Genetic Variations in Two Edible Frog Species (Crowned Bullfrog (*Hoplobatrachus occipitalis*) and African Clawed Frog (*Xenopus muelleri*)) in Ibadan, Nigeria Using Allozyme Markers

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Abstract
Attempts to determine interspecific differences in patterns of protein variation in edible frog species have been relatively few. Therefore, this study determined the genetic variation in populations of *Hoplobatrachus occipitalis* and *Xenopus muelleri* in two locations [University of Ibadan (UI) and Igbo Oloyin area (IO)] in Ibadan, Nigeria, using five allozyme loci. Eighty (80) edible frogs comprising twenty (20) live samples each of *H. occipitalis* and *X. muelleri* species from IO and UI were collected. 1ml of blood was drawn per sample via cardiac venipuncture. Plasma fractions were obtained and subjected to Cellulose Acetate Electrophoresis to determine the genetic variations at Haemoglobin (HB), Carbonic anhydrase (CA), Transferrin (TF), Albumin (AL) and Esterase (EST) loci. Test for Hardy–Weinberg Equilibrium (HWE; $\alpha_{0.05}$), Heterozygosity [observed (H_o) and expected (H_e)] and Genetic differentiation (F_{ST}) were estimated. Significant deviations from HWE were observed at several loci in all the populations. H_o was higher (than H_e) for both populations of *H. occipitalis* but lower for both populations of *X. muelleri*. F_{ST} was 0.0559 and 0.0264 for the populations of *H. occipitalis* and *X. muelleri*, respectively. There is an indication of evolutionary forces in operation in the IO populations of *H. occipitalis* and *X. muelleri*.

Keywords Allozyme, Biodiversity, Conservation, Genetic diversity, *Hoplobatrachus occipitalis, Xenopus muelleri*

1. Introduction

Humans have depended on natural resources for their survival and this is often seen as one of the strongest political arguments to preserve the global biodiversity [1, 2]. However, the changing environment, human reliance and over-exploitation of these resources is one of today's major threats to biodiversity, leading for example to habitat degradation, erosion of genetic diversity, species decline and loss, destabilization and destruction of ecosystems and hence jeopardizing present and future livelihoods [2]. No thanks to the booming human population which further put a lot of pressure on these resources.

Amphibian populations are declining worldwide; and over-exploitation is identified as one of the major reasons behind reduction in their number [3]. According to Niasse *et al*. [4], utilization and overuse is one of the main threats for 281 amphibian species, 54% of which are already listed as vulnerable, endangered or critically endangered when IUCN Red List categories are applied. Mohneke [5] estimated that over 2.7 million individual frogs are being collected by thirty-two (32) frog collectors annually in Nigeria.

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Hoplobatrachus occipitalis and *Xenopus muelleri* are part of the many frog species valued by humans as alternative sources of proteins, traditional medicine, pet trade and have many cultural roles in literature, symbolism and religion [6-8]. It was estimated that between 31% and 56% of all amphibian species has been listed as threatened by the IUCN Red List [9]. *H. occipitalis* and *X. muelleri* being utilized as alternative source of protein in Nigeria are sourced from the wild. This is unsustainable ecologically and conservation-wise, as it puts a lot of pressure on these species and the ecosystem services being rendered by them. Therefore, conservation efforts on these populations of frogs' species are paramount. Maintaining genetic diversity is an insurance package against future adverse conditions [10]. And so, understanding the genetic structure of these frogs' species is necessary in order to understand their dynamics as it relates to their diverse habitat types. These depend on the provision of genetic information that will foster formulating appropriate management and conservation strategies [11].

Future research, development and protection of frog species require a wholistic approach where more urgent needs and appropriate methods are identified and prioritized, and research resources utilized efficiently. Allozyme electrophoresis has become an important technique and is now used routinely for anuran species [12, 13, 14, 15]. The advantage of using electrophoresis is that this technique can detect higher heterozygosity per locus than can be detected using dominant Random Amplified Polymorphic RAPD markers [16].

A documentation of the blood protein variations is necessary and could thus serve as an important tool in elucidating genetic variations and for better implementation of practical conservation actions as a basis for all other studies on biology of frogs. Presently, there is dearth of information on genetic variations in *H. occipitalis* and *X. muelleri* in Nigeria. Therefore, this study seeks to provide baseline data and information on the genetic variation at five allozyme loci in *H. occipitalis* and *X. muelleri* from two different locations (Igbo Oloyin and University of Ibadan Awba dam) in Ibadan, Nigeria.

