POPULATION STRUCTURE AND GENETIC DIVERSITY OF *OREOCHROMIS SHIRANUS CHILWAE* (TREWAVAS, 1966) IN LAKE CHILWA

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Abstract

Lake Chilwa has gone through several water recessions which have adversely affected fishes biomass and species diversity leading to restocking of *O. shiranus chilwae* at Kachulu in 1969. The study used six microsatellite loci to assess the genetic variability and population structure of *O. shiranus chilwae* populations and whether the restocked fish radiated to all sites in the lake. The populations exhibited considerably low allelic variability with a total allele number of 58, a mean of 10 alleles per locus and allele number range of 4-18. Observed number of alleles (na), and effective number of alleles (ne) revealed that Chinguma and Chisi were the least and most genetically diverse populations respectively. Based on all allelic diversity indices used in the study, western, northern and southern clusters were not significantly different (p>0.05). The populations in study were discrete, moderately structured (F_{ST}=0.12) and not genetically related to each other on the basis of isolation by distance model (Mantels test, r=0.28; p=0.97). Future

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fish restocking should be done on several sites of the lake because *O. shiranus chilwae* has low migration rate (*Nm*=1.8) hence did not radiate to all sites of the lake from Kachulu in 1969. Conservation efforts need to concentrate on each population since the populations are still distinct.

Keywords: *O. shiranus chilwae*, microsatellite loci, populations, migration, Lake chilwa, water recession, genetic diversity

Introduction

Lake Chilwa is an endorheic lake considered twelfth largest lake in Africa and second largest in Malawi after Lake Malawi, located in southern Malawi. At high levels the lake is one of the most productive lakes in Africa. In the 1970s, the lake contributed up to 33% of Malawi annual fish production (Furse et al., 1979). This contribution reduced to 20% in the 1990s and fish catches data of 2000-2009 from major water bodies in Malawi showed that Lake Chilwa' contribution was down to 11% (EAD, 2010). This decline is due to mainly overfishing which result from high human population growth rate (CIA, 2010).

The Lake Chilwa basin has recorded twenty-eight species of fish over the years and of the twenty-eight species, only thirteen have been found in the lake itself while the others are believed to be riverine and only enter the lake in wet season when the salinity of the waters is reduced (Kirk, 1967). The three most saline tolerant species namely; *Oreochromis shiranus chilwae*, *Clarias gariepinus* and *Barbus species* in habit the open water, forming the lake' fishery (EAD, 2001). Six fish families have been recorded from Lake Chilwa namely; Alestiidae, Cichlidae, Clariidae, Cyprinidae, Mormyridae and Schilbeidae (Delaney *et al*., 2006). The lake has five major influent rivers, Domasi, Likangala, Phalombe, Sombani (Malawi side) and Mnembo, the largest source of freshwater to Lake Chilwa, (Mozambique side) (Figure 1).

The lake has gone through several episodes of minor and major water recessions due shallowness of the lake (Mean=2m) and below average rainfall for two or three successive years (EAD, 2001). The lake went through moderate recessions in 1900, 1923, 1931/33, 1943, 1949, 1953/55, 1960/61 and severe recessions in 1914/15, 1966/67 (Morgan, 1971). In recent years, in 1995 and 2012 the lake experienced severe recessions, however, the basin refills rapidly after heavy rain. Fish species diversity and biomass is highly affected particularly during severe recessions due to

increased salinity which prompts fishes to find refuge in pools in inflowing rivers and surrounding marshes (EAD, 2001).

In some severe cases, the lake fishery required restocking, for instance during the 1966/67 recession, the lake was restocked with 300,000 progenies of *O. shiranus chilwae* in the early months of 1969 at Kachulu (Figure 1) (Morgan, 1971; Mathotho, 1975). The restocking was done at one point with the assumption that the fish would migrate to various parts of the lake. Since then, it was not known whether the fish indeed had migrated to the different parts of the lake as assumed, therefore this study used microsatellite markers to determine whether the *O. shiranus chilwae* stocked at Kachulu migrated to the various part of the lake and also to assess the populations structure and genetic diversity given the bottle necks experienced by the populations. This has management implication in that if the stocks migrated continuously, then stock recovery programme in future should restock species in one area but if the stock has limited migration, then restocking should be done in various areas of the lake.

