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Evolutionary relationship among two catfish species by Single Nucleotide Polymorphisms (SNPs) DNA markers

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Abstract
The three dams in Niger State, Nigeria, that were investigated for evolutionary relationship among two catfish species (*Clarias gariepinus* and *Heterobrachus bidosalis*) are Agaie/Lapai dam, Shiroro dam and Tagwai dam. Ten samples of each species were collected from the study dams and they were subjected to single nucleotide polymorphic genotyping. Four SNPs primers, which consist of growth hormone and early growth response gene were utilized to amplified the DNA. Bands were generated from all the samples from the study dams in all the primers used for the amplification, which gives strong indications that, all the samples been genotyped are rich in both growth hormone and early growth response gene. Further-more, two samples from each species from the study dams were sequenced, then subjected to Bio-edit software to draw the evolutionary relationship tree. The samples were grouped into two main branches by the dendrogram in Bio edit software that draws the evolutionary relationship tree, the first branch are made up of all the *C. gariepinus* samples and the second branch comprises of all the *H. bidosalis* samples from the three dams, the two main groups show by the dendrogram reveals that both species are evolutionarily not very close to each other and this also give the reassurance of the two species belonging to different species and genera.

Keywords: Growth gene, evolution, sequencing, stock enhancement, dendrogram

Introduction
In Africa and South East Asia, catfishes are highly distributed across their wild waters and they serve as one of the highest numbers of wild fresh water fish caught in those regions. 14 genera of catfish species have been reported by (Teugels, 1986) [15] in Africa region and two genera in South East Asia with high genetic variability. Catfishes have surpassed Tilapia in global aquaculture production and helped in part by the sheer number of their extensive geographical range (Lutz 2021) [10].
Catfish species especially *Clarias gariepinus* (African catfish), is widely culture in Africa and globally because of its high commercial value (Fagbenro *et al*., 2013) [6]. In 2015, a total of 160, 265 tons of catfish were produced globally and Malaysia taking the lead, followed by Nigeria as catfish producing countries (FAO, 2017) [7]. Global ranking has placed Nigeria as the highest producer of catfish with total production of 1.2 million tonnes (Malomo, 2023) [11]. Catfish species have become a model fish for aquaculture due to its commercial value and favourable characteristic such as ability to withstand hash weather condition, omnivorous feeding, high reproduction, resistance to diseases and high growth rate (Agbebe *et al*., 2013) [1]. The two species of catfish that was investigated on are *Clarias gariepinus* and *Heterobranchus bidorsalis* and the genus *Clarias* can easily be differentiated from genus *Heterobranchus* by the presence of adipose fin in *Heterobranchus* and no adipose fin in *Clarias* (Moses & Olufeagba 2005) [12].
Molecular markers are being referred to as any steady differences, that can be transfer, quantify or detected by a suitable experimental process. These differences can be manifested at various levels such as, morphological level, genetical level, chromosomal level, biochemical or genomic level. Any markers that show differences that is seen at the DNA level are called molecular markers and they are found at specific location in the genome and have the ability to "mark" the location of a particular gene or the transfer of a specific trait (Dudu *et al*., 2015) [3]. Single nucleotide polymorphisms (SNPs) are variations in the genomic DNA as a result of