2. Materials and methods

Eighty (80) edible frog species were collected, which include twenty (20) live samples each of *Hoplobatracus occipitallis* and *Xenopus muelleri* species, obtained from Igbo Oloyin, Akinyele Local Government Area, and University of Ibadan Awba dam, in Ibadan North Local Government Area with geographical coordinates 7°33'42.7"N/3°57'58.7"E and 7°26'33.2"N/ 3°53'11.2"E, respectively. The study was conducted in Ibadan Oyo State, Southwestern Nigeria between August 2017 and February, 2018. The study areas lie within the rain forest vegetation zone which is charaterised by an average annual rainfall of between 1524mm and 2032mm and relative humidity of 74.55% [17]. The specimens were captured at night from their spawning sites using net traps and hand nets to prevent injury to them.

Table 1. Electrophoresis Protocol for Haemoglobin, Carbonic Anhydrase, Transferrin, Albumin and Esterase

Protein	Sample	Buffer	Time (mins)	pH	Voltage	Stain	Destain
Haemoglobin	RBCs	Tris EDTA Borate	30	8.4	250	Ponceau	5% Acetic Acid
Carbonic Anhydrase	RBCs	EDTA Sodium Acetate 1:4 dilution	35	5.6	200	Ponceau	1% Acetic Acid
Transferrin	Plasma undiluted	Tris Glycine	30	8.5	150	Ponceau	5% Acetic Acid
Esterase 1	RBCs	0/01M Phosphate	30	6.8	140	Ponceau	5% Acetic Acid
Albumin	Plasma undiluted	Tris Citrate	30	7.6	180	Ponceau	5% Acetic Acid

RBCs = Red Blood Cells
Source: Riken [19]

About 1ml of blood was drawn per sample via cardiac venipuncture following the approved minimum safe blood draw for healthy amphibians [18] into EDTA treated bottles. Plasma fractions were separated from erythrocyte fractions by centrifugation and were subjected to Cellulose

Acetate Electrophoresis to determine the genetic variants at Haemoglobin (HB), Carbonic anhydrase (CA), Transferrin (TF), Albumin (AL) and Esterase 1 (ES1) loci as described by Riken [19] (Table 1). Genetic distance (D), Genetic differentiation (F_{ST}), Heterozygosity (H_o and H_e) and test for Hardy–Weinberg Equilibrium (HWE) at $\alpha_{0.05}$ were estimated, using POPGENE Version 1.32 [20] and Tools for Population Genetic Analyses [21].

3. Results and discussion

The allelic frequencies and Hardy-Weinberg Equilibrium (HWE) of the populations are presented in table 2. In *H. occipitalis* (UI population), there were two alleles A(0.5500), B(0.4500) at Haemoglobin (HB) locus; three alleles A(0.7250), B(0.2500), C(0.0250) at Carbonic anhydrase (CA) locus; four alleles A(0.5750), B(0.1000), C(0.1250), D(0.2000) at Transferrin (TF) locus; and three alleles each at Albumin (ALB) [A(0.3500), B(0.5500), C(0.1000)] and Esterase (EST) [A(0.7250), B(0.1000), C(0.1750)] loci. The Igbo Oloyin population of *H. occipitalis* had two alleles each at HB[A(0.3750), B(0.6250)], CA[A(0.6750), C(0.3250)] and ALB[B(0.500), C(0.500)] loci; three alleles at EST[A(0.5500), B(0.3000), C(0.1500)] locus and four alleles at TF [A(0.4000), B(0.2000), C(0.2000), D(0.2000)] locus. The chi square ($\chi 2$) test for HWE shows significant difference at HB (p=0.0082) locus in UI population and at HB (p=0.0099), CA (p=0.0000) and ALB (p=0.0000) in Igbo Oloyin population of *H. occipitalis* (Table 2).