Materials and methods

Sample collection and tissue extraction

The study was carried out at Lake Chilwa where a total of ten sites were sampled (Figure 1). Fish (*O. shiranus chilwae*) samples were caught in 2001 using a beach seine net that was cast in all sites. The number of samples collected from each site are shown in Table 1. Muscle tissue of 5-10mm^2 was extracted from the left side of each fish and preserved in 95% ethanol in well labelled 2ml vials. Tissue samples were transferred to the Molecular Biology and Ecology Research Unit (MBERU) laboratory, Department of Biological Sciences, Chancellor College for DNA analysis.

DNA Extraction

Genomic DNA was extracted using the Phenol/Chloroform protocol as outlined by Sambrook et al. (1989). Muscle tissue of 3mm^2 was minced and placed in a 2ml microfuge tube containing 1.0ml TNE {5µl 1M Tris-HCl (pH 8.0), 10µl 5M NaCl, 1µl 0.5M EDTA, 484µl distilled water} on ice. The tissue was homogenised in the buffer with a handy homogeniser. Lysis buffer (975µl) {150µl 10% SDS, 10µl 1M Tris-HCl (pH 8.0), 27.5µl 5M NaCl, 15.5µl 0.5M EDTA, 772µl distilled water} and 25µl of 20mg/ml Proteinase K (-20°C) were added to the microfuge

tube and incubated at 50°C in an automated Advantec water bath for three hours with intermittent mixing until the tissue was completely digested. Half volume of the mixture was

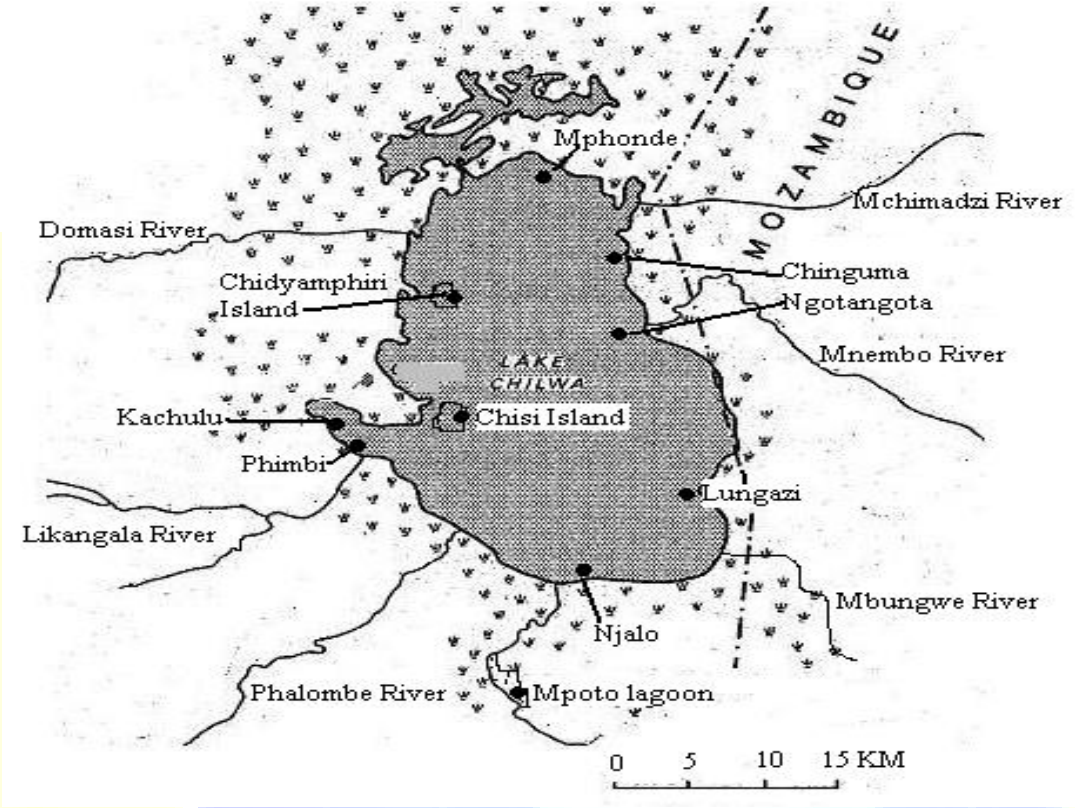

Figure 1: Map of the Lake Chilwa showing sampling sites

Table 1 Sampling sites and samples size

Sample site	Cluster	No of samples collected
Kachulu (KU)	Western	39
Chisi (CS)		40
Phimbi (PH)		40
Chidyamphiri (CM)		23
Mpoto Lagoon (ML)	Southern	40
Njalo (NJ)		40
Lungadzi (LG)		34
Ngotangota (NA)	Northern	24
Chinguma (CA)		12

