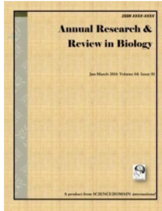

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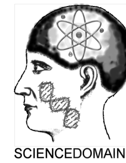

Microsatellite Analysis of Six Populations of *Chrysichthys nigrodigitatus* from Nigeria

S. A. Nwafili[1,2*], Ren Guijing[1] and Gao Tian Xiang[1]

[1]Laboratory of Mariculture, Ocean University of China, 266003 Qingdao, China.
[2]Department of Animal Science and Fisheries, Faculty of Agriculture, University of Port Harcourt, Nigeria.

Authors' contributions

All the authors contributed equally to the manuscript. All authors have read and approved the final manuscript.

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ABSTRACT

Aim: To investigate the patterns and levels of genetic polymorphism and population structures of wild *C. nigrodigitatus* using the microsatellite DNA in Nigeria.
Place and Duration of Study: in Nigeria between 2008 and 2009, and Laboratory of Mariculture, Ocean University of China, 2009-2010.
Methodology: A total of 93 individuals of *Chryscichthys nigrodigitatus* obtained from 6 sites in Nigeria were used for the study. DNA extracted from alcohol preserved muscle tissue was amplified by PCR. Amplified products were detected using the silver staining technique to visualize bands.
Results: The four microsatellite loci indicate high genetic variation in all 6 populations of the species with the number of alleles and H_O varying from 2-10 and 0.800-1.00 per locus, respectively. The NJ tree revealed a marginal genetic differentiation between two clades, which were not well supported. Significant genetic differences were detected between most samples. Freshwater or brackishwater habitat, limited long-distance dispersal of the adult and juveniles may account for breakdown of gene flow.
Conclusion: *C. nigrodigitatus* maintains sufficient intra population genetic variability and moderate inter population differentiation in the studied area.

**Corresponding author: Email: nwafili4@gmail.com;*

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1. INTRODUCTION

Chrysichthys nigrodigitatus is a euryhaline fish of tropical Africa which supports thriving commercial fisheries with great potentials for aquaculture in West Africa. It occurs in a variety of fresh and brackish waters habitats such as rivers, mangrove swamps, lakes, and estuaries and low salinity coastal areas [1-3]. The species is characterized by spawning migration undertaken in the rainy season from more saline brackishwaters to freshwaters where spawning occurs. In the reverse, the juveniles follow the flood water back to the saline environment to feed and grow [1]. However, the wild population of *C. nigrodigitaus* is observed to be declining due to destructive fishing methods, environmental pollution and overfishing. Most water bodies in the Niger Delta are polluted from petroleum extraction and allied activities, urbanization and agriculture. Fishermen target migrating gravid females thereby disrupting the reproductive and recruitment cycle. Furthermore, culture of the species still relies on the capture of fry from the wild for stocking. All these may be interacting to decrease the genetic diversity of the populations. As an important commercial and aquaculture species understanding the level of genetic diversity and patterns of population genetic structure are of paramount significance.

There are several molecular markers for assessing genetic diversity. Among all the types of molecular markers, the microsatellites DNA are widely relied on for the analysis of genetic diversity and population structure in fish. Microsatellites DNA are characterized by a core sequence that consists of a number of tandemly repeated units with a length of 1-6 base pairs. Microsatellites would potentially produce higher values of polymorphism than mtDNA sequences, which mutate at a higher speed than mtDNA sequences [4].

The population genetic structure and genetic diversity of *C. nigrodigitatus* obtained from the Niger Delta was analyzed using four microsatellite markers, in order to address the following questions: What is the current level of genetic diversity and how is the population structured? Is the genetic diversity higher or lower in the Niger Delta populations when compared with populations from other regions in Nigeria?

The present study aims to investigate the patterns and levels of genetic polymorphism and population structures of wild *C. nigrodigitatus* using the microsatellite DNA to [1] estimate the level of genetic diversity in the *C. nigrodigitatus* populations in Nigeria, [2] calculate the distribution of variability within a population and among populations and [3] examine the genetic relationship between the populations.