Table 2. Allele frequencies and Hardy-Weinberg Equilibrium at five blood protein loci in populations of *Hoplobatrachus occipitalis* and *Xenopus muelleri* in Ibadan, Nigeria

	Allele				HWE	
Loci	A	B	C	D	Chi-square ($\chi 2$)	Probability (p)
Hoplobatrachus occipitalis (University of Ibadan population)						
HB	0.5500	0.4500			6.9865	0.0082
CA	0.7250	0.2500	0.0250		2.9795	0.3948
TF	0.5750	0.1000	0.1250	0.2000	5.8548	0.4396
ALB	0.3500	0.5500	0.1000		7.2727	0.0637
EST	0.7250	0.1000	0.1750		6.7685	0.0797
Hoplobatrachus occipitalis (Igbo Oloyin population)						
HB	0.3750	0.6250			6.6500	0.0099
CA	0.6750		0.3250		16.8889	0.0000
TF	0.4000	0.2000	0.2000	0.2000	12.3491	0.0546
ALB		0.5000	0.5000		19.0000	0.0000
EST	0.5500	0.3000	0.1500		14.2727	0.0026
Xenopus muelleri (University of Ibadan population)						
HB	0.5750	0.4250			1.9222	0.1656
CA	0.7500	0.0750	0.1750		1.9655	0.5796
TF	0.3250	0.2500	0.1250	0.3000	18.7576	0.0046
ALB	0.3750	0.5500	0.0750		7.1818	0.0663
EST	0.6750	0.2750	0.0500		3.6646	0.3000
Xenopus muelleri (Igbo Oloyin population)						
HB	0.8250	0.1750			5.5674	0.0183
CA	0.6750		0.3250		16.8889	0.0000
TF	0.4750	0.3500	0.1000	0.0750	7.2961	0.2943
ALB	0.3750	0.6250			0.4397	0.5073
EST	0.6500	0.2000	0.1500		14.3057	0.0025

HB-Haemoglobin; CA-Carbonic anhydrase; TF-Transferrin; ALB-Albumin; EST-Esterase

In *X. muelleri* (UI population), there were two alleles at HB[A(0.5750), B(0.4250)] locus; three alleles each at CA[A(0.7500), B(0.0750), C(0.1750)], ALB[A(0.3750), B(0.5500)C(0.0750)] and EST[A(0.6750), B(0.2750), C(0.0500)] loci; four alleles at TF[A(0.3250), B(0.2500), C(0.1250), D(0.3000)] locus. The Igbo Oloyin population of *X. muelleri* had two alleles each at HB[A(0.8250), B(0.1750)], CA[A (0.6750), C(0.3250)] and ALB[A(0.3750), B(0.6250)] loci; three alleles at EST[A(0.6500), B(0.2000), C(0.1500)] locus and four alleles at TF [A(0.4750), B(0.3500), C(0.1000), D(0.0750)] locus. The chi square ($\chi 2$) test for HWE shows significant difference at TF (p=0.0046) locus in UI population and at HB(p=0.0183), CA(p=0.0000) and EST(p=0.0025) in Igbo Oloyin population of *X. muelleri* (Table 2).

According to Lewontin [22] the amount of genetic variation in a population can be estimated if there is information about the number of loci at which variation occurs. In this study, all the loci tested both populations were polymorphic, even though the number of alleles varied based on location. Populations from UI had higher average number of alleles than the population from Igbo Oloyin for the two species. Another obvious observation for the chi square values, is the fact that, four and three loci were not in Hardy-Weinberg equilibrium (HWE) for the Igbo oloyin populations of *H. occipitalis* and *X. muelleri*, respectively, as against only one locus each from the UI populations of both species. Random mating and other/or any other condition HWE seems to have been distorted in the populations at Igbo oloyin. This indicates the influence of evolutionary forces, such as migration and selection. Usually, the populations of frogs in Igbo oloyin are exposed to exploitation while UI populations are not. It is expected that a disturbed population would have a lower diversity than an undisturbed population [23, 24]. This is being played out here. The allele diversities (A) were 3.0000, 2.6000, 3.0000 and 2.6000 for the *H. occipitalis* (UI) *H. occipitalis* (Igbo Oloyin), *X. muelleri* (UI) and *X. muelleri* (Igbo Oloyin), respectively (Table 3). The average effective number of alleles (n_e) was higher in the Igbo Oloyin population (2.3290±0.7344) of *H. occipitalis* than the UI population (2.0534±0.3519), while that of the UI population (2.2762±0.7953) of *X. muelleri* was higher than Igbo Oloyin population (1.9762±0.4941). The Shannon information index showed that most of the loci were highly informative indicating polymorphism across the loci with an overall mean of 0.8371 ± 0.1924, 0.8584 ± 0.2983, 0.8782 ± 0.2664 and. 0.7576 ± 0.2640 in *H. occipitalis* (UI) *H. occipitalis* (Igbo Oloyin), *X. muelleri*(UI) and *X. muelleri* (Igbo Oloyin), respectively (Table 3). Average Observed heterozygosity (H_o) was higher than the expected heterozygosity (H_e) for both populations of *H. occipitalis* but H_e was higher than H_o for both populations of *X. muelleri* (Table 3).