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single nucleotide differences or single nucleotide addition or removal and they are inherited in a co-dominant fashion (Okumu and Çiftci, 2003) [13]. SNPs are found in both coding and noncoding locations of the genome and SNP substitution polymorphisms alter the amino acid while SNP of similar polymorphisms alter the codon but not the amino acid and SNPs serving to regulate (regulatory polymorphisms) can change gene regulation (Dunham, 2011) [4]. Hess *et al*. (2011) [8] noted that, SNPs have high processing efficiency, ease to score and highly consistence between laboratories, which makes SNPs a marker choice for various genetic investigations. DNA sequencing, primer extension typing and the designing of allele-specific oligo and gene-chip technology are the techniques that can be used to determine the single base variation or SNPs in species (Dunham, 2004) [5]. The important of genetic information cannot be over emphasized, it helps in revealing the evolutionary relationship among species, in-depth and better presentation of taxonomy, evolution, species identification, species genetic variability and diversity in natural populations, compare genetic diversity and variation of natural and farmed populations, assessment of demographic bottleneck in natural population and stocking and restocking enhancement programmes (Chauhan & Rajiv, 2010) [2]. This has made it imperative that data from genetic analysis gives better knowledge in management and conservation strategies. Therefore, the knowledge of evolutionary relationship among *C. gariepinus* and *H. bidorsalis* based on genetic data in Niger State, Nigeria is important for management and conservation approach because presently is not available.

Materials and Methods
Sampling of experimental fish
Ten samples of *C. gariepinus* and *H. bidosalis* were sampled from three dams, namely Agaie/Lapai dam, Shiroro dam and Tagwai dam in Niger State, Nigeria for the single nucleotide polymorphisms (SNPs) amplification and out of these ten samples, two samples were picked from each species (*C. gariepinus* and *H. bidosalis*) from the three study dams for the cycle sequencing. The caudal fin of all the fish sampled for the experiment were cut into well-labelled specimen bottles and kept in the refrigerator at -17 °C for further processing.

Isolation, purity and quantification of genomic DNA
The protocol described in Quick-DNA[TM] Miniprep Plus kit was followed to isolate the DNA from the caudal fin, revised 2021(www.zymoresearch.com), by following the guidelines step by step with little changes. The purity of the DNA isolated was checked by using a Nano-drop spectrophotometer and measure the absorbance at 260 nm to the absorbance at 280 nm which gives reading that range from 1.7-2.0. The quantification of isolated DNA was carried out by 1% agarose gel electrophoresis with buffer solution of 0.5x TBE at 80 volts for 1hr 30 mins and stained with 5 μl ethidium bromide. The gel pictures ware captured under UV light (Thermo Scientific, USA), photographed and saved in the system. The total volume of DNA isolated from each sample was 200 μl and these were kept in the refrigerator at -17 °C before being utilized for single nucleotide polymorphisms amplification. These experiments were done at the Bioscience center of the International Institute of Tropical Agriculture (IITA) in Ibadan, Nigeria.

Single nucleotide polymorphisms amplification, electrophoresis and cycle sequencing
The Single nucleotide polymorphisms amplification was

conducted by utilizing one growth hormone and three early growth response gene single nucleotide polymorphisms primers, the amplification reaction contained 2.5 μl of 10x PCR buffer,1 μl of 25 mM MgCl$_2$, 1 μl each of forward and reverse primers, 1μl of DMSO, 2 μl of 2.5 mM DNTPs, 0.1 μl of 5 ng/μl Tag DNA polymerase and 3 μl of 10 ng/μl DNA, the final reaction volume was raised to 25 μl by adding 13.4 μl of deionized water. The electrophoresis of the amplifies generated was carried out using 2% agarose gels with buffer solution of 0.5 x TBE at 80 volts for 1hr 30 mins and stained with 5 μl ethidium bromide, the gel picture was captured under UV light (thermos scientific, USA), photographed and saved in the system. The gel picture shows the presence of the growth hormone and early growth response gene in the experimental fish. The cycle sequence was conducted by following the protocol for BigDye terminator v 3.1 cycle sequence kit in an automated sequencing machine (ABI 3500). The cycle sequencing amplification reaction contained 2 μl of BigDye sequencing buffer, 4 μl of ready reaction premix, 3 μl of PCR product, 2 μl of primer and the final reaction volume was raised to 20 μl by adding 9 μl of nuclease free water. These were conducted at the Bioscience center of the International Institute of Tropical Agriculture (IITA) in Ibadan, Nigeria. The evolutionary relationship tree (dendrogram) was drawn by utilizing BioEdit software and tree view X software to view the tree.