2. MATERIALS AND METHODS

2.1 Sample Collection

Chrysichthys nigrodigitatus specimen for this study were collected between December 2008 and July, 2009) from 8 sites in Nigeria (Fig. 1). These sites were 3 Niger Delta locations, namely; Benin River at Koko (KO), Warri River (WA) and Buguma Creek (BU). The remaining populations were obtained from Lagos lagoon (LA) and the River Niger at the downstream (LN) and upstream (UN), respectively. Muscle tissue was excised from each individual fish and preserved in 95% alcohol until DNA extraction. Total genomic DNA was extracted using standard phenol-chloroform method [5]. Genomic DNA was suspended in 100µl distilled water.

2.2 Microsatellite Genotyping

Four microsatellite loci CN13, CN25, CN45 and CN67 listed in Table 1 [6] were PCR amplified with conditions comprising 50µL volumes containing 1.25 U T*aq* DNA polymerase (Takara Co., Dalian, China), 10-100ng template DNA, 200nmolL-1 forward and reverse primers, 200µmolL-1 of each dNTPs, 10mmolL-1 Tris, PH 8.3, 50mmolL-1 KCl, 1.5mmolL-1 $MgCl_2$. The PCR conditions consisted of 30cycles of denuturation at 94℃, 1min; annealing at 62-65℃, 1min and extension at 72℃, 45s on thermal cycler 9700 (Applied Biosystems, Foster City, CA, USA). Amplified products were checked for yield on an agarose minigel before loading, along with size standards internal to every lane, onto a 6% denaturing polyacrylamide gel ran for 2h at 50℃ using Sequi-Gen GT Sequencing cell (Bio-Rad, USA) and finally detected using the silver staining technique to visualize bands. A 50 bp DNA ladder was used as a reference marker to enable the determination of allele sizes.

2.3 Scoring Microsatellite Markers

Data collation was done manually. All microsatellite loci were scored by visually. Microsatellite loci amplified by PCR are characterized by a distinct banding pattern consisting of the main alleles together with fainter stutter bands. Genotypes from scored loci were entered into the Excel spreadsheets.

2.4 Statistical Analyses

Descriptive statistics of microsatellite loci used to quantify genetic diversity included observed heterozygosity (H_O), expected heterozygosity (H_E), number of alleles, polymorphic information content (PIC) and Wright's F-statistics. H_O and H_E for each population across the loci and those for each locus across populations were calculated using the GENEPOP programme Version 3.3 [7], according to Weir and Cockerham's F_{IS} [8]. Expected heterozygosity is the heterozygosity that would be obtained given the allele frequencies at a particular locus under conditions of HWE. The discrepancies between

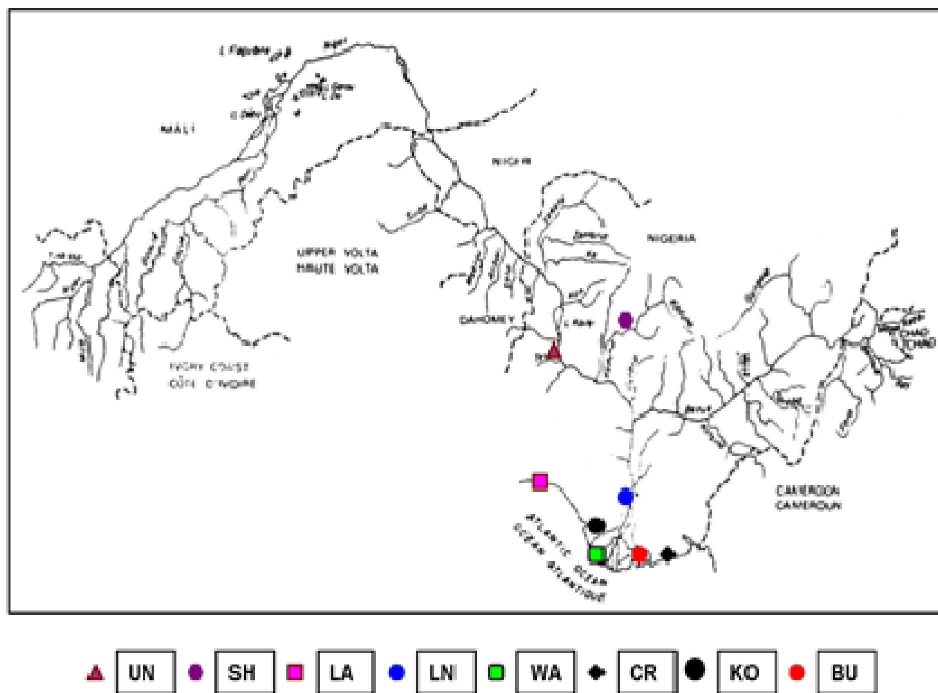

Fig. 1. Map of study area: KO (17), LA (11), BU (22), CN (15), LN (15), and WA (13). Numbers in parenthesis are sampling sizes for each population.