One of the most important attributes of a population in view of our constantly changing environment is genetic diversity [25]. This is necessary for a population to be able to evolve and adapt to changes in the environment. The average expected heterozygosities (H_e) obtained from this study [0.5146 (*H. occipitalis*(UI), 0.5564 (*H. occipitalis* (Igbo), 0.5403(*X. muelleri* (UI) and 0.4815 (*X. muelleri* (Igbo)] are higher than that obtained by Islam and Hossain [26] for by *Hoplobatrachus tigerinus* (0.4690), by Rafinski and Babik [27] for *Rana arvalis* (0.156) and by Formas and Breva [28] for *Batrachyla leptopus* (0.051). All of these studies also used allozyme markers as used in the present study. H_o in this study reveals that the populations are healthy conservation-wise, and inbreeding is not a threat in the two species at the time of the study.

The pairwise Nei's Unbiased Measures of Genetic Identity and distance were as shown in Table 4. Genetic distance between *H. occipitalis* (UI) and *H. occipitalis* (Igbo Oloyin) populations was 0.1238 and that between *X. muelleri* (UI) and *X. muelleri* (Igbo Oloyin) populations was 0.0409. Genetic distance between the two species was 0.0603 (Table 4). The dendograms highlighting the genetic distance are shown in figure 1.

In the present studies, the genetic distance (D) [29] between the two populations of *H. occipitalis* is 0.1238 while that between the two populations of *X. muelleri* is 0.0409. Genetic distance between the two species (*H. occipitalis* and *X. muelleri*) was 0.0603. In their study of the genetic variation of *H. tigerinus*, Islam and Hossain [26], recorded an average D of 0.115 in three populations. Hoshan *et al*. [30] recorded genetic distance (D) [29, 31, 32] varied from 0.008 to 0.017 among three populations of *H. tigerinus* in Bangladesh. In eight populations of the Chilean frog *Batrachyla* *leptopus*, the average genetic distance was 0.063 [28]. Nei [29] opined that, the genetic distance (D) is approximately 1.0 for inter species comparisons, 0.1 for subspecies and 0.01 for local races.

Table 3. Measures of genetic diversity at five blood protein loci in populations of *Hoplobatrachus occipitalis* and *Xenopus muelleri* in Ibadan, Nigeria

					Heterozygosity	
Locus	N	n_a/A	n_e	I	H_o	H_e
Hoplobatrachus occipitalis (University of Ibadan population)						
HB	40	2	1.9802	0.6881	0.8000	0.5077
CA	40	3	1.6985	0.6719	0.4000	0.4218
TF	40	4	2.5237	1.1303	0.7000	0.6192
ALB	40	3	2.2989	0.9265	0.8000	0.5795
EST	40	3	1.7660	0.7684	0.4000	0.4449
Mean	40	3.0000	2.0534	0.8371	0.6200	0.5146
St. Dev		0.7071	0.3519	0.1924	0.2049	0.0847
Hoplobatrachus occipitalis (Igbo Oloyin population)						
HB	40	2	1.8824	0.6616	0.7500	0.4808
CA	40	2	1.7817	0.6306	0.0500	0.4500
TF	40	4	3.5714	1.3322	0.6500	0.7385
ALB	40	2	2.0000	0.6931	1.0000	0.5128
EST	40	3	2.4096	0.9746	0.4000	0.6000
Mean	40	2.6000	2.3290	0.8584	0.5700	0.5564
St. Dev		0.8944	0.7344	0.2983	0.3616	0.1162
Xenopus muelleri (University of Ibadan population)						
HB	40	2	1.9560	0.6819	0.3500	0.5013
CA	40	3	1.6701	0.7151	0.5000	0.4115
TF	40	4	3.6530	1.3330	0.7000	0.7449
ALB	40	3	2.2284	0.8909	0.8000	0.5654
EST	40	3	1.8735	0.7701	0.3500	0.4782
Mean	40	3.0000	2.2762	0.8782	0.5400	0.5403
St. Dev		0.7071	0.7953	0.2664	0.2043	0.1269
Xenopus muelleri (Igbo Oloyin population)						
HB	40	2	1.4060	0.4637	0.1500	0.2962
CA	40	2	1.7817	0.6306	0.0500	0.4500
TF	40	4	2.7491	1.1456	0.8000	0.6526
ALB	40	2	1.8824	0.6616	0.5500	0.4808
EST	40	3	2.0619	0.8865	0.3000	0.5282
Mean	40	2.6000	1.9762	0.7576	0.3700	0.4815
St. Dev		0.8944	0.4941	0.2640	0.3054	0.1292