Results and Discussion
Single Nucleotide Polymorphisms (SNPs) amplification products generated by utilizing one growth hormone and three early response growth gene sequence which were gotten from GenBank (https://www.ncbi.nlm.nih.gov) with version number Af416488.1, JX470096.1, JX470060.1 and JX470024.1. The primers were design using primer premier version 6.1 software (Premier Biosoft international, USA) to show the presence or not of these growth-relate gene in the indigenous cat fish species (*C. gariepinus* and *H. bidosalis* from three dams as shown in plate 1-8. The four SNPs primer designed that were used for the experiment generated bands at different loci in both species. SNPs primer 1(GH) generated bands at 350 bp loci, SNPs primer 2 (EGR 3) generated bands at 250 bp, SNPs primer 3(EGR 1) generated bands at 500 bp and SNPs primer 4 (EGR 2B) generated bands at 300 bp as presented in table 1.
The various loci reveal by the primers in all the samples collected for the analysis from the study dams, shows the presence of all the four growth related genes in the catfish species that was investigated on and this is an indication that there is a high potential for growth when water temperature, dissolve oxygen, PH and turbidity are in optimum and availability of food.

Evolutionary relationship
The evolutionary relationship among *C. gariepinus* and *H. bidosalis* was drawn by utilizing the Unweighed Paired Group Method of Arithmetic Mean (UPGMA) dendrogram in BioEdit software. In each primer, the UPGMA dendrogram divides the two species into two main group. The first group consist of six (6) samples of *C. gariepinus* and the second group consist of six (6) samples of *H. bidosalis,* as presented in fig 1.
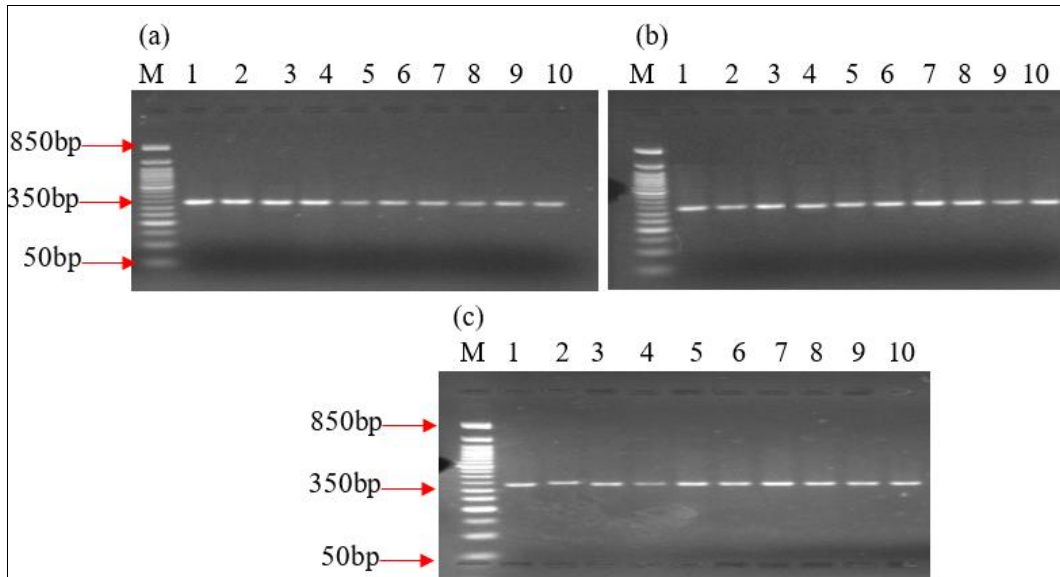
Plate 1: SNPs primer 1(GH) genotyping, (a) Agaie/Lapai site, (b) Shiroro site, (c) Tagwai site, M: DNA ladder (50bp), 1-10: *Clarias gariepinus* samples.
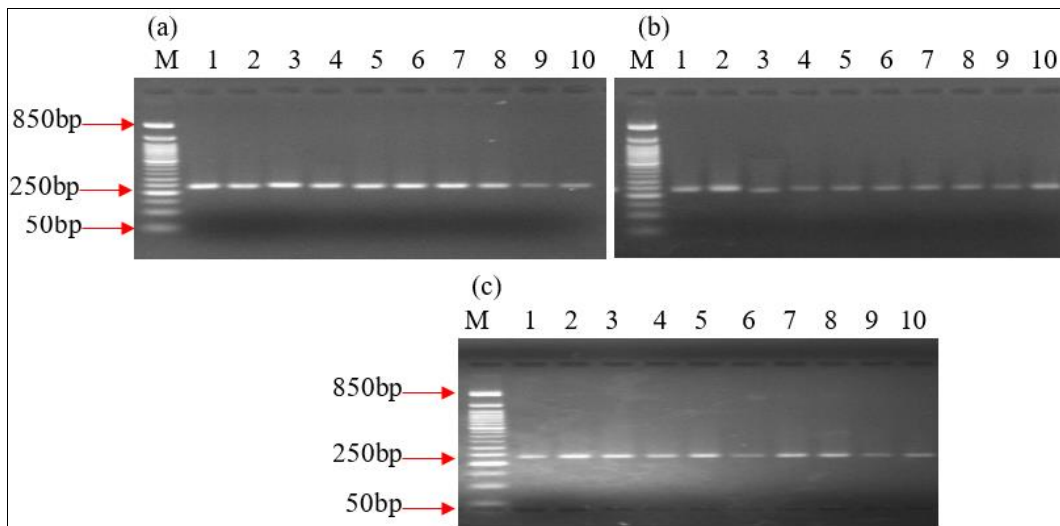