transferred into a new 2ml microfuge tube into which 750µl phenol was added and mixed on shaker for 2h. The sample was centrifuged at 8000rpm for 5min and the upper layer transferred into a new 2ml microfuge tube and 380µl Phenol and 380µl Chloroform were added before the mixture was left on a shaker for 2h. Then the mixture was centrifuged again at 8000rpm for 5min, the supernant mixed with750µl Chloroform in a new 2ml centrifuge tube and left on a shaker for 2h. This was followed by centrifugation of the mixture at 8000rpm for 5min. The supernant was precipitated in 1000µl cold absolute Ethanol in 1.5ul microfuge tube placed under -20°C for one hour. The DNA pellet was separated from the suspension by centrifugation at14000 rpm for 10min at 4°C and decanting the supernatant. The pellet was rinsed in 750µl 70% ethanol and centrifuged again at 14000 rpm for 5 min at 4°C. The ethanol was decanted and the DNA air-dried for 15min and rehydrated in 50µl high TE buffer {10mM Tris-HCl (pH8.0), 1mMEDTA (pH8.0)} and stored at -20°C.

DNA amplification and PCR products detection

DNA was amplified in 12.5 µl final volume at six polymorphic microsatellite loci (Table 2). The template DNA (2µl) was placed in 0.2ml PCR tube to which 10.5µl of the PCR master mix cocktail was added. The PCR master mix consisted of 5.7µl PCR grade water, 1µl of 10mM dNTP mix, 1.25 µl of 10 x PCR buffer, 1µl of 25mM Magnesium Chloride ($MgCl_2$), 0.2µM (0.75 µl) each of both forward and reverse primers, 0.06µl of 5U AmpliTaq-Gold. The amplification were carried out in a MJ Research Minicycler™ with the following PCR conditions: one AmpliTaq-Gold activation cycle at 95°C for 12 min, followed by 10 amplification cycles, each consisting of a 30s denaturing step at 94°C, 15s annealing step at primer specific temperature, and a 30s extension step at 72°C. This was followed by another 25 cycles, each consisting of denaturing step at 89°C for 30s, annealing step at primer specific temperature for 15s and extension step at 72°C for 15s. The final extension was at 65°C for 20min followed by a soaking temperature of 4°C.

Amplified microsatellite bands were visualized using silver sequence staining procedure on 6% polyacrylamide gels (26 ml of ultra pure water, 7.5 ml of 40% acrylamide:bis (19:1) solution, 5.0

ml of 10 X TBE, 14µl of TEMED (N,N,N',N'-tetramethylethylenediamine), and 357µl of 10% Ammonium persulphate) which were poured in BIORAD Sequi-Gen® GT nucleic acid electrophoresis cell. Two band size standard markers {pGem DNA marker and X174 DNA *Hinf* 1(Promega, USA)} were used to score the bands over a light box.

Table 2 Microsatellite primer sequences and their annealing temperatures

Primer	Sequence	T_{ann}(°C)
Os-08 F	5`- AGAGGAAATGAGCAGCCTC -3`	54
Os-08 R	5`- GATGCGGCAACAGTTATGTC -3`	
Os-64 F	5`- CAGTGTCTTCAGTTCCTTGC -3`	54
Os-64 R	5`- CAGAAGCATCTTATTGATGAC -3`	
Os-75 F	5`- AGCCTAAAATAATGGAATCAC -3`	49
Os-75 R	5`- CCACAGAGTCATGGTTCAC -3`	
Os-25 F	5`- TTGTGAAATTGAATTGCACTC-3`	54
Os-25 R	5`- ACCTCCCTTTGATCCTCTGC -3`	
UNH-146 F	5`- CCACTCTGCCTGCCCTCTAT -3`	54
UNH-146 R	5`- AGCTGCGTCAAACTCTCAAAAG -3`	
UNH-154 F	5`- ACGGAAACAGAAGTTACTT -3`	54
UNH-154 R	5`- TTCCTACTTGTCCACCT-3`	

Data analysis

GENEPOP (Raymond & Rousset 1995) was used to conduct the following analyses: test for conformity to Hardy-Weinberg Equilibrium (Haldane, 1954; Weir, 1990; Guo & Thompson, 1992), test for genotyping linkage equilibrium, test of genic and genotypic differentiation, estimation of effective number of migrants using Slatkin's private allele method (Slatkin, 1985) and computation of Wrights statistics (Wright, 1969).