Table 1. Primer sequences used for microsatellite analysis of *C. nigrodigitatus*

Primer	Sequence	Annealing Temp	$MgCl_2$ (mM)
Cn13	F:aagcacagatttggccctac		
	R:ttcgtgtgtacaggcttag	64°C	0.60
Cn25	F: tcagcacagaatacagcatg		
	R:ggttatcaccagttattctattgtg	62°C	0.60
Cn45	F:gcatgccgactcccactc		
	R:cattttctccgggaaaagcc	65°C	0.60
Cn67	F:tgagtgaggaggttattctcacc		
	R:agtaaatgccaaaaatgtacatgc	63°C	0.60

H_O and H_E were tested using Fisher's exact test with the level of significance determined by a Markov chain method (10,000 dememorisation steps, 100 batches and 500 iterations) implemented in GENEPOP v3.3 [7]. Any such deviation from HWE at specific loci may indicate a high null allele frequency, sex-linked loci or linkage to other loci under selection. If departures from HWE are present in the majority of loci, this may indicate some violation of the HWE conditions, such as the presence of population structuring, migration or population bottlenecks.

Allelic richness (AR) was also calculated using FSTAT v.2.9.3 [9]. Allelic richness was computed to allow for a comparison among samples of different sizes [10].

Genic differentiation between populations was estimated using unbiased estimate of P-value of the probability test (or Fisher's exact test) as described by Raymond and Rousset [7]. The genetic differentiation among populations was also measured by F_{ST} and analyzed by a hierarchical analysis of genetic diversity (AMOVA) implemented in ARLEQUIN version 3.11 [11]. We also conducted the AMOVA analysis with two groups representing the freshwater group and brackishwater group. The division of groups is shown in Table 4.

Genetic distances of Cavalli-Sforza and Edwards [12] between populations were calculated using the programme POPULATIONS 1.2.30 [13]. A hybrid neighbour-joining tree was constructed with the software MEGA [14]. The tree topology was based on Da genetic distance [15] with statistical support from 1000 bootstrapped data sets for phylogenetic relationships [16].

Multilocus estimates of the effective number of migrants (Nm) between populations were calculated using the private allele method of Slatkin [17] and were corrected for sample size as given in Barton and Slatkin [18].

3. RESULTS

Genetic diversity at four microsatellite loci was determined using the primers CN67, CN45, CN25 and CN13 reported as useful for amplification studies in *C. nigrodigitatus* [6]. The four microsatellite loci used in this study exhibited high polymorphism in the 6 populations under investigation (Table 2). Across the populations, the number of alleles ranged from 2 to 10 per locus. The locus CN67 had the highest number of alleles [10] and the locus CN25 had the least number of alleles [2]. The PIC values were higher than 0.5 except for population LN at CN25 locus.

The highest average number of alleles was recorded in KO and LN populations (7.5), followed by BU (7.25), LA (7.0), UN (6.75) and WA (6.25). The allelic richness calculated by FSTAT taking into consideration differences in sample size showed that across populations LA had the highest value (6.51), followed by UN(6.22), KO (6.08), LN (5.62), WA(5.51) and BU (5.45) while across the 4 loci, CN67 had the highest value of 6.75 followed by CN45 (6.62), CN13 (6.07) and CN25 (4.15). The observed heterozygosity values (H_O) were consistently higher than the expected heterozygosity (H_E) values across all populations. For example, the H_E for population BU was 0.880 and the H_O was found to be 0.962 at locus CN67. However, at locus CN13 H_E (0.853) was higher than H_O (0.800). No significant departure from HWE was detected at the locus level. However, at the population level KO deviated significantly from HWE due to excess heterozygosity.

