Genetic Diversity Studies in Toxicant Stressed Populations of *Tilapia zillii* in three Nigerian Reservoirs.

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Abstract: Genetic diversity is essential in natural populations of fishes as it confers fitness on the fishes thus enhancing their conservation. Unfortunately, genetic diversity has been greatly threatened in fish populations due to high concentration of toxicants in their aquatic environment amongst other factors. In this study, three natural populations of *Tilapia zillii* (Opa, Osu and Igun reservoirs) with varying degrees of heavy metal contamination were evaluated in order to determine the effect of the toxicants (heavy metals) on their genetic diversity. Samples of *T. zillii* from each of the reservoirs were collected; fish organs (gill) were taken from each of the samples for determination of heavy metal concentration. Caudal fin tissue of the samples was also taken for Random Amplified Polymerase DNA (RAPD) analysis in order to determine the amount of genetic diversity in each of the populations. Result showed that the difference between the amounts of heavy metal contamination between the three populations was statistically significant ($P< 0.05$). Population of *T. zillii* with the highest degree of heavy metal contamination (Igun reservoir) had the lowest amount of genetic diversity (35% polymorphism and H=0.36) compared to the other two populations (Opa and Osu) with 58.9% polymorphism, H =0.57 and 72.5% polymorphism, H =0.67 respectively. The trend follows that the higher the degree of heavy metal contamination, the lesser the amount of genetic diversity in such population. Our results thus suggest that that the availability of toxicants in high concentration, in aquatic environments has a potential to substantially reduce the within-population genetic diversity of fishes there.

[Awodiran MO, Oladimeji TE, Komolafe OO. **Genetic Diversity Studies in Toxicant Stressed Populations of *Tilapia zillii* in three Nigerian Reservoirs.** *Nat Sci* 2016;14(8):54-60]. ISSN 1545-0740 (print); ISSN 2375-7167 (online). http://www.sciencepub.net/nature. 9. doi:10.7537/marsnsj14081609.

Keywords: Genetic diversity, Heavy metal, Heterozygosity, Polymorphism

1. Introduction

Genetic diversity, which is the differences among individuals within a population or species that is determined by genotype variation (Ribeiro and Lopes, 2013) is very important in natural populations as a high genetic diversity provides a population the potential to adapt to selective forces (Gillespie and Guttman, 1989). It is likely that genetically similar organisms would all be equally susceptible to biological forces and natural phenomena such as drought, floods, or drastic fluctuations in temperature. When there is sufficient variability in the population of a species, some individuals would possess alleles that would be able to withstand pressure, survive and reproduce thus preventing extinction of such a population. It is important for individuals within populations to be able to adapt to changing environments; this adaptive potential is the basis for natural selection and is largely genetically determined (Bader, 1998). In order for a species to persist, there is need for sufficient genetic diversity within that population; this will enhance its ability to adapt to the changing environment. Spontaneous mutation of a gene or immigration from a population of genetically different individuals usually introduces new genetic variation into a population.

The number and relative abundance of alleles in a population is a measure of genetic diversity. Unfortunately, genetic diversity has been greatly threatened in natural populations. One of the greatest threat to reduction in genetic diversity in natural aquatic populations is the high concentration of toxicants (heavy metals) which has not only led to reduction of fishes in these water bodies but has also led to "genetic erosion" (Ungherese *et al.,* 2010; Van Straalen and Timmermans, 2002).

Anthropogenic toxicants in the environment can affect natural populations in many ways; one of the more subtle effects is genetic change which has potential large long term consequences (Van Straalen and Timmermans, 2002). Some of the ways by which toxicants may affect genetic diversity in populations is by increasing mutation rates, causing bottleneck events and by altering migration.

Metals are introduced into our aquatic system as contaminants via several ways which include weathering of rocks, dissolution of aerosol particles from the atmosphere and from several human activities such as mining, agricultural activities, industrial activities amongst others (Adefemi *et al.*, 2004). Toxic level of these metals in fish is as a result of bioaccumulation and magnification (Ipinmoroti *et al.,* 1997b; Ekeanyawu *et al.,* 2010).