POPGENE version 1.31 computer program (Yeh *et al*., 1999) was used to compute a number of measures of genetic variation within and between sample populations. The following variables were computed to determine the genetic diversity among populations: observed number of alleles (na), effective number of alleles (ne) (Crow and Kimura, 1970), expected and observed

homozygosity and heterozygosity (Ne, 1973), Shannon's (1949) Information Index (I) (Shannon and Weaver, 1949), Estimates of migration per generation (Nm) and Nei's (1978) unbiased genetic distance.

NTSYS pc version 2.1 (Rohlf, 2001) was used to generate symmetric pair wise dissimilarity matrix from Nei's (1978) unbiased genetic distance which was used to construct a phylogenetic tree from the Sequential Agglomerative Hierarchical and Nested (SAHN) clustering method using the Unweighted Pair-Group Method with Arithmetical averages (UPGMA) (Sneath and Sokal, 1973).

Mantel's test was done to determine correlations between geographical and genetic distance matrices among the *O. shiranus chilwae* populations. The MXCOMP programme of NTSYS pc version 2.1 (Rohlf, 2001) was used to compute a product-moment correlation coefficient (i.e. normalized mantel's statistics Z) for the two matrices (Rohlf, 2001). In order to determine if the correlation was significant, actual coefficient was compared to the values produced by randomly permuting the matrix pair 3000 times.

Graph pad PRISM version 3.00 for Windows (GraphPad Software, San Diego, California, USA) www.graphpad.com.) was used to plot standard error graphs for mean observed number of alleles (na), effective number of alleles (ne) observed heterozygosity and Shannon's Information Index (I) per among the clusters. The program was also used to compare means at 95% level of significance using unpaired *t* test with Welch's correction.

Results and Discussion

Allelic variability

A composite genotypic linkage disequilibrium analysis for all locus pair across all ten populations at six loci, using Fisher' method showed that 3 (20%) locus pairs out of 15 locus pairs were in significant linkage disequilibrium ($p \leq 0.05$) (data not shown). This implies that genotypes at 80% of all loci were not linked and assorted independently therefore the polymorphism of the majority (80%) of the loci was not influence by other loci.

The populations revealed some allelic variability at the six loci. A total of 58 alleles with a mean of 10 per locus and a range of 4 to 18 alleles per locus were generated (Table 3). Chimenya (2001) using the same primers on 11 populations of *O. mossambicus* from Shire river obtained more alleles (128) with a mean of 21 alleles per locus. The reduced polymorphism of the loci

among the *O.shiranus chilwae* compared to *O. mossambicus* populations could be due to varying fishing pressures and reduce numbers in the former because of recurrent water recessions and heavy overfishing. Such pressures on the species result in inbreeding depression in small populations which reduce genetic diversity (Meffe, 1986). Ambali et al., (2000), using microsatellite markers, concluded that reduce genetic diversity among *O. shiranus* in Malawi was caused by decreased effective population size.

Table 3 Total number of alleles per locus (A), and allele size range (SR) in base pairs (bp)

Locus	A	SR
Os-08	18	80-142
Os-25	10	78-122
Os-64	4	128-138
Os-75	11	112-142
UNH-146	7	120-200
UNH-154	8	114-174
Total number of alleles	58	
Mean number of alleles	10	

Genetic diversity indices

Indices of genetic diversity are presented in Table 4 and plots of mean observed number of alleles (na), mean effective number of alleles (ne), mean Shannon Information Index (I) and mean observed heterozygosity are presented in Figure 2. The observed number of alleles (na) ranged between 3.17±0.83-5.67±1.56 while the effective number of alleles (ne) ranged between 2.18±0.48-2.87±0.99 and Shannon's information index (I) ranged between 0.85±0.41-1.19±0.41 (Table 4). Observed heterozygosity ranged between 0.49±0.36-0.87±0.12 and expected heterozygosity ranged between 0.49±0.37-0.64±0.10 (Table 4). The allele number ranges among the three indices suggest relatively low degree of genetic diversity among the populations. But the observed heterozygosity mean (0.7) is close to the expected mean of 0.6 and indicative of moderate to high levels of genetic variation in all populations. Based on na and ne, Chinguma (northern cluster) was the least genetically diverse among the populations while Chisi (western cluster) was the most diverse. Shannon information index also showed that Chinguma is the least diverse while Lungadzi is high in diversity. In terms of diversity of genotypes (heterozygosity),

Mphonde was revealed as the most heterozygous while Kachulu population was the least in heterozygosity (Table 4). However, all the populations were not significant different on the basis of observed number of alleles and effective number of alleles ($p>0.05$). Generally, the pooled populations in three clusters, though of different sizes, {western (142), southern (114) northern (66)} did not exhibit significantly different allelic diversity based on comparison of mean± SE of observed number of alleles, effective number of alleles, Shannon Information Index and observed heterozygosity ($p>0.05$) (Figure 2).