Plate 2: SNPs primer 2 (EGR 3) genotyping, (a) Agaie/Lapai site, (b) Shiroro site, (c) Tagwai site, M: DNA ladder (50bp), 1-10: *Clarias gariepinus* samples.
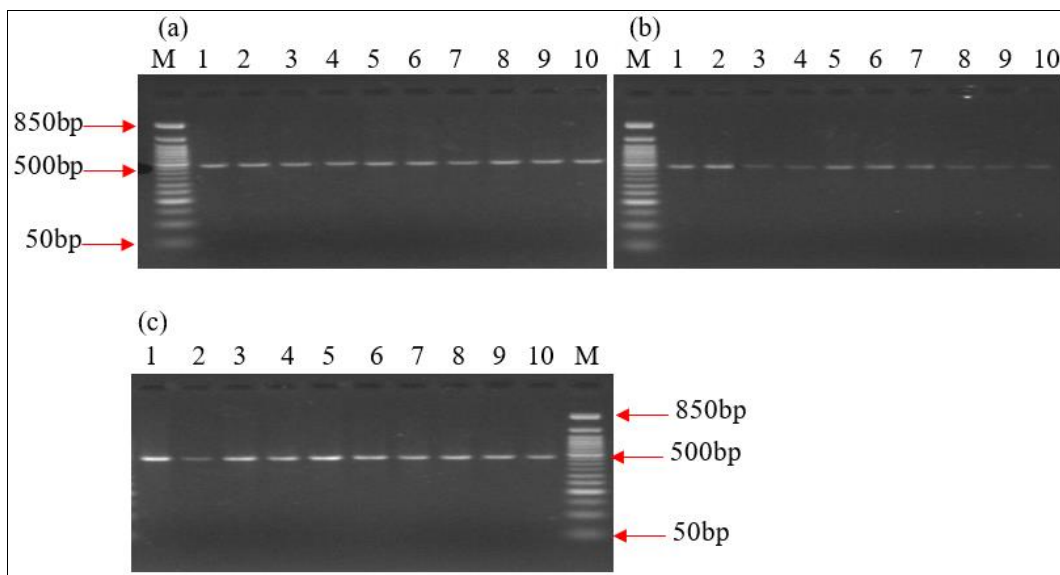