Concern with reduction of genetic resources in fish is part of a larger global concern for the genetic resources of the biosphere; this has stimulated strong interest in molecular genetics research, which is vital to the long-term management of fisheries resources (Park and Moran, 1995). Few studies have reported changes in allelic frequency within natural populations exposed to pollution, using both allozymes and DNA markers (Sloss *et al*., 1998; Bader, 1998; Park *et al.,* 1999; Kovatch *et al*., 2000; Kim *et al.,* 2003; Ross *et al*., 2002) as well the loss of genetic diversity as a consequence for availability of toxicants. (Bickham *et al.,* 2000; Gardeström *et al.,* 2008). One measure of genetic diversity using RAPD marker is the proportion of polymorphic loci. This is simply the proportion of loci examined that shows evidence of more than one allele (Bader, 1998). A more reliable measure is by estimating the average frequency of heterozygous individuals per locus (H) of the population (Bader 1998).

This study therefore aims to determine the effect of heavy metal contamination on the amount of genetic diversity of *Tilapia zillii* populations from three reservoirs in Osun state, Nigeria.

2. Material and Methods

Sample Collection

Fresh adult samples of *Tilapia zillii* were collected from Opa reservoir, Osu reservoir and an abandoned gold mining reservoir in Igun, Osun state, Nigeria. Fish samples were put in an ice-chest and brought to the laboratory for analysis. A minimum of six individuals from each reservoir were examined for heavy metal contamination and standard morphometric parameters such as total length, standard length and weight of each fish were measured and recorded. Each sample collected was dissected for its gill tissues which were put in properly labeled separate specimen bottles and kept in freezer until heavy metal analysis was carried out. The caudal fin tissue of the fishes were cut and preserved in 95% ethanol for RAPD analysis.

Description of study areas

The Opa Reservoir Basin was established in 1978 by the impoundment of Opa River which took its source from Oke-Opa Hills. It lies between longitudes 4°30' to 4°40'East of the Greenwich and latitudes 7°27'to7°35'North of the Equator.

Osu reservoir is newly impounded in1995, located at Osu, Atakunmosa West Local government area of Osun State. Osu reservoir was formed by the impoundment of River Sasa. It lies between longitude 004°38.32′ E to 004°38′49.1″E and latitude 07°35.3′N and 07°35′17.9″N.

Igun reservoir is located in an abandoned gold mine area of Igun village in Atakumosa West Local Government area of Osun State. It extends over longitudes $4^0$30'E to $4^0$45'E and latitude $07^0$35'N to $07^0$38'N. The reservoir was impounded in order to meet the needs of the Nigerian mining cooperation which started in December 1941, however, illegal miners still engage in small scale gold mining around the reservoir till date.

Digestion of fish Samples

Samples of fish organ viz gill were oven dried at 50°c for about ten hours. The dried samples obtained were pounded and pulverized into powdery form. 0.5g of each dried powdery sample were weighed accurately and digested with aquaregia, a mixture of concentrated hydrochloric acid and nitric acid in ratio 3:1 respectively. To each sample, 5 ml of concentrated nitric acid and 15 ml of hydrochloric acid were added and the mixture was allowed to slowly react for about an hour. The mixture was then heated at 60^0c until near dryness. 3ml of concentrated nitric acid was added again for complete dissolution. 10ml of distilled water was then added and boiled to remove excess acid. The process was repeated again and boiled until the value of mixture in the flask reduced to about 5ml. The mixture was allowed to cool and then filtered. The filtrates were made up with distilled water up to 20 ml mark. The heavy metal concentrations were measured using Atomic Absorption spectrometer (AAS), Model LAAS-210.

Statistical analyses: The differences in the heavy metal contamination of the fish of the three studied sites were evaluated by Analysis of Variance (ANOVA) using SPSS 21

