Study: The study was carried out in the Biotechnology Centre, Federal University of Agriculture, Abeokuta Nigeria between January and July 2014.
Results: We observed a dimorphic expression pattern of the three marker genes in relation to strains and sex differentiation, indicating that sox9a retained its function in testis, Figα was highly expressed in the female and Cyp19a1b was up-regulated in male *C. gariepinus* than male *H. bidorsalis* catfish species. The phylogenetic tree showed that male Heterobranchus and female Clarias were closer irrespective of male or female while male Clarias differed from the two.
Conclusion: To date, these three genes, Sox 9a, Figα and Cyp19a1b have been detected in many fishes, but little or no data has been reported in African catfishes. The findings from this study might be used as the target gene for catfish gender regulation.

Keywords: Oligonucleotide; dimorphism; clariid species; polymerase; sequences.

1. INTRODUCTION

The culture of species of the catfish belonging to the Clariidae family is fast gaining global attention. In Africa, especially Nigeria, the species mostly cultured are *Clarias gariepinus, Heterobranchus* species and their hybrids [1]. They are widely cultured owing to their high market price, fast growth rate and ability to withstand adverse pond conditions especially low oxygen content [2].

Genetic enhancement of farmed fish has advanced to the point that it is now having an impact on aquaculture worldwide, but potential maximum improvement in overall performance is not close to being achieved [3]. As space for aquaculture becomes more limiting, the necessity for more efficient production or increased production within the same amount of space will further increase the importance of genetic improvement of aquaculture species. Genetic research and its application have had a significant role in the development of aquaculture, and this role and impact will become increasingly important as aquaculture develops further [3].

The polymerase chain reaction (PCR) is a technology in molecular biology used to amplify a single copy or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. A primer is a strand of short nucleic acid sequences (generally about 10 base pairs) that serves as a starting point for DNA synthesis. It is required for DNA replication because the enzymes that catalyze this process, DNA polymerases, can only add new nucleotides to an existing strand of DNA. The polymerase starts replication at the 3'-end of the primer, and copies the opposite strand [4].

Sox9 is a member of the Sry-related HMG box (Sox) gene family and is conserved in vertebrates. Among the mammalian Sox family, Sox9 has been extensively analyzed and is known to be critical for many aspects of cell differentiation such as chondrocyte specification, neural crest differentiation, heart valve development and male sex determination [5,6]. In mammals, the sex determining gene, Sry, is on the Y chromosome and directly upregulates the transcription of Sox9 in the supporting cells of the XY gonad only. Once Sox9 expression is established in the XY supporting cells, it is both functionally required and sufficient for testis determination [7].

Figα is a molecular marker for production of primary oocytes. Figα (gene factor in the germ line), a female specific gene marker, has also been implicated as playing important roles in early sex determination [8]. Like many other genes in the sex determination and differentiation pathways, figα appears to be conserved as it encodes a protein similar to that produced by figα in mice [9].

Cytochrome P450 aromatase (P450arom) is a member of the cytochrome P450 superfamily and is the terminal enzyme in the pathway responsible for generating sex steroid [10]. Aromatase is the only enzyme responsible for the irreversible conversion of androgens into estrogens [11]. In most vertebrates, P450arom is encoded by a single copy of the *cyp19* gene; however, two forms of the P450arom protein have sometimes been reported: P450aromA, which is encoded by *cyp19a1a* and mainly expressed in the ovary, and P450aromB, which is encoded by *cyp19a1b* and primarily expressed in the brain [6]. *Cyp19a1a* and *cyp19a1b* have different tissue distribution and expression patterns.

Therefore, the aim of the study is to investigate and characterize the expression patterns of three oligonucleotide markers on the African catfish species. The findings from this study might be used as the target gene for catfish gender regulation.

2. METHODOLOGY

2.1 DNA Extraction

Total DNA isolation was carried out on the whole blood of the two strains of Clariid species – 100 spp *Clarias gariepinus* male and female (1.2 – 1.5 kg, 34 – 52 cm) separately as well as 100 spp *Heterobranchus bidorsalis* (1.7 – 2.2 kg, 38 – 60 cm) respectively using the Quick-gDNA Zymo research kit, Epigenetic company USA. Deoxyribonucleic Acid was extracted from total volume of 1 ml whole blood of the two Clariid species in 1.5 ml microcentrifuge. These were carried out in the Department of animal breeding and Genetics laboratory, Federal University of Agriculture, Abeokuta Nigeria following the manufacturer instruction manual (www.Zymoresearch.com ver1.0.0).

