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Full Length Research Paper

Preliminary characterization of genetic strains in clariid species, *Clarias gariepinus* and *Heterobranchus bidorsalis* using microsatellite markers

O. T. Agbebi[1]*, D. E. Ilaboya[1] and A. O. Adebambo[2]

[1]Department of Aquaculture and Fisheries Management, Federal University of Agriculture, PMB 2240, Abeokuta, Ogun state, Nigeria.
[2]Department of Animal Breeding and Genetics, Federal University of Agriculture, PMB 2240, Abeokuta, Ogun state, Nigeria.

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Two Clariid species, *Clarias gariepinus* and *Heterobranchus bidorsalis* were compared at four loci using *C. gariepinus* microsatellite markers (*Cga01*, *Cga02*, *Cga03*, *and Cga05*). The heterozygosities observed were found to be 0.450 ± 0.050 and 0.442 ± 0.127, while 100% polymorphism was observed in both species. Overall, 95% of the samples amplified upon polymerase chain reaction (PCR) amplification and 44.3% of the total alleles observed for all the loci were heterozygote. Conformity to Hardy-Weinberg equilibrium using the Chi-Square test showed that 81.25% of locus-population relationship conformed to Hardy-Weinberg equilibrium. The neighbor-joining phylogenetic dendrogram obtained gave a bootstrap value of 72, indicating that the genetic distance between the two species is distinct.

Key words: Microsatellite, *Clarias gariepinus*, *Heterobranchus bidorsalis*.

INTRODUCTION

The family Clariidae belongs to the order Siluriformes and contributes significantly to annual freshwater fish production in South and Southeast Asia and Africa (Na Nakorn, 1999). This family is naturally distributed all over Africa, South and South-East Asia with the highest genetic diversity found in Africa. Nearly one-fifth of all known catfish species occur in Africa and South-East Asia; however, the highest diversity is found in Africa with 14 genera and 92 species (Teugels, 1986a). *Heterobranchus bidorsalis* (Geoffroy Saint-Hilaire, 1809) and *Clarias gariepinus* (Burchell, 1822) contribute immensely to the annual fresh water fish production in Nigeria (Fagbenro et al., 1993). They are commonly referred to as mud fishes or African catfish in various parts of Nigeria, and are important source of animal protein. Among the freshwater species for culture in Nigeria, *H. bidorsalis* and *C. gariepinus* are the most common and have received much attention and acceptability because of their economic importance and desirable attributes such as hardiness, high palatability, high fecundity, disease resistance and fast growth. Hence, they command high commercial values (Agbebi et al., 2009).

C. gariepinus culture started almost 50 years ago in Africa and in 1994, Garibaldi (1996) reported a production of 3,978 metric tons in Africa, whereas *Heterobranchus* was recently introduced in aquaculture and has been reported to show promising results (Teugels and Gourène, 1997). Legendre et al. (1992) demonstrated that under identical conditions, *Heterobranchus longifilis* has a growth rate which doubles that of *C. gariepinus*. Experiments with *H. bidorsalis* have recently been conducted in Nigeria (Fagbenro et al., 1993), but data on the production *of Heterobranchus* species are not yet available. Despite the popularity of these two species and the great market

*Corresponding author. E-mail: agbebi20@yahoo.com.

potentials, the production is still low, basically at subsistence level due majorly to inadequate availability of seed for stocking, feed and marketing problems. The fingerlings supplied by hatcheries in Nigeria are not sufficient to meet farmers' fingerlings needs, hence, there is need for improvement of fingerlings production majorly through genetically modified fish breeding that ensures a high success rate of quality fingerlings production. The precise description and characterization of strains in these species is *sine qua non* to the sustainable management of their cultivated and natural stocks and to guide conservation efforts of these economically important resources.

Microsatellite markers are highly polymorphic genetic markers used to identify locations on the chromosome associated with specific strains in an organism. Microsatellites have a small locus size that allows them to be easily assayed by polymerase chain reaction (PCR). The use of microsatellites markers gives deeper knowledge about the domestication and improvement of desirable traits in breeds. Microsatellites have a major drawback of being expensive and labor-intensive. They are widely used in diversity and phylogeny studies, assessment of inbreeding level in a population, gene mapping, verify cytogenetic treatments such as induction of polyploidy or gynogenesis (Magoulas et al., 1997). The use of microsatellites has become a standard method to estimate natural genetic diversity in livestock (Erhardt and Weimann, 2007). Microsatellite loci characteristically exhibit high levels of length mutation, resulting in extensive allelic variation and levels of heterozygosity in fish ranging from 24 to 90% (O'Connell and Wright, 1997).