DNA Extraction and RAPD Analysis

Total genomic DNA was extracted from caudal fin tissue of seven (7) *T. zillii* fishes in each reservoir population, following standard CTAB method (Saghai *et al.,* 1984). Spectrophotometer was used to measure the concentration of all the DNA samples at 260nm and 280nm and their quality was detected by agarose gel electrophoresis. The integrity of the DNA was visualized and photographed on UV light source. The DNA extracts were subjected to PCR amplification following the PCR protocol for RAPD analysis as described by Williams *et al*. (1990) and Plotsky *et al.* (1995). In order to ensure there were no spurious bands, PCR blanks were run and all the bands that showed up consistently in the negative controls were removed from the final analysis. Also some portion of the samples were run in triplicates and spread across gel in order to ensure consistent measures. All the bands that showed up in triplicate (and not on the negative control) were considered good for the analysis. The primers used were purchased from Operon Technologies, U.S.A. Ten different primers were tested on fish samples and five, better responding ones, exhibiting the highest quality

banding patterns and sufficient variability were selected for population analysis. A 100 bp ladder Fermentas and markerll Roche (Germany) were used as molecular standard size markers. The primers identities are presented in Table 3. Gel products were visualized and documented using the Gel Documentation system, Gel-Pro Analyzer (Media Cybernetics) and subsequently analyzed for polymorphism. The genotypes were detected by scoring the presence (1) or absence (0) of distinct reproducible bands and faint bands were neglected. Genetic diversity in the populations was measured by estimating the proportion of polymorphic loci observed from the RAPD profiles and Heterozygosity (H) values was estimated as

$$H = \frac{2(1-q)}{2-q}$$

Where the average frequency of a band,

$$q = 1 - \sqrt{(1-Pb)}$$

$$Pb = \frac{2NXY}{NX + NY}$$

Pb = band-sharing coefficients while NX and NY are the number of bands for individuals X and Y and NXY is the number of bands that both individuals have in common. (Smith *et al.,* 1996; Olowofeso and Aro, 2006).

The relationship between the heavy metal contaminations in each reservoir population and the average heterozygosity across all loci was determined using regression analysis by PAST software

3. Results

The mean total length, standard length and weight of the fishes collected in each of the reservoirs is presented in Table 1. The mean concentration of all the heavy metals analysed in the gills of *T. zillii* from the three locations is shown in Table 2. The metals analysed in each of the fishes include Arsenic, Cadmium, copper, Lead, Nickel and Zinc.

In the gills of *T. zillii* from Igun reservoir, Zinc was the most bio accumulated heavy metal (146.672 ± 27.936 µg g-1 dry weight (DW)) while Nickel was the least bioaccumulated heavy metal (7.585 ± 1.606 µg g-1). Cadmium and lead were below the detectable limit of the equipment. Likewise, in the gills of *T. zillii* from Opa reservoir Zinc was the most bio accumulated heavy metal (0.036 µg g-1) while Nickel was the least bioaccumulated (0.003± 0.0004 µg g-1). Copper was the most bioaccumulated metal in samples from Osu reservoir while Arsenic was least bioaccumulated (0.001 ± 0.0020 µg g-1). The mean concentrations of heavy metals analysed in the gills of *T. zillii* was generally highest in Igun reservoir, followed by Opa reservoir and least in Osu reservoir. The differences were statistically significant at P< 0.05. Comparing the mean concentrations of these heavy metals with that of World Health Organization (WHO) permissible limit, it was found that the *T. zillii* fishes from Osu reservoir had very low concentrations of heavy metals in their tissues while those from Igun reservoir had very high heavy metal concentrations, above WHO permissible limit.

Table 1: Morphometric parameters of *Tilapia zillii* sampled in the study areas

	Number of Samples	Mean Total Length of samples (cm)	Mean Standard Length of samples (cm)	Mean Weight of samples (g)
Opa	16	18.4	14.4	116
Osu	6	23.5	18.9	217
Igun	10	18.7	14.6	164

Table 2: Mean concentration of heavy metals (µg/g) in the gills of *Tilapia zillii* from Igun, Opa and Osu Reservoir

Location		Mean Heavy metal concentration (µg/g))				
	As	Cd	Cu	Pb	Ni	Zn
Igun	41.639	0.00	31.982	0.000	7.585	146.672
Opa	0.016	0.008	0.02	0.012	0.003	0.036
Osu	0.001	0.004	0.015	0.001	0.003	0.006

Rapd Result

Five primers were used to initiate PCR amplifications which produced good RAPD bands. The bands ranged in molecular size from approximately 200 to 4500 bp. No artifacts were observed and the number of loci amplified per primer varied. On the overall, 99 loci were amplified by the five primers, 59 of them were polymorphic (59.6%). The total number of RAPD bands produced in each population varies. There was no band that was

population specific. 20 amplified loci were obtained in Igun, of which 7 were polymorphic (35 %). In Opa, 39 DNA loci were amplified of which 23 were polymorphic (with 58.9 %) and 40 loci were amplified in Osu, of which 29 were polymorphic (72.5 %) (Table 4). The number of polymorphic loci obtained in the three populations ranged from 7 to 40. The average heterozygosity values across all loci in each population of *T. zillii* studied (Igun, Opa and Osu) were 0.36, 0.57 and 0.67 respectively (Table 4).