2.2 PCR Analysis, Gel Electrophoresis and Sequencing

Polymerase chain reaction (PCR) reagents consisting of (Master mix having 10 X PCR buffer, DNTP, Reversed and Forward primer, Taq polymerase) and sterile water aliquot to 25 ul including the 1ul the species DNA while the RT-PCR consist of equal PCR reagent with the exception of PET 15e LMM DNA was ran on Thermocycler.

The PCR amplification was performed in a Biorad I-cycler programmed for initial denaturation step at 94°C for 3 minutes followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 43°C for 45 seconds and extension at 72°C for 1 mins, 15sec and terminal extension at 72°C for 10 minutes was carried out. The samples were cooled at 4°C.

Agarose gel electrophoresis was used for quantification of the amplified DNA. Agarose gel of 1.5%, weight per volume (w/v) by dissolving 1.6 gm of agarose (BP 1356-100) in 200 ml of 1xTBE (Tris-borate/EDTA electrophoresis buffer). The mixture was heat up on hot plate with magnetic stirring capability for 2 mins at 100°C. After boiling and melting, the gel was cooled to 50°C before adding 0.5 µg of Ethidium bromide (10 mg ml-1).

The gel (at about 40°C) was poured into the horizontal gel electrophoresis chamber with the well-forming comb and the agarose gel was allowed to solidify for about 30 minutes. The DNA samples were grouped into Clarias and Heterobranchus (male and female) respectively for each oligonucleotide primers. Minimum of 6 wells were created for each PCR sample, a ladder, the controls (positive and negative reactions). The loading consisting of 5 ul per DNA sample, 1ul blue juice, 1ul ladder and thereafter connected to power supply capable of providing 100V/50min.

After the electrophoresis, the gel was stained for 30 minutes with Coomassie blue consist of (10% acetic acid, 10% isopropanol and 0.25% Coomassie brilliant blue) and thereafter destained overnight in 100-150 ml destain solution consist of (10% acetic and 10% isopropanol). The gels were then viewed under UV Typhoon transilluminator.

3. RESULTS

3.1 Alignment and Phylogenetic Tree

The alignment of the raw sequences obtained from the DNA of fish sample using Sox 9a was done by CLC bioinformatics software, the alignment generated was later used to create the phylogenetic tree.

4. DISCUSSION

4.1 PCR Analysis and Sequencing

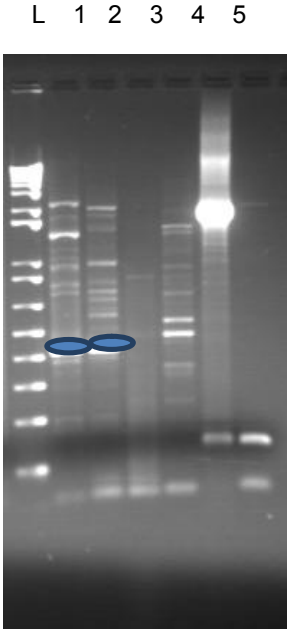
In Plate 1, there were eleven alleles found on Male Clarias sample, out of the 11, there are 3 major distinct bands and their sizes are 450 bp, 1200bp and 1500bp, the remaining bands are minor bands. Female Clarias had 9 alleles with 3 prominent bands and their sizes are 450 bp, 1200 bp and 1400 bp, the remaining bands are minor bands. Female Heterobranchus did not amplify and therefore had no major band while the male Heterobranchus had 9 alleles with 2 major bands and their sizes are 1500 bp and 1750 bp, the remaining are minor bands. The number of nucleotides obtained from the sequences of the Sox 9a for the Clarias and Heterobranchus ranged from 345- 400 nucleotides.

Two samples was amplified with the second primer Figα, the two samples were Clarias female and Heterobranchus female. There were 9 alleles found on female clarias sample, out of 9, there were 5 major distinct bands and their sizes were 1500 bp, 3000 kb and 3500 bp, 5000 bp, 5500 bp the remaining were minor bands. The Heterobranchus female had 2 alleles with 1 major band and the size is 750 bp, the other bands were minor. Three samples were amplified in the third primer, they were male Heterobranchus, female Heterobranchus and male Clarias. There were 7 alleles found in male clarias with 6 major distinct bands and their sizes were 500 bp, 1000 bp, 1750 bp, 2000 bp, 2250 bp, and 3000 bp, the remaining were minor bands. Male Heterobranchus had 9 minor bands and female Heterobranchus had 7 minor bands.