The index of inbreeding F_{IS} was negative for all populations across loci except for KO at locus CN13 (Table 2). Negative F_{IS} imply there is an excess of heterozygotes relative to Hardy-Weinberg expectations. The exact test for genic differentiation revealed highly significant differentiation between the studied populations at three loci at 0.05% level (CN25, $P<0.001$; CN67, $P<0.001$ and CN13, $P<0.001$). AMOVA shows that population specific F_{ST} indices (Table 2) varied from 0.0281 in BU to 0.0357 in LA, indicating that 2.81 to 3.57% of total genetic variation comes from intrapopulational variation and 97.19-96.43% of the variations are due to variation within populations. The mean F_{ST} was estimated at 0.0309. Thus, about 97% of total genetic variation resides within each population. The AMOVA analysis based on freshwater and brackishwater group (Table 3) showed that 7.37% of the genetic diversity was found between freshwater and brackishwater groups ($P<0.05$). A small (3.55%) but significant ($P<0.01$) amount of genetic diversity was found among populations within groups. The AMOVA analysis also showed that a large and significant genetic differentiation (89.08%, $P<0.01$) accounts for individuals within populations.

Table 2. Information includes: number of fish in bracket, Allelic richness (AR), number of alleles (A), Observed heterozygosity (H_O), Expected heterozygosity (H_E), Polymorphic information content (Bolstein et al 1980) and coefficient of inbreeding (F_{IS}) (Weir and Cockerham, 1984) for each locus in each population MF_{ST} and MFIS are mean FST and F_{IS}, respectively. Sampling abbreviation is given in Fig 1

locus	Populations					
	LA	BU	KO	WA	LN	UN
CN25						
AR	6.220	3.590	3.920	4.400	2.000	4.780
A	7.000	5.000	4.000	5.000	2.000	5.000
H_O	1.000	1.000	1.000	1.000	1.000	1.000
H_E	0.842	0.797	0.742	0.517	0.817	0.517
PIC	0.773	0.746	0.651	0.653	0.375	0.733
F_{IS}	-0.200	-0.565	-0.374	-0.374	-1.000	-0.241
CN67						
AR	7.250	7.150	6.510	5.530	6.850	7.190
A	8.000	10.00	9.000	7.000	10.000	8.000
HO	1.000	0.962	0.867	1.000	0.867	1.000
HE	0.889	0.880	0.853	0.779	0.846	0.892
PIC	0.820	0.848	0.802	0.710	0.796	0.834
FIS	-0.134	-0.095	-0.017	-0.300	-0.025	-0.128
CN45						
AR	6.560	6.360	7.200	7.110	6.980	5.490
A	7.000	8.000	8.000	8.000	9.000	6.000
HO	1.000	0.957	1.000	0.909	1.000	1.000
HE	0.884	0.863	0.895	0.879	0.862	0.827
PIC	0.819	0.824	0.832	0.820	0.812	0.758
FIS	-0.139	-0.111	-0.125	-0.036	-0.167	-0.222
CN13						
AR	6.000	4.710	6.670	4.980	6.640	7.420
A	6.000	6.000	9.000	5.000	9.000	8.000
HO	0.857	0.840	0.800	0.875	1.000	1.000
HE	0.835	0.788	0.853	0.808	0.844	0.905
PIC	0.741	0.734	0.803	0.722	0.793	0.848
FIS	-0.029	-0.068	0.064	-0.089	-0.193	-0.111
MFST	0.0357	0.0281	0.0297	0.0330	0.0300	0.0286
MFIS	-0.0276	-0.0946	-0.0168	-0.3005	-0.0254	-0.1282

Table 3. Percentages of variation between groups, among populations and within population

Source of variation	Percentage	Fixation index	*F*-statistics	Possibility or P value
Between groups	7.373	0.074	F_{CT}	0.047
Among populations	3.546	0.038	F_{SC}	0.000
Within populations	89.081	0.109	F_{ST}	0.000

Pairwise F_{ST} comparisons of different populations of Silver catfish, *C. nigrodigitatus* for population structure are shown in Table 4. The microsatellite markers revealed significant F_{ST} differences in many population pairs. The F_{ST} values were not significant between populations KO and WA, LN and UN, BU and KO and LA and WA. The largest F_{ST} distance value was between BU and LN (0.120).

Barton & Slatkin's [18] estimates of number of migrants were 0.826235, 0.450384, and 0.324012 respectively. Overall populations and loci, the number of migrants using the private alleles method according to [18] was 0.618; which is less than 1, indicating moderate levels of gene flow.