HB-Haemoglobin; CA-Carbonic anhydrase; TF-Transferrin; ALB-Albumin; EST-Esterase; N-Sample size; na-Number of alleles; ne- Number of effective alleles; A- Allelic diversity; I-Shannon's information index; H_o-Observed heterozygosity; H_e- Expected heterozygosity

Table 4. Pairwise Nei's Unbiased Measures of Genetic Identity (above diagonal) and Genetic distance (below diagonal) between populations and species of *Hoplobatrachus occipitalis* and *Xenopus muelleri* in Ibadan, Nigeria

	H. occipitalis (UI)	*H. occipitalis* (Igbo)	*X. muelleri* (UI)	*X. muelleri* (Igbo)
Populations				
H. occipitalis (UI)	-	0.8836	0.9722	0.9285
H. occipitalis (Igbo)	0.1238	-	0.9086	0.8340
X. muelleri (UI)	0.0282	0.0959	-	0.9599
X. muelleri (Igbo)	0.0742	0.1815	0.0409	-
Species				
	H. occipitalis	*X. muelleri*		
H. occipitalis	-	0.9415		
X. muelleri	0.0603	-		

UI- University of Ibadan; Igbo- Igbo Oloyin

Ayala [33] reported that the D-value between subspecies is approximately 0.20 though the present study recorded similar genetic distance values with other frog species in some earlier studies, a particular attention is drawn to the D between the two populations of *H. occipitalis* which seems too high for populations of the same species. A more robust DNA analysis is suggested to ascertain this result. The Wright's F-statistics and Gene flow analyses for *Hoplobatrachus occipitalis* and *Xenopus muelleri* populations were shown in table 5. In *H. occipitalis*, the average F_{IS}, F_{IT} and F_{ST} were -0.1396, -0.0758 and 0.0559, respectively. In *X. muelleri*, the average F_{IS}, F_{IT} and F_{ST} were 0.0866, 0.1107 and 0.0264, respectively. Average gene flow in the studied populations of *H. occipitalis* and *X. muelleri* are 4.2192 and 9.2245, respectively (Table 5).

Table 5. Wright's F-statistics and Gene flow analyses at five blood protein loci in *Hoplobatrachus occipitalis* and *Xenopus muelleri* in Ibadan, Nigeria

Locus	N	F_{IS}	F_{IT}	F_{ST}	Nm*
Hoplobatrachus occipitalis populations					
HB	80.0000	-0.6083	-0.5588	0.0308	7.8673
CA	80.0000	0.4706	0.5148	0.0836	2.7419
TF	80.0000	-0.0198	-0.0023	0.0172	14.3108
ALB	80.0000	-0.6901	-0.4907	0.1180	1.8684
EST	80.0000	0.2147	0.2413	0.0338	7.1491
Mean	80.0000	-0.1396	-0.0758	0.0559	4.2192
SE	0.0000	0.2274	0.2072	0.0190	2.2188
Xenopus muelleri populations					
HB	80.0000	0.3569	0.4048	0.0744	3.1100
CA	80.0000	0.3452	0.3581	0.0197	12.4444
TF	80.0000	-0.1009	-0.0681	0.0298	8.1343
ALB	80.0000	-0.3235	-0.3163	0.0055	45.3333
EST	80.0000	0.3376	0.3430	0.0082	30.1923
Mean	80.0000	0.0866	0.1107	0.0264	9.2245
SE	0.0000	0.1414	0.1432	0.0125	7.8380

*Nm—Gene flow estimated from Fst= 0.25(1 − Fst)/Fst; HB-Haemoglobin; CA-Carbonic anhydrase; TF-Transferrin; ALB-Albumin; EST-Esterase; N- Sample size