A negative correlation was observed between the heavy metal contaminations and average heterozygosity values in the selected fishes from the three reservoir populations, although the correlation were not statistically significant (Figure 1).

Table 3: List of primers used and their sequences

S/N	Primer Name	Sequence	Fragment size
1	OPAE 05	CCTGTCAGTG	250bp - 2500bp
2	OPAF 09	CCCCTCAGAA	200bp - 4500bp
3	OPAF 07	GGAAAGCGTC	250bp - 3500bp
4	OPAD 09	TCGCTTCTCC	200bp - 2500bp
5	OPAE 04	CCAGCACTTC	200bp - 2500bp

Table 4: Estimates of genetic variations

Population	Total number of Loci	Number of Polymorphic loci	Proportion of polymorphic Loci (%)	Average Heterozygosity across all loci.
Igun	20	7	35.0	0.36
Opa	39	23	58.9	0.57
Osu	40	29	72.5	0.67

4. Discussion

Several factors are responsible for the availability of metals in aquatic organisms; these include the concentration of the metal in the aquatic environment, time of exposure of the organism, mode of metal uptake, excretion rate, as well as homeostasis (Giesy and Wiener, 1977). Aquatic environment with reported high concentration of heavy metals are usually incriminated in having aquatic organisms (especially fish) which had bioaccumulated the heavy metals. However, differential uptake and bioaccumulation potential in the fishes could bring about differences in heavy metal concentrations of tissues and organs (Holcombe *et al.,* 1979).

The concentration of heavy metals in the tissues of selected fishes from Opa, Osu and Igun reservoirs varies from each other while the estimates of genetic diversity from the sites also varies. Igun reservoir has been reported to have high concentration of heavy metals due to the illegal mining activities going on around it (Aderinto, 2013). The population of selected fishes in Igun reservoir with the highest concentration of heavy metals (above WHO permissible limit) compared to the population from Opa and Osu has the lowest estimate of genetic diversity. This is consistent with the report of Bourret *et al*. (2007) who demonstrated using basic population genetics parameters that metal contaminants associated with mining activities have evolutionary impacts on the genetic makeup of yellow perch populations from northern Ontario and Qu´ebec. The concentration of heavy metals in selected fishes from Osu reservoir is lowest relative to Igun and Opa reservoirs. This compares favorably with the report of Ungherese *et al.* (2010) that populations from sites with low availability of toxicants have the highest values of genetic diversity and vice-versa. Kim *et al*. (2003) also reported a decreased genetic diversity within heavy-metal polluted populations of *Littorina brevicula* compared to the unpolluted ones. Similarly, Fratini *et al*. (2008) found that populations of intertidal crab, *Pachygrapsus marmoratus* from polluted sites have significantly less genetic variability, than populations from unpolluted sites. In a review by Mussali-Galante *et al.* (2014) they reported how several molecular markers were used by different authors to assess genetic diversity in impacted populations, the order of the most common ones of which were SSR's > allozyme > RAPD's > mtDNA sequencing> other molecular markers. They found that genetic diversity was reduced for nearly all animal populations that were exposed to a single metal, or a mixture of metals in aquatic ecosystems.

In conclusion, the findings in this study further confirm the report that availability of toxicants in high concentration could reduce genetic diversity in natural populations. However, it must be borne in mind that environmental contamination by heavy metals is not the only factor affecting the genetic diversity of *T. zillii* populations. Therefore, this result must be considered preliminary for further detailed investigations of this topic. Also, assessment of the

genetic diversity of populations could be a valuable addition, as ecological indicator for determining the effects of environmental pollution on aquatic ecosystems. It is therefore recommended that introduction of toxicants to aquatic environments should be controlled by strict regulations in other to preserve the genetic diversity of fishes and other aquatic organisms which is very imperative to their conservation. Additional methods such as microsatellites may be used to maximize the efficiency of genetic diversity studies.