The neighbour-joining tree clearly showed a marginal genetic differentiation between two groups (Fig. 2). This clade is differentiated by a bootstrap support value of 69.

Table 4. Differentiation between populations. Pairwise F_{ST} (below diagonal) and Cavalli-Sforza chord distances (above) among six populations of *Chrysichthys nigrodigitatus*

	LA	BU	KO	WA	LN	UN
LA	0.000	0.369	0.404	0.421	0.438	0.525
BU	0.040*	0.000	0.328	0.410	0.394	0.478
KO	0.023	0.025	0.000	0.372	0.371	0.463
WA	0.025	0.058**	0.038	0.000	0.421	0.506
LN	0.082*	0.120**	0.081*	0.090**	0.000	0.371
UN	0.048*	0.094**	0.051*	0.071**	0.036	0.000

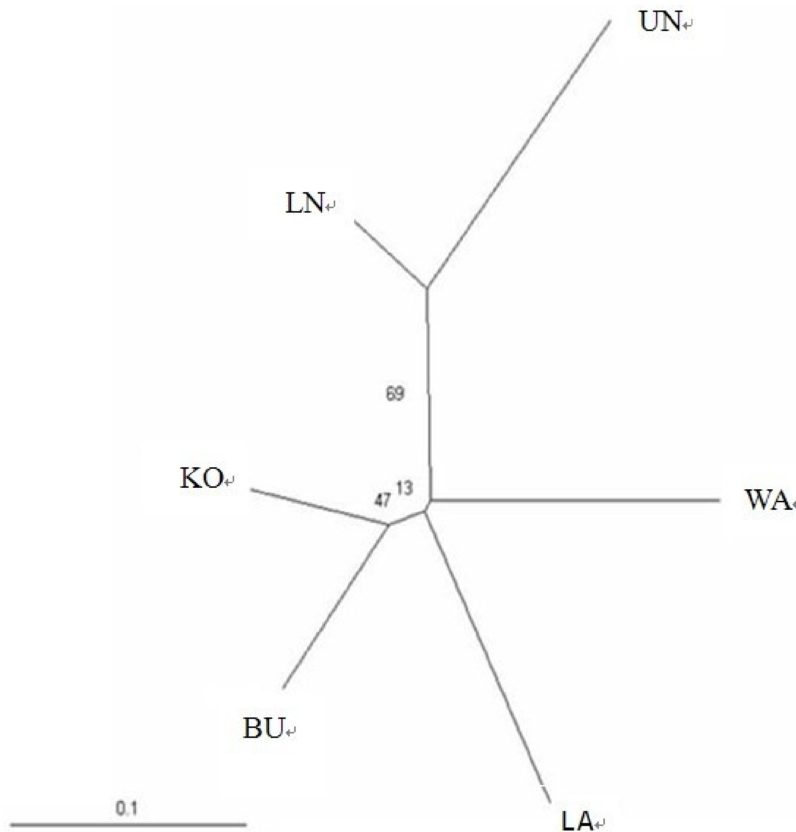

Fig. 2. NJ tree based on D_A distances (Nei et al. 1983) for 6 populations of *C. nigrodigitatus* using 4 microsatellite loci

4. DISCUSSION

A total of eight populations were screened for microsatellite variation. However, two populations, Cross River Basin (CR) and Shiroro Lake (SH) were excluded from analyses because most individuals were monomorphic for all the loci and in others the bands were poor due to degraded DNA. Earlier mtDNA and AFLP analysis of the two populations showed they possess very low diversity [19]. The Niger Delta region of Nigeria is one of the ten most important wetlands in the world. Oil spill and discharges from many other industries is common in the area resulting in environmental contamination. Environmental contamination result in loss of biodiversity and destruction of habitats. In line with the aim of this study, that is, to determine whether genetic diversity in the Niger Delta populations of *C. nigrodigitatus* have been reduced as a result of long-term environmental pollution and degradation, microsatellite analysis was performed on the same set of individuals as in mtDNA and AFLP [19]. Among the populations, mtDNA and AFLP polymorphisms was high except in SH and CR whose