Results from previous studies have shown that microsatellite is the tool of desire for population monitoring, preservation of genetic diversity and breeding in aquaculture (Kotoulas et al., 1997). *C. gariepinus* primers have been used to amplify microsatellite loci in other species including *Clarias anguillaris, H. longifilis*, *Clarias alluaudi* and Cyprinids (Galbusera et al., 1996). The aim of this study was to therefore compare *C. gariepinus* and *H. bidorsalis* based on their genetic composition, which is an important tool in fish breeding and genetics.

MATERIALS AND METHODS

Sample collection

The study was carried out on 10 domestic samples each of *C. gariepinus* and *H. bidorsalis* from an earthen pond in Abeokuta, Ogun State, Nigeria. Blood samples were collected from each sample from the lateral line at the dorsal end and immediately transferred to FTA® Classic Cards (Whatman Bioscience, Maidstone, UK) and left to air dry.

DNA extraction

Genome DNA was extracted from the FTA® Classic Cards as follows: five 1.2-mm discs of the FTA® classic cards were punched into 1.5 ml Eppendorf tubes using Harris micro-punch® and 1000 µL of 100 mM Tris-Base and 0.1% sodium dodecyl sulphate (SDS) buffer was put into each Eppendorf tube. The tubes were vortexed for 30 min, after which the supernatant was discarded. Thereafter, 500 µL of 5 M guanidine thiocyanate was added, vortexed for 10 min and discarded. Next, 500 µL nuclease free water was added and vortexed for 10 min, and then the supernatant was discarded. The same volume of nuclease free water was added and left to stand for 10 min, after which the supernatant was again discarded. Finally, 50 µL of nuclease free water was added to the discs and heated at 90°C for 10 min in a thermocycler to complete the extraction. The DNA concentration was further diluted in ratio 1:20 to remove impurities and prevent smearing.

Polymerase chain reaction (PCR) amplification

Four microsatellite markers (Cga01, Cga02, Cga03 and Cga05) were selected from the seven microsatellite markers isolated in *C. gariepinus* by Galbusera et al. (1996). The genome DNA was amplified through polymerase chain reactions carried out in 10 µL reaction volumes containing 20 to 40 ng genomic DNA, 2 µL 5× Taq master mix of Taq DNA polymerase, dATP, dCTP, dGTP, dTTP, $(NH_4)_2SO_4$, $MgCl_2$, Tween-20, Nonidet P-40, red dye, gel loading buffer, stabilizers (Jena Bioscience, LÖbstedter, Germany), 0.3 µL each of the forward and reverse primer sets and 5.9 µL of PCR grade water (Jena Bioscience, LÖbstedter, Germany). Amplifications were carried out using GeneAmp PCR thermocycler programmed as follows: 30 cycles each of denaturation, annealing and extension temperatures at 95°C for 20 s, 58 to 60°C for 25 s and 72°C for 45 s, respectively, and a final extension temperature of 72°C for 60 s to complete the amplifications.

The PCR condition for each marker was optimized and differs from those described by Galbusera et al. (1996). The initial denaturation time was not included and the thermocycler was loaded at 95°C and the annealing temperature for each marker was adjusted to yield clear bands. Electrophoresis was conducted on 2% agarose gel and scored by comparison to 8 bp standard DNA ladder (Jena Bioscience, LÖbstedter, Germany), having the following values 75, 154, 220, 298, 344, 396, 504 and 1632 bp, with the aid of gel analyzer.

Statistical analysis

Statistical analysis of the data was done using Microsatellite Toolkit version 3.1 (Park, 2001) to compute input data for population genetics analysis. GenAlEx 6.1 (Peakall and Smouse, 2006) was used to compute the following statistics for each population: the mean number of alleles, mean number of effective alleles, allele frequency per population, fixation index and heterozygosity deficits showing genetic variation partitioned into total (F_{IT}), within (F_{IS}) and among (F_{ST}) population components, while NJPLOT 2.3 (Bio-Soft.Net) was used to plot and view the rooted phylogenetic tree with bootstrap value.

RESULTS

Allelic variability

Upon PCR amplification, 44.3% overall heterozygosity was observed and the number of alleles ranged from 21 to 25 (Table 1); all the four loci assayed were polymorphic. Measures of allelic variability are presented in Table 2. The mean number of alleles (MNA) was 12.25

Table 1. The four microsatellite primers set including locus name, repeat array, primer sequence, annealing temperature, allele size and number of alleles.