Plate 3: SNPs primer 3 (EGR 1) genotyping, (a) Agaie/Lapai site, (b) Shiroro site, (c) Tagwai site, M: DNA ladder (50bp), 1-10: *Clarias gariepinus* samples.
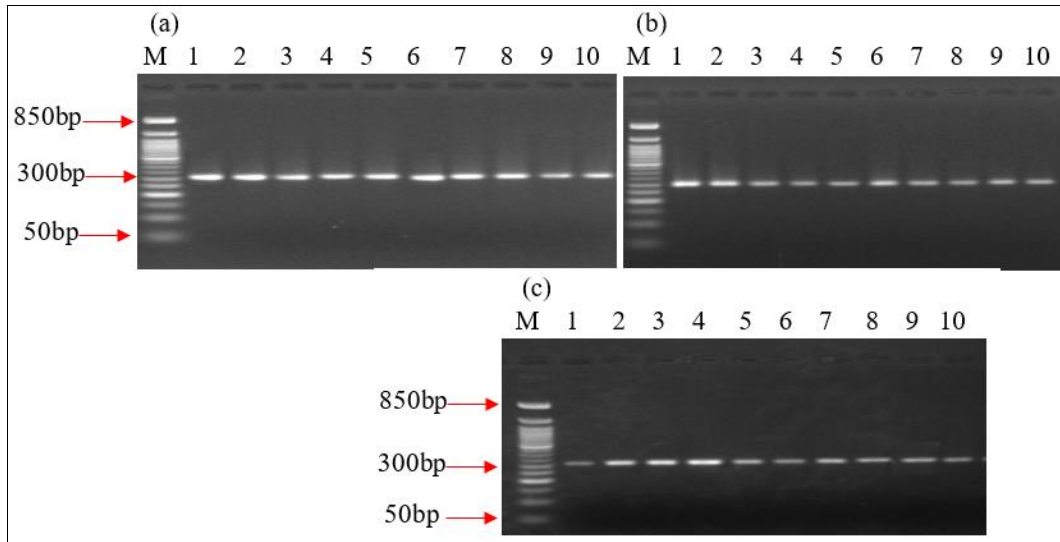
Plate 4: SNPs primer 4 (EGR 2B) genotyping, (a) Agaie/Lapai site, (b) Shiroro site, (c) Tagwai site, M: DNA ladder (50bp), 1-10: *Clarias gariepinus* samples.
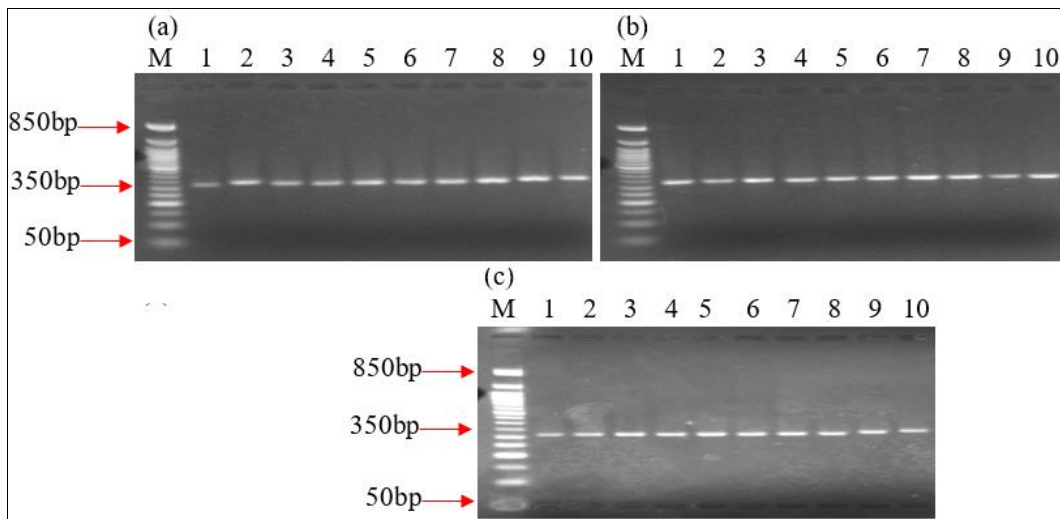