populations experienced a bottleneck (19). Only two mtDNA haplotypes were found in SH due to the isolating effect of the Shiroro Dam coupled with overfishing [20]. Three mtDNA haplotypes were also found in CR attributable to overfishing and possible environmental degradation. The mtDNA and AFLP analysis did not show any less genetic diversity in the Niger Delta populations (BU, KO, and WA) when compared with LA, UN and LN populations. Mulvey et al. [21] did not also find any evidence of decreased diversity in the mtDNA control region of *Fundulus heteroclitus* populations in a highly contaminated environment. Microsatellite analysis of five populations of *C. nigrodigitatus* excluding any Nigerian population revealed high levels genetic variability comparable to those of marine species [6]. The microsatellite data in the present study revealed a similar scenario. However, it is surprising that while mtDNA sequencing was able to detect some variation in SH and CR; microsatellite analysis could not detect any. The probable reason for this lack of variation in SH and CR could be genotyping or scoring errors and the few mtDNA haplotypes have been excluded because of poor genomic DNA.

The range of heterozygosity values of 80-100% and the number of alleles per locus (2-10 per population) in this study was reported for some catfish e.g., [22-24]. All the loci had high values of heterozygosity in all the populations of *C. nigrodigitatus*. However, Kotoulas et al. [6] in a study of four natural populations of *C. nigrodigitatus* detected 29-30 alleles at three microsatellite loci. The small sample sizes may have limited the detection of more number of alleles in the present study. The microsatellite PIC values higher than 0.5 in this study indicate that more genetic information can be provided by SSR loci [25].

The level of genetic differentiation detected between pairs of population was moderate. This may suggest limited level of gene flow and limited dispersal between collection sites. The same conclusion was reached by Song et al. [19]. Indeed, fish which distribute in freshwater and brackishwater environment may show genetic differentiation somewhat in between those that inhabit barrier free marine environment and barrier isolated freshwater [26-29]. Significant ($P< 0.05$) population differentiation was found in more of the pairs of populations F_{ST}'s, showing obviously that the populations were genetically structured. Although the pairwise F_{ST} between populations LA/KO, BU/KO, LA/WA, and KO/WA were large, they were insignificant due to the low bootstrap values. The low support values may be due to the small number of individuals and few loci examined. More number of individuals per population and more number of microsatellite loci are expected to give a better definition of genetic diversity and population structure.

The inbreeding coefficient (F_{IS}; Table 2) defined as the probability that two homologous alleles present in the same individual are identical by descent showed non-significant negative values for all populations, indicating the presence of more heterozygous individuals in these populations. Overall, *C. nigrodigitatus* populations were found to be generally out crossing with little or no inbreeding. The AMOVA analysis showed that 7.37% of the genetic diversity was found between freshwater and brackishwater groups (P=0.047). A small (3.55%) but significant (P=0.000) amount of genetic diversity was found among populations within groups. The AMOVA analysis also showed that a large and significant genetic differentiation (89.08%, P=0.000) accounts for individuals within populations. Unrooted NJ trees based on microsatellites revealed populations UN and LN were differentiated from other populations. The result was consistent with hierarchical analysis of molecular variance above. Freshwater or brackishwater habitat, limited long-distance dispersal of the adult and juveniles may account for breakdown of gene flow.

On the whole, the obtained data show that *C. nigrodigitatus* maintains sufficient intrapopulation genetic variability and moderate interpopulation differentiation in the studied area. Similar conclusions were reached with mtDNA and AFLP analyses [19]. The potential loss of genetic diversity in the Niger Delta due to disturbances, such as environmental pollution from oil extraction, destruction of breeding sites, capture of fry for aquaculture and overexploitation, are reasons for concern and the conservation of genetic diversity may be one of the most important issues facing the future of *C. nigrodigitatus.* However, because of limited sampling size, and lack of representative populations from most of the distribution area, further investigation need to be carried out.

5. CONCLUSION

Freshwater or brackishwater habitat, limited long-distance dispersal of *C. nigrodigitatus* adults and

juveniles may account for breakdown of gene flow and consequently responsible for the current genetic structure. Furthermore, genetic diversity of the species in the Niger Delta was comparable with populations from outside the polluted Niger Delta aquatic environment, maintaining sufficient intrapopulation polymorphism and moderate interpopulation diffentiation. However, an extensive studies comprising more populations and increased population size are needed to make far reaching conclusion.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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