The low genetic diversity among *O. shiranus chilwae* population observed in this study supports the findings of Ambali (1996) where *O.shiranus chilwae* sampled at Kachulu showed considerably low genetic diversity despite the fact that this was the point of restocking in 1969 according to Morgan (1971). Reduced genetic diversity at Kachulu and other sites may be a reflection of the effects of bottlenecking due to water recessions over the years. Ambali et al., (1999) demonstrated in their microsatellite study of *O.shiranus* species in reservoirs in Malawi that genetic diversity was high in more recently stocked and managed reservoirs. This suggests that the genetic diversity of *O.shiranus chilwae* stocked at Kachulu has been eroded over time since 1969.

Table 4 Summary of genetic variation statistics for all populations at six microsatellite loci

Population	na*	ne*	I*	Obs-Het #	Exp-Het**
Kachulu (KU)	5.00±1.23	2.28±0.66	0.99±0.39	0.49±0.36	0.51±0.23
Chisi (CS)	5.67±1.56	2.87±0.99	1.09±0.61	0.58±0.44	0.55±0.32
Phimbi (PH)	4.33±0.89	2.56±0.51	1.05±0.36	0.63±0.32	0.58±0.18
Chidyamphiri (CM)	3.33±0.66	2.24±0.46	0.90±0.35	0.71±0.36	0.53±0.22
Mpoto Lagoon (ML)	5.33±0.59	2.82±0.78	1.16±0.35	0.58±0.44	0.60±0.17
Njalo NJ)	4.00±1.14	2.41±0.76	0.91±0.57	0.64±0.45	0.49±0.37
Lungadzi (LG)	5.17±1.19	3.00±0.78	1.19±0.41	0.64±0.33	0.62±0.19
Ngotangota (NA)	4.00±0.84	2.60±0.27	1.08±0.24	0.79±0.27	0.62±0.09
Chinguma (CA)	3.17±0.83	2.18±0.48	0.85±0.41	0.58±0.48	0.51±0.27
Mphonde (ME)	3.67±0.54	2.80±0.35	1.09±0.23	0.87±0.12	0.64±0.10

na* = mean number of alleles, ne* = mean effective number of alleles (Kimura and Crow, 1964); I* = mean Shannon's information index (Lewontin, 1972); Obs-Het#=Mean observed heterozygosity; ** = mean expected heterozygosity (Levene, 1949)