Plate 5: SNPs primer 1(GH) genotyping, (a) Agaie/Lapai site, (b) Shiroro site, (c) Tagwai site, M: DNA ladder (50bp), 1-10: *Heterobranchus bidosalis* samples.
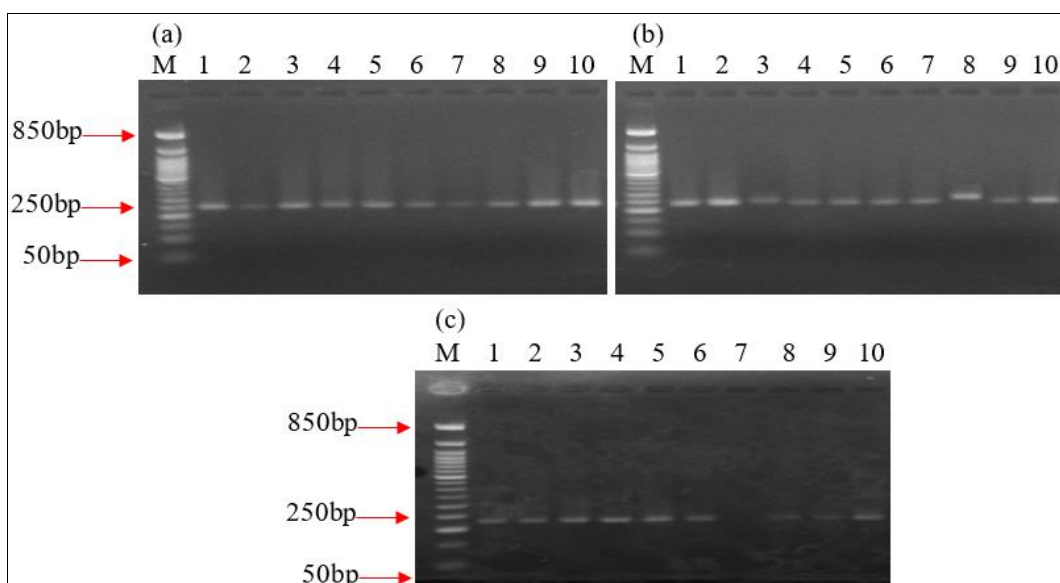

Plate 6: SNPs primer 2 (EGR 3) genotyping, (a) Agaie/Lapai site, (b) Shiroro site, (c) Tagwai site, M: DNA ladder (50bp), 1-10: *Heterobranchus bidosalis* samples.

Plate 7: SNPs primer 3 (EGR1) genotyping, (a) Agaie/Lapai site, (b) Shiroro site, (c) Tagwai site, M: DNA ladder (50bp), 1-10: *Heterobranchus bidosalis* samples.