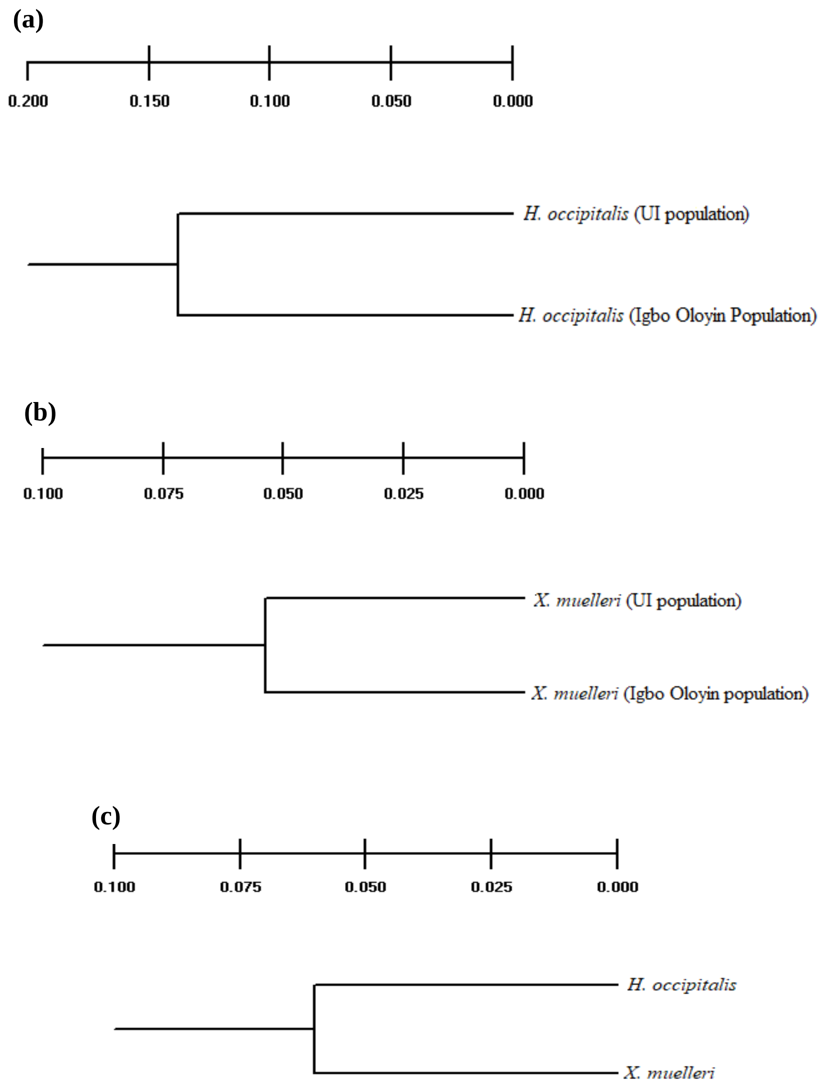

Figure 1. Dendograms showing similarities between the two populations of *H. occipitalis* (a); two populations of *X. muelleri* (b); the two frog species (*H. occipitalis* and *X. muelleri*) (c) in Ibadan, Nigeria based on five blood proteins analysis

The F statistics developed by Wright [34, 35] summarises genetic structure within and between populations. It partitions genetic variability using levels of heterozygosity [36]. The negative values of F_{IS} in all the loci as revealed for the populations of *H. occipitalis* is an indication of excess heterozygosity, while a deficit of heterozygosity was indicated in the populations of *X. muelleri* (having positive value) [24]. The F_{ST} value of *Hoplobatrachus occipitalis* (0.0559) populations as obtained in this study is comparable to that obtained for *Hoplobatrachus tigerinus* (0.043) [26]. The fixation index (F_{ST}) between the populations of both species are closer to zero than 1. Therefore, the populations have allele frequencies that are not too divergent from each other and inbreeding is not a problem in all the populations at the two locations for the two species.

4. Conclusions

The study examined the genetic variation in populations of *H. occipitalis* and *X. muelleri* in two locations in Ibadan, Nigeria. There is evidence of the influence of evolutionary forces, such as migration and selection in the Igbo oloyin populations of the two species. The Igbo oloyin population of *X. muelleri* may be undergoing inbreeding as suggested by their heterozygosity in this study. It can then be concluded that there is considerable genetic variation among the natural population of *H. occipitalis* and *X. muelleri* in the study area. And to some extent, the effect of exploitation (for the booming frog markets and other factors (including climate change) is playing out, especially in the IO populations. But the results are somewhat limited based on the limitation of the allozyme marker used. Therefore, further extensive research works on genetic variability of the frog populations involving more individuals and populations using DNA markers would be necessary to further decipher the genetic variation in the natural ranges of the species.

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