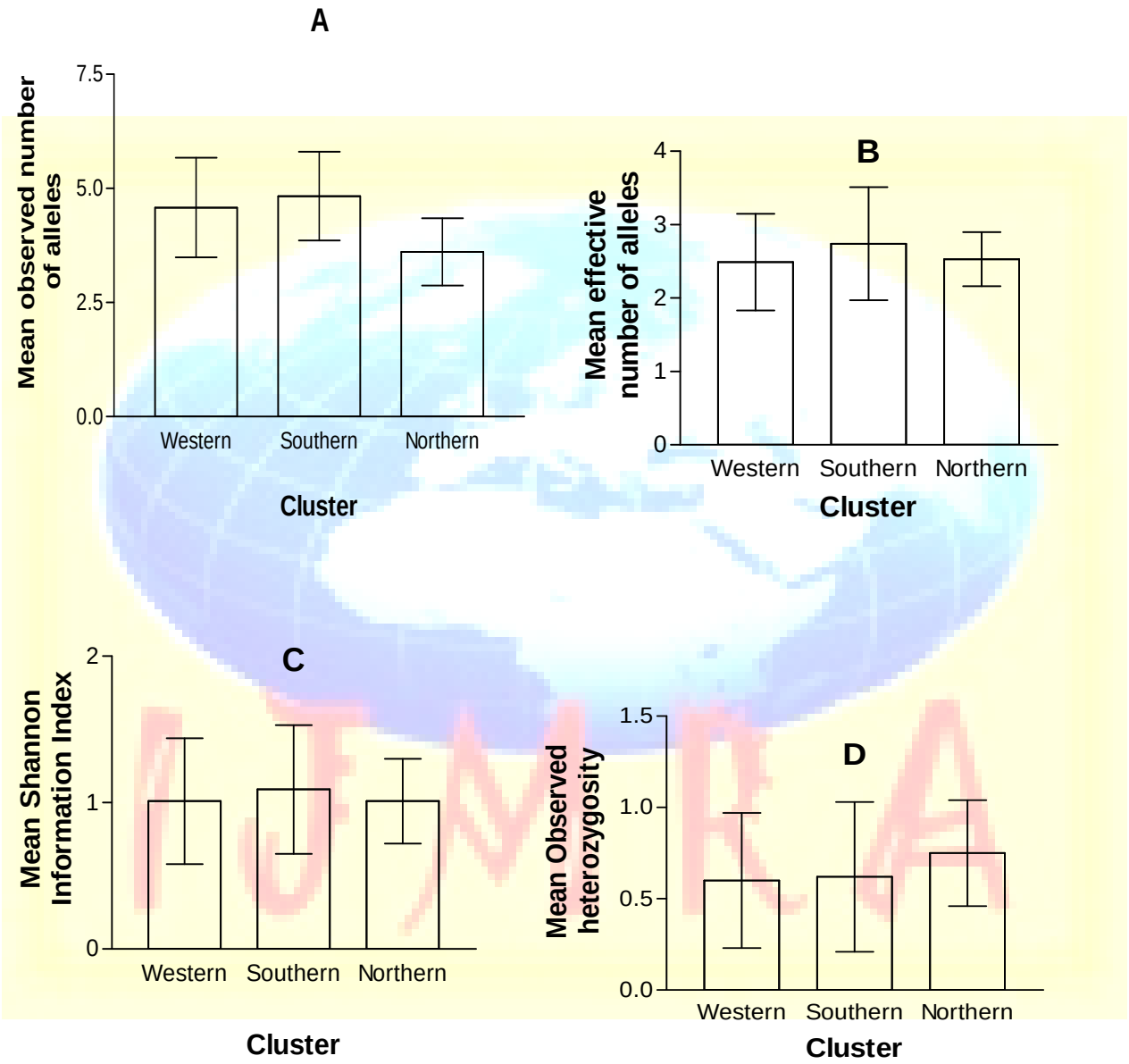

Figure 2: Genetic diversity of the populations clustered by area; A=mean±SE observed number of alleles; B= mean±SE effective number of alleles; C=mean±SE Shannon Information Index and D=mean±SE Observed heterozygosity

Conformity to the Hardy-Weinberg Equilibrium and heterozygosity

Table 5 shows probability values for test for Hardy-Weinberg Equilibrium (HWE). Thirty two percent of the population-locus combinations were in significant departure from HWE ($p < 0.05$) while 68% of the population-locus combinations were in HWE ($p > 0.05$). There were more loci-population combinations that were in HWE contrary to expectations, given that the populations in Lake Chilwa are known to experience bottlenecks which result in inbreeding depression because of severe water recession and heavy overexploitation of fish. Therefore the populations are neither infinitely large nor experiencing random mating. Gene flow is evident in the lake though overall it is low (*Nm=1.8*) but within clusters, it is generally high (Table 7). This observation supports the argument that the test of HWE is less efficient in markers like microsatellites which are highly polymorphic (Kamonrat, 1996).

Table 5 Probability values of test for Hardy-Weinberg Equilibrium at six microsatellite loci

Population	Os-08	Os-25	Os-64	Os-75	UNH-146	UNH-154
Kachulu	0.0108	0.2418	1	0.0000	0.4204	0.3581
Mpoto	0.0132	1	1	0.0000	0.0000	0.0000
Lagoon						
Chisi	0.0000	1	1	0.0000	1	0.6202
Lungadzi	0.0151	0.9981	1	0.0000	0.9083	0.0023
Ngotangota	0.1943	1	1	0.0096	1	0.0005
Chinguma	0.0000	1	1	0.3721	1	1
Mphonde	0.7055	0.9991	1	0.0864	1	1
Chidyamphiri	0.9945	1	1	0.0276	1	0.0929
Njalo	0.0023	1	1	0.0052	1	1
Phimbi	0.0485	0.9811	1	0.0000	0.8152	0.1633

Heterozygosity excess or deficiency values (F_{IS}) are presented in Table 6. Mean F_{IS} values were negative in all populations except Kachulu indicating heterozygosity excess (Table 6). In general, all populations and all clusters experienced heterozygosity excess (Table7). Heterozygosity excess was expressed by 68% of population-locus combinations (Table 6). This

observation is probably due to selection pressures operating in Lake Chilwa that favour heterozygotes also referred to as overdominance or heterozygote superiority.