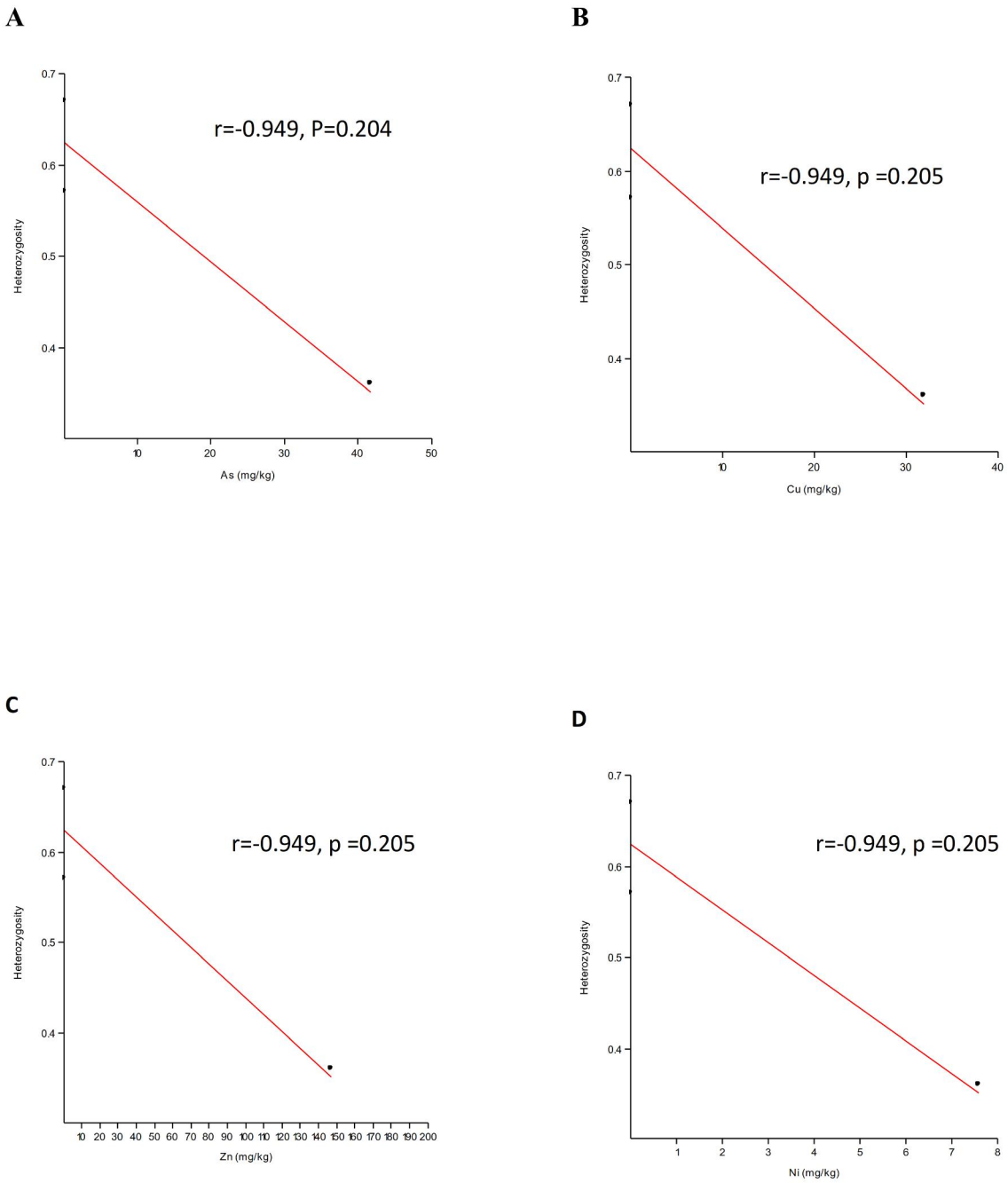

Figure 1: Relationship between heavy metal concentrations in the gills of *T. zillii* (expressed in µg/g) and average heterozygosity across all loci in each population.

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