Locus	Repeat array	Primer sequences (5' → 3')	Annealing temperature (°C)	Range of allele size (bp)	Number of alleles
Cgao1	$(GT)_{15}$	GGCTAAAAGAACCCTGTCTG TACAGCGTCGATAAGCCAGG	59	107-212	22
Cgao2	$(GT)_{10}N_2(GT)_8$	GCTAGTGTGAACGCAAGGC ACCTCTGAGATAAAACACAGC	60	129-234	21
Cgao3	$(GT)_{21}$	CACTTCTTACATTTGTGCCC ACCTGTATTGATTTCTTGCC	58	94-278	25
Cga05	$(GT)_{11}N_2(GT)_2$	TCCACATTAAGGACAACCACCG TTTGCAGTTCACGACTGCCG	60	102-206	22

Table 2. Basic indicators of allelic variability between *C. gariepinus* and *H. bidorsalis*.

Population	N	MNA	NE	H_O	H_E	F	PIC
C. gariepinus	10.000 0.000	12.250 0.750	9.986 1.090	0.450 0.050	0.896 0.011	0.499 0.053	0.788
H. bidorsalis	9.750 0.250	11.500 0.957	10.022 0.932	0.442 0.127	0.897 0.011	0.513 0.138	0.782

Table 3. Conformity to Hardy-Weinberg equilibrium.

Parameter	DF	CHISQ	Probability	Significance
Population 1				
Cga01	55	74.722	0.040	P<0.05
Cga02	55	90.400	0.002	P<0.01
Cga03	78	107.778	0.014	P<0.05
Cga05	91	120.000	0.023	P<0.05
Population 2				
Cga01	55	91.111	0.002	P<0.01
Cga02	78	91.111	0.147	Ns
Cga03	78	90.000	0.166	Ns
Cga05	36	80.000	0.000	P<0.001

± 0.75 and 11.50 ± 0.96, while the effective number of alleles (NE) contributing to the population was 9.99 ± 1.09 and 10.02 ± 0.93 for *C. gariepinus* and *H. bidorsalis*, respectively.

Genetic diversity

The summary of the observed (H_O) and expected heterozygosity (H_E), polymorphic information content (PIC), and fixation index (F) for the two populations are shown in Table 2.

Population structure

Levels of intra- and inter-population variation based on Weir and Cockerham (1984) estimation of F_{IT}, F_{ST} and F_{IS} are shown in Table 3. The within population heterozygosity

Table 4. Global F-statistics estimates for each microsatellite locus.

Locus	F_{IS}	F_{IT}	F_{ST}
Cga01	o.494	0.524	0.058
Cgao2	0.496	0.514	0.036
Cga03	0.360	0.388	0.043
Cga05	0.664	0.683	0.056
Mean	0.504 ± 0.062	0.527 ± 0.060	0.048 ± 0.005

deficit, also known as inbreeding coefficient (F_{IS}) was highest at Cga05 (0.664) and least at Cga03 (0.360), with an average of 0.504 ± 0.062 at all loci. The among population heterozygosity deficit (F_{ST}) ranged from 0.036 to 0.058 at loci Cga02 and Cga01, respectively, with an average value of 0.048 ± 0.005, while the overall heterozygosity deficit (F_{IT}) ranged from 0.388 at loci cga03 to 0.683 at loci Cga05, with an average value of 0.527 ± 0.060 for the four loci.

Genetic distance

The Cavalli-Sforza and Edwards, (1967) neighbor joining phylogenetic dendrogram plotted showed a bootstrap value of 72 between the two species.

DISCUSSION

Allelic variation

The four loci assayed were polymorphic for all the populations, with each having a least number of allele of four for a population. The range of alleles observed per locus, four to eight was similar to the three to eight range observed by Volckaert and Agnese (1996) in the analysis of five microsatellite loci in ten samples of *C. gariepinus*. However, Galbusera et al. (1996) observed a range of five to 14 numbers of alleles in the characterization of seven microsatellite loci in *C. gariepinus.* The genetically homogenous samples were observed to maintain the same allele frequencies for a microsatellite locus (Table 4). This could be because both samples are under the same selection pressures, hence they are forced to maintain the same allele frequencies or because of extensive exchange of migrants in the populations. Allele frequency in heterogeneous populations may be established as a result of random drift due to the long term isolation or because the populations are subjected to different selection pressures.

Genetic diversity

The genotypic data obtained showed a good level of informativeness having a polymorphic information content (PIC) value higher than 0.5 for both population, which was above the threshold for which genetic markers begin to be informative. The PIC value depends on the number of alleles detected per locus and their frequencies. The heterozygosity percentage observed for all the alleles (44.3%) fell within the observed levels of heterozygosity in fish, which range from 24 to 90% (O'Connell and Wright, 1997). The average observed heterozygosity (H_O = 0.446 ± 0.063) was higher than values of H_O in *C. gariepinus* populations collected across Africa (0.06 to 0.15) (Teugels et al., 1992; Agnèse et al., 1997), while the expected heterozygosity observed in this study (He = 0.896 ± 0.111) fell within the range observed from a previous study by Rognon et al. (1998) (He = 0.05 to 0.15).