Table 6 Heterozygosity excess and deficiency (F_{IS}) using Weir and Cockerham method*

Pop.\Locus	Os-08	Os-25	Os-64	Os-75	UNH-146	UNH-154	Mean F_{IS}
Kachulu	0.404	0.075	-0.753	0.383	0.066	-0.025	0.025
Mpoto Lagoon	0.282	-0.702	-0.951	0.638	0.404	0.284	-0.008
Chisi	0.456	-0.642	-1.000	0.527	-0.219	-0.021	-0.150
Lungadzi	0.186	-0.371	-0.763	0.525	-0.234	0.213	-0.074
Ngotangota	-0.070	-0.510	-1.000	-0.040	-0.706	0.395	-0.322
Chinguma	0.771	-0.571	-1.000	-0.054	-0.100	-0.222	-0.196
Mphonde	-0.081	-0.413	-1.000	0.048	-0.493	-0.480	-0.403
Chidyamphiri	-0.274	-0.739	-1.000	-0.028	-0.158	-0.041	-0.373
Njalo	-0.057	-0.496	-0.906	-0.000	-0.147	-0.057	-0.277
Phimbi	0.156	-0.296	-0.951	0.311	-0.086	0.214	-0.109

* negative F_{IS} values indicate heterozygosity excess while positive values indicate heterozygosity deficiency. Heterozygosity excess was exhibited by 68% of the population-locus combinations while 32% showed heterozygosity deficiency.

Genetic structure and differentiation among populations

The populations were significantly differentiated as depicted by tests for a genic and genotyping differentiation ($p \leq 0.05$; data not shown). Wright' statistics presented in Table 7 showed overall moderate differentiation of 12% among the populations which is supported by low gene flow of 1.8 individuals per generation (Table 7). Wright (1978) considered F_{ST} values ranging from 5-15 as demonstrating moderate differentiation. Comparison of within cluster population differentiation indicates that the northern cluster populations had the least differentiation (F_{ST}=0.06) and western cluster populations had the highest differentiation (F_{ST}=0.11). This is supported by highest within cluster gene flow (*Nm* =4.1) in the northern cluster and lowest within cluster migration (*Nm*=2.0) in the western cluster. Mills and Allendorf (1996) reported that studies have shown that $Nm \geq 1$ leads to considerable homogeneity among populations while population structuring and divergence can occur when $Nm \leq 1$. This implies that rates of gene flow found in this study are therefore high and could lead to homogeneity of populations in Lake Chilwa over time. However, this study has shown that the *O. shiranus chilwae* populations in Lake Chilwa are significantly differentiated and moderately structured signifying that though gene flow is a potent force acting against genetic divergence resulting from genetic drift among subpopulation (Hartl and Clark, 1989), the seemingly low rate of migration among the populations (*Nm*=1.8) may have resulted into discrete populations as evidenced by the UPGMA clustering (Figure 3).

Table 7: Estimates of number of migrants per generation (*Nm*) and fixation index (F_{ST})

Populations/Cluster	Nm	F_{ST}	F_{IS}
For all populations	1.8	0.12	-0.19
Within western cluster	2.0	0.11	-0.15
Within southern cluster	2.9	0.08	-0.12
Within northern cluster	4.1	0.06	-0.31

Genetic relationships and cluster analysis of the populations

Figure 3 shows a dendrogram depicting genetic relationships among the populations based on Nei's (1978) genetic distances. The dendrogram has three distinct clusters; cluster 1 consists of all populations under western cluster (top of the dendrogram), cluster 2 has Mpoto lagoon population and cluster 3 has all the remaining populations from southern and northern clusters. In cluster 1, Kachulu population is genetically distant from the other populations in cluster which are geographically close to it suggesting that Kachulu may not have been the source for restocking the other populations even within this cluster in 1969. Mantel's test, which determined the correlation between geographical distance (data not shown) and genetic distance (data not shown) among the populations showed low insignificant positive correlation (r = 0.28; p=0.97). The results suggest that isolation by distance model did not influence the degree of genetic differentiation (F_{ST}=0.12) among the Lake Chilwa *O. shiranus chilwae* populations in this study. Isolation by distance model (Wright 1943) stipulates that gene flow is highest among close populations, therefore is expected that close populations should have similar genetic composition which is not widely the case in these populations.