Plate 8: SNPs primer 4 (EGR 2B) genotyping, (a) Agaie/Lapai site, (b) Shiroro site, (c) Tagwai site, M: DNA ladder (50bp), 1-10: *Heterobranchus bidosalis* samples
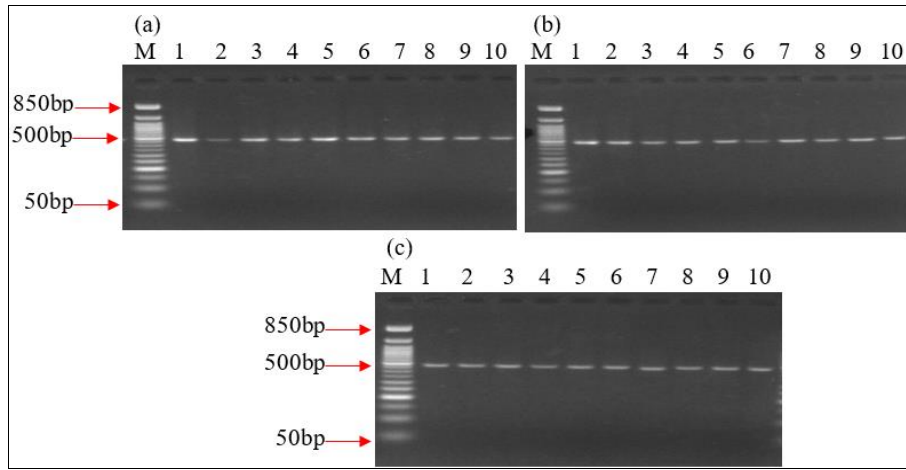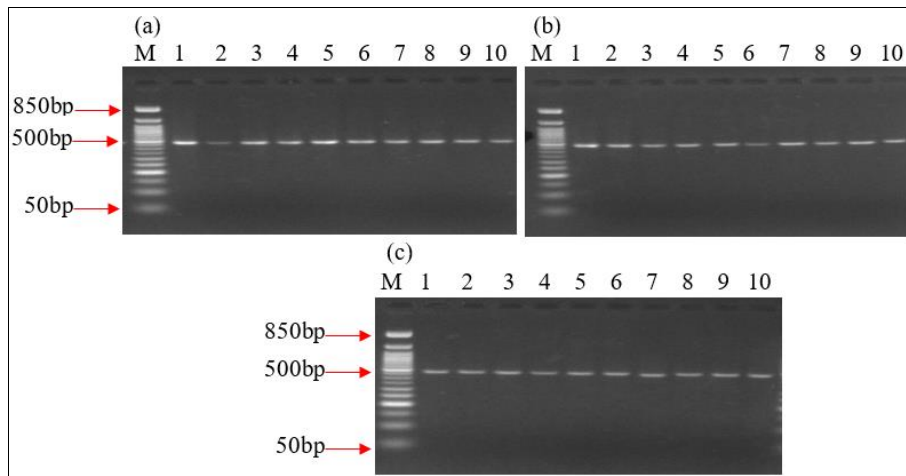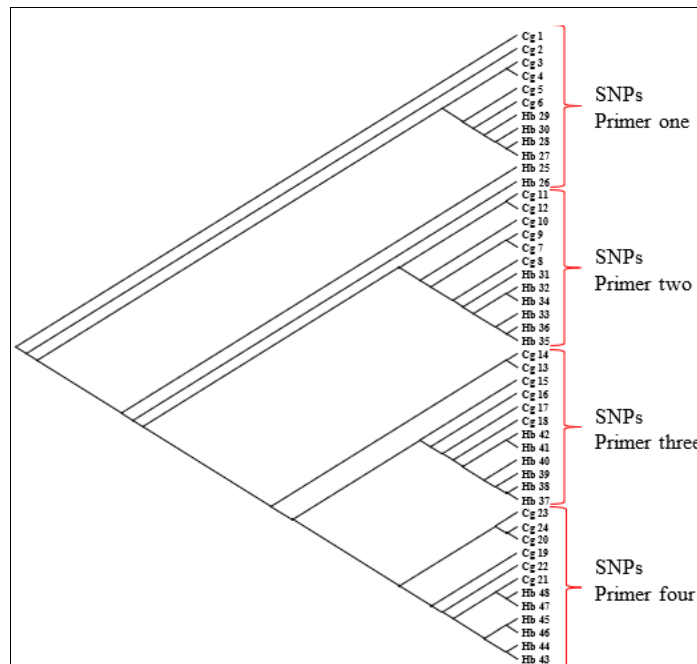