4.2 Alignment and Phylogenetic Tree Using Sox 9a Primer

There were 75 nucleotides generated by the alignment and out of the 75 nucleotides seven had 100% consensus (Fig. 1). The phylogenetic tree showed that male Heterobranchus and female Clarias were closer using the oligonucleotide markers while male Clarias differed from the two (Fig. 2).

The three oligonucleotide markers of Clariid spp revealed that there were similarities and differences in the genomes of the fish. Sox-9a had a band of 500 bp common to most of the samples, alignment showed that there were differences within the band extracted. Sox-9a also revealed that male Heterobranchus and female Clarias are genetically similar. The two varieties were closely related irrespective of male or female. Figα marker only amplified with female Clarias and female Heterobranchus, the marker did not amplify male Clarias and male Heterobranchus. There was a lot of differences that existed using Figα marker. This observation corroborated with the findings [11], who stated that Figα was upregulated in the female of both catfish species and was an oocyte-specific gene marker, making it a useful tool for determining the development of ovarian tissue.



L - Ladder

1) CM Clarias male
2) CF Clarias female
3) HFHeterobranchus female
4) HMHeterobranchus male
5) Positive reaction (+)
6) Negative reaction (-)

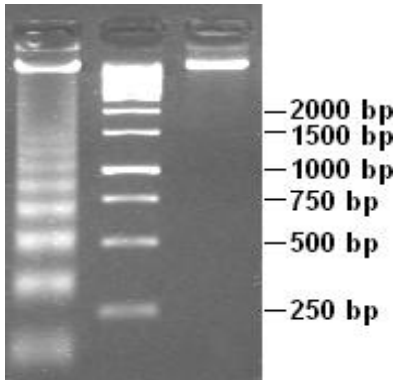


Plate 2. Bio-rad molecular marker

Plate 1. 1.5% Agarose PCR Gel for only sox9a Primers

--Sliced DNA fragments

Table 1. Three oligonucleotides sequences

Primer name	Forward primer sequences 5' - 3'	Reverse primer sequences 5' - 3'
Sox9a	CGGTGAAGAACGGCCAGAGC	CTGTAGAGTCAGCAATGGGT
Figα α	ATGTCGTGTGAAATGACCGGC	CTAGGATGGGAGTGAACTTGG
Cyp19a1b	AACATTGGACGCATGCATAA	TGTTTGATGGTGCTGATGGT

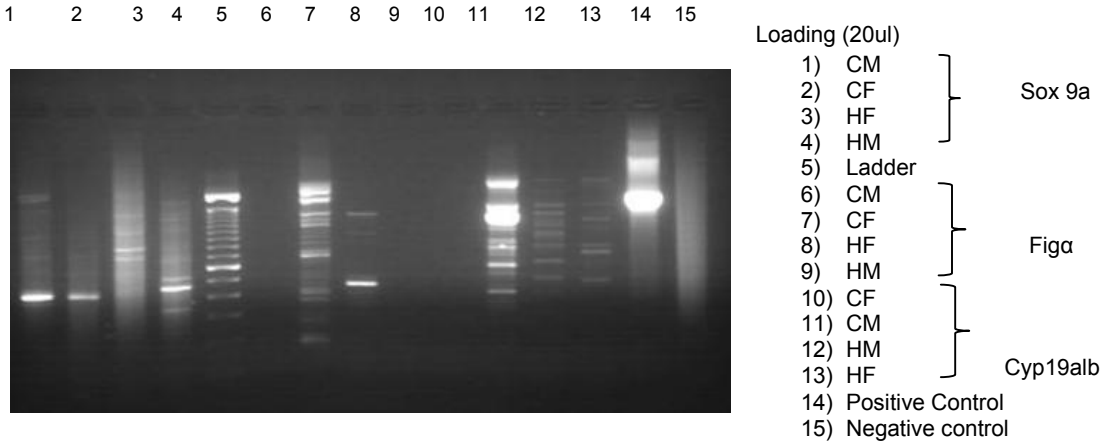


Plate 3. 1.5% Agarose PCR Gel of 3 markers; Sox9a, Figa and Cyp 19a1b

CM - Clarias male; CF - Clarias female; HF - Heterobranchus female; HM - Heterobranchus male.