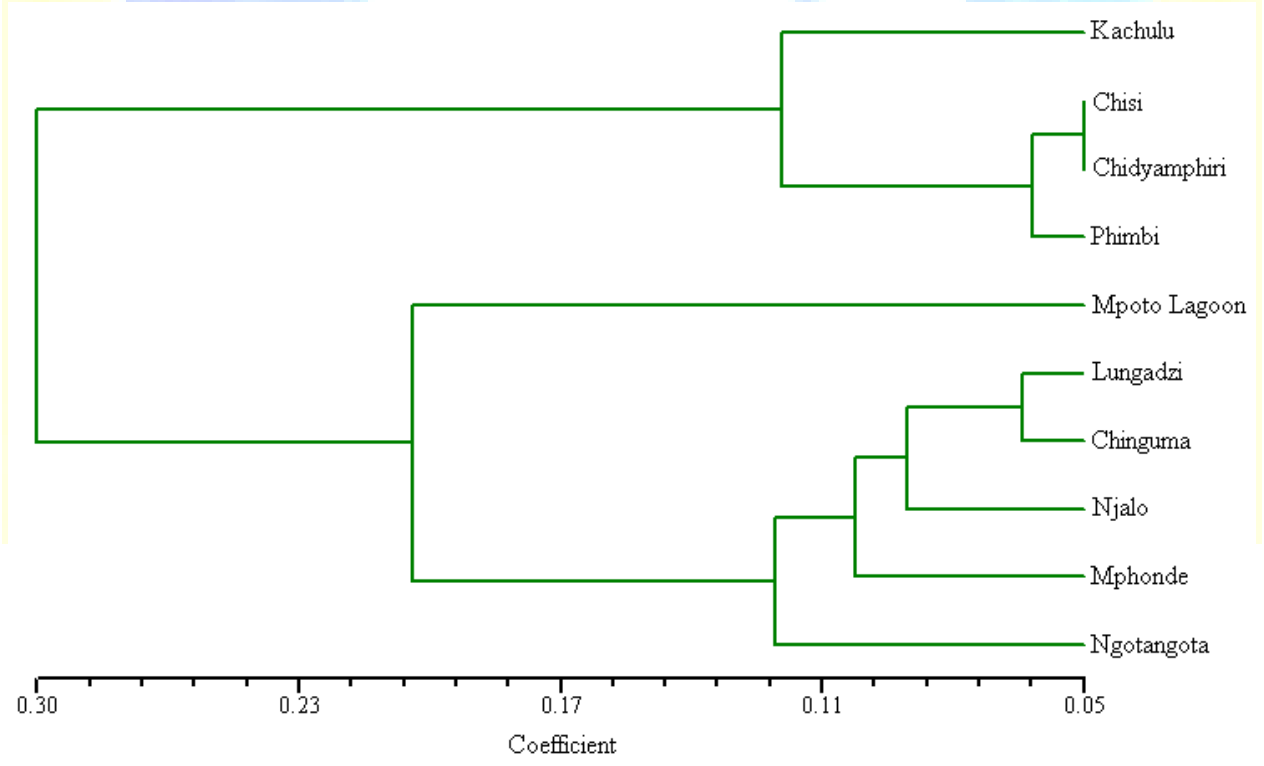

Figure 3: Dendrogram based on Nei's (1978) Genetic Distance using SAHN clustering method

Conclusions

O. shiranus chilwae populations in the study showed a considerably low allelic variability at six loci though heterozygosity was moderate among the population. The three clusters exhibit similar genetic variability. The study found that the populations are discrete, moderately structured and not related to each other by isolation by distance model. Conservation efforts, therefore, need to concentrate on each population since the populations are still distinct though going toward homogeneity given the high gene flow within the clusters.

The study recommends that future restocking after severe water recession should be done at more than one site because *O. shiranus chilwae* has low migration rate (*Nm*=1.8). Analysis of genetic relationships among the populations shows that the populations are differentiated from Kachulu population and did not probably radiate from the Kachulu restocking of 1969 but rather from other sources given the genetic distances (Figure 3).

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