Fig 1: Evolutionary relationship tree (dendrogram) among *C. garipinus* and *H. bidosalis* from three dams. For *C. gariepinus* primer one Agaie/Lapai (1 and 2), Shiroro (3 and 4) and Tagwai (5 and 6) and *H. bidosalis* Agaie/Lapai (25 and 26), Shiroro (27 and 28) and Taqwai (29 and 30). For *C. gariepinus* primer two Agaie/Lapai (7 and 8), Shiroro (9 and 10) and Taqwai (11 and 12) and *H. bidosalis* Agaie/Lapai (31 and 32), Shiroro (33 and 34) and Taqwai (35 and 36). For *C. gariepinus* primer three Agaie/Lapai (13 and 14), Shiroro (15 and 16), Taqwai (17 and 18) and *H. bidosalis* Agaie/Lapai (37 and 38), Shiroro (39 and 40), Tagwai (41 and 42). For *C. gariepinus* primer four Agaie/ Lapai (21 and 22), Shiroro (23 and 24), Tagwai (25 and 26) and *H. bidosalis* Agaie/Lapai (43 and 44), Shiroro (45 and 46), Tagwai (47 and 48).

Table 1: Primer name and nucleotide sequence use in Single Nucleotide Polymorphisms genotyping with their annealing temperature and band sizes

SNPs	Primer name Primer sequence Ta(^{0}C) Band size (bp)
1	GH Sense: AGAGCAACGGATGAGTAAC 53.2 350 Anti-sense: TGAAGCAAGACAGCAGAC
2	EGR 3 Sense: CCTCCTCATCACTGTCCTA 56.5 250 Anti-sense: GCTGCTGCTTGTCCTTAA
3	EGR 1 Sense: GTCCTCTTCCTCCTCTTCT 57.1 500 Anti-sense: TGTGGTGTCTTACTTG
4	EGR 2B Sense: GAGCATCAGCACATCTACT 58.6 300 Anti-sense: GTGTTGAGTGGCGTAAGA

Ta: Annealing temperature, bp: base pair

The dendrogram separates the population into two main groups in each primer, the first group comprises of *C. gariepinus* and the second group comprises of *H. bidosalis* from three dams. The two main groups shows that both species are evolutionarily not very close genetically, which shows that they are of different species and different genera. The dendrogram also reveals that Agaie/Lapai dam catfish species are closer genetically to Shiroro dam catfish species than Tagwai dam catfish species. Tagwai dam catfish species are closer genetically to Shiroro dam catfish species than Agaie/Lapai dam catfish species. The genetic closeness and distant exhibited by the species in various sampling sites can be attributed to gene flow, genetic drift, source of water into the dams and geographical location of the dams. Sokenu *et al.* (2020) [14] reported genetic distant between *Oreochromis niloticus* and *Sarotherodon melanotheron* by utilizing SNPs growth gene (IGF-I) which agrees with the results obtained in this research.

The significant of SNPs cannot be over stated, SNPs locations exist in the whole genome (30, 000000 various locations of SNP have been detected in humans), they exhibit high polymorphism and it is the only analysis that reveal every single variation or polymorphism between populations (Dunham, 2011) [4]. SNPs can be detected at every 76 to 2000 bp in different organisms and at every 76 bp SNPs were detected in channel catfish, also the myostatin gene in channel catfish has both microsatellites and many SNPs (Kocabas *et al.*, 2002) [9].

Conclusion
In conclusion, the dendrogram shows that the two catfish species are not very close genetically and this also gives the reassurance of the two species belonging to different species and genera. In stocking enhancement program for management and conservation, samples from Shiroro dam may be used to enhance stock in both Agaie/Lapai dam and Tagwai dam because they are of the same level of genetic similarity to both sites and when this is being embark on, genetic variability among catfish species in these dams needs to be checked.

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