Note: *Plate 1 is 1.5% Agarose PCR Gel for only Sox 9a marker extracted from plate 2 for clarity and was the only marker possessing equal species' weight sliced for alignment, therefore only Sox 9a could undergo alignment. Plate 2 is a combination of 1.5% Agarose PCR Gel for the three markers sox 9a, Figα and Cyp 19a Ib*

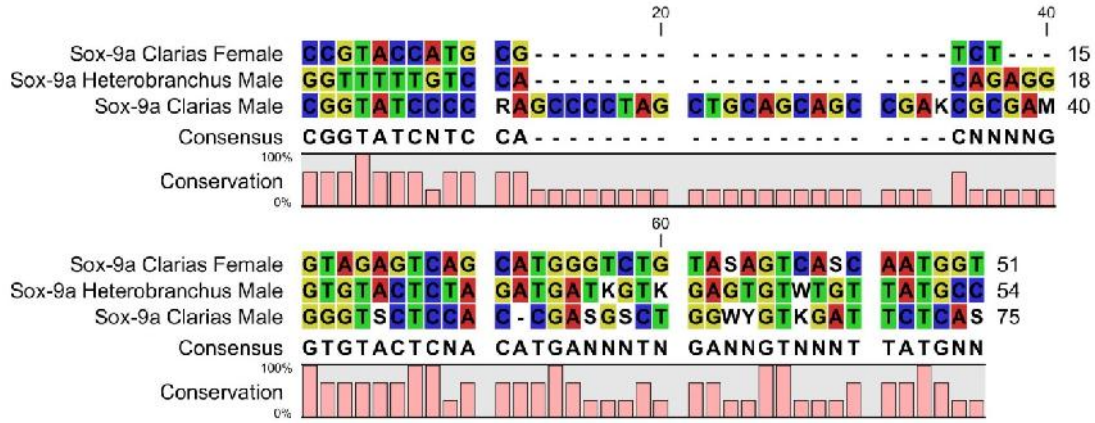


Fig. 1. Alignment for Sox-9a primer for fish samples

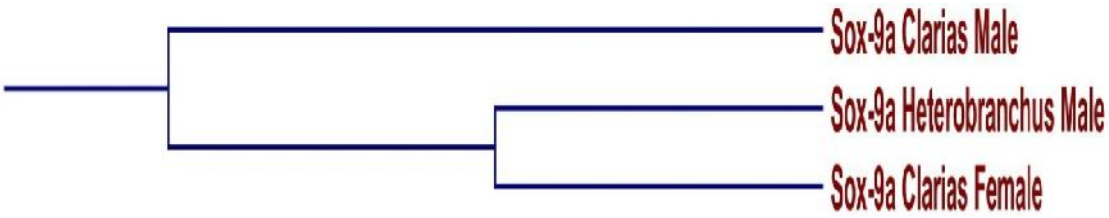


Fig. 2. Phylogenetic tree for fish samples

Sox 9a was much dominant in male catfish species having major bands in both Clarias and Heterobranchus. This could be supported by [6,12] who revealed that Sox 9 was first identified as the gene responsible for autosomal XY sex reversal associated with campomelic dysplasia, a human skeletal malformation syndrome. Sox9 was the direct target of the testicular sex determining gene, Sox9 transcription was up-regulated in the presence of Sry in developing pre-Sertoli cells during male development [13,14]. To date, the sox9 gene has been identified in several vertebrate species including mammals [6,12,13], birds [15], reptiles [7,16,17], amphibians [18], and fishes [19-21].

The Cyp19a1b was expressed in both Heterobranchus and Clarias species, up-regulated in Clarias male with 7 major distinct bands but down-regulated in male Heterobranchus. This was in support of [10] who observed cytochrome P450 aromatase (P450arom) as a member of the cytochrome P450 superfamily and is the terminal enzyme in the pathway responsible for generating sex steroids, which plays an important role in maintaining the physiological balance between the sex steroid hormones. P450aromB, which is encoded by *cyp19a1b* and primarily expressed in the brain [5].

5. CONCLUSION

To date, these three genes have been detected in many fishes, including *Danio rerio* [20], *Carassius auratus* [22], *Oreochromis niloticus* [23], *Oncorhynchus mykiss* [24], *Cyprinus carpio* [25] but few or no information has been reported in African catfishes.

Therefore the oligonucleotide markers - Sox 9a. Figα, *cyp19a1b* are likely to play an important role in gonad-differentiation. The expression of *these* genes could be used as markers to detect the gonad differentiation and also might be used as the target gene for catfish gender regulation.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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