Hardy-Weinberg equilibrium

Generally, a deviation from Hardy-Weinberg equilibrium can occur due to the mixing of heterogeneous gene pools (Ferguson et al., 1996). Since the populations are domesticated, their parents may not be from the same natural stock. Such deviation is as a result of one or more of the following factors (evolutionary forces): non-random mating, random change in allele frequency in a population (genetic drift), mutation, migration and natural selection. A deviation from Hardy-Weinberg showed by a significant deficit ($P< 0.05$) in heterozygosities at loci Cga02 and Cga05 in populations 1(CM), 3(HM) and 4(HF), respectively, may be due to non-random mating of individual samples or substructing of samples (Wahlund effect); a case where two more sub populations have different allele frequency, thus causing a reduction in overall heterozygosity. This deviation shows a homozygosity excess for the populations at loci Cga02 and Cga05.

Genetic distance

The phylogenetic tree across both species shows a separation of the species into a clade with a bootstrap value of 72. A bootstrap value of 72 shows the length of genetic distance between the two sampled species and implies they are significantly different, although they are from the same family (Figure 1).

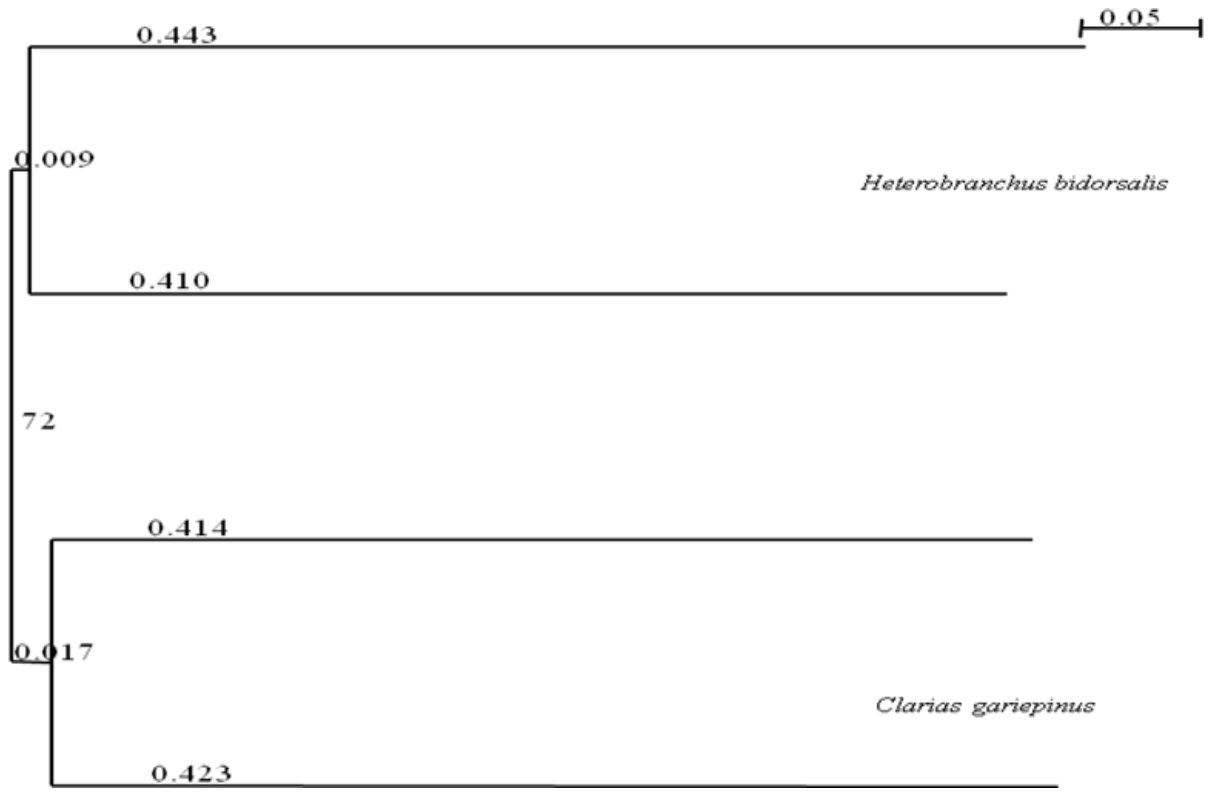

Figure 1. Genetic distance trees (Cavalli-Sforza) among the fish populations studied.

Conclusion

Microsatellites have been observed to be a powerful tool in fish identification, and for genetic conservation and management purposes. Analysis of the genetic diversity of the two species revealed two clearly different strains and their respective population structures. Since this is a preliminary study, further studies should be carried out with larger sample size and more microsatellite loci to provide more information about the population structure and conservation genetics of Clariid and other fish species.

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