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POPULATION AND STOCK CHARACTERIZATION OF LAKE VICTORIA TILAPINE FISHES BASED ON RAPD MARKERS

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Two sister groups of cichlids, the Tilapiines and the Haplochromines, have historically comprised the majority of the ichthyfauna of the Lake Victoria basin of eastern Africa. Tilapias are exclusively African and Levantine in their natural range and are thought to have diverged from a complex ancestral mixture, which also included the ancestors of the haplochromines, more than 10 million years ago (Fryer and Iles, 1972; Trewavas 1983). The tilapias, particularly members of the genus *Oreochromis*, have been extensively introduced all over the world, and are now among the most economically important species in global aquaculture. The tilapias offer a fertile field for study of complex ecology, behavior, and evolutionary attributes as a result of their rich evolutionary history and relationship to the Haplochromines, a sister group that is even more ecologically complex and diverse (Trewavas, 1983). Tilapias belong to the tribe Tilapiini (subfamily: Tilapiinae) of the family Cichlidae in the order Labroidea of the Perciformes (Nelson, 1994). Trewavas (1983) describes ten genera within the Tilapiini, including three major genera, *Tilapia*, *Oreochromis* and *Saratherodon*, represented in the Lake Victoria basin. Species of *Tilapia* are substrate spawners; both parents guard the young. *Oreochromis* species are maternal mouthbrooders, only females carry eggs and young in their mouths. *Saratherodon* are biparental mouthbrooders, both female and male parents carry eggs and young in their mouths, and .

Species classification has been largely based on variation in dentition, bone structure, pigmentation, squamation characteristics and general body morphology (Fryer and Iles, 1972; Kornfield et al., 1979; Stiassny, 1991, 1992; Trewavas, 1982). However, most or all of these characters overlap and may fail to unambiguously identify species owing to interpopulation variation and small differences among species. Molecular techniques have been employed in an attempt to characterize and identify Tilapiine species (Seyoum and Kornfield, 1992; Frank et al., 1992). Bardakci and Skibinski (1994) used protein electrophoresis to discriminate tilapia species and their hybrids. Mitochondrial DNA markers have been used by Capili (1990) and by Seyoum and Kornfield (1992) to identify subspecies of *Oreochromis niloticus*. Only a few studies (Frank et al., 1992, Bardakci and Skibinski, 1994) attempted to analyze nuclear DNA markers, and no study has been reported concerning variation within and between natural populations.

The aquatic ecosystem of Lake Victoria has been greatly modified by overfishing, increased human activity in the drainage basin, and the introduction of exotic species. The Nile perch, *Lates niloticus*, a voracious predator, and several non-native Tilapiine species (including the ecologically versatile Nile tilapia, *Oreochromis niloticus*) were deliberately introduced between

1930 and 1965. These exotic species rapidly displaced many of the indigenous Haplochromine cichlids from the lake, perhaps driving over 50% of the species to extinction. In addition to the Haplochromine cichlids, two endemic Tilapiine species, *Oreochromis variabilis* and *O. esculentus*, were extirpated from Lake Victoria, and their only remaining natural refugia are satellite lakes near Lakes Victoria and Kyoga (Ogutu-Ohwayo, 1990, 1992, Balirwa, 1992). In a majority of the satellite lakes, the remaining populations of the endemic Lake Victoria Tilapiines are threatened due to the occurrence of exotic Tilapiines.

We have explored the extent of genetic variation and of gene introgression between several species of tilapia found in the Lake Victoria region. The markers developed can be used in the identification of wild stocks and species, and in the monitoring of wild or managed stocks to apply appropriate fisheries management techniques. They also can be applied in aquaculture, to identify individuals, families, species and to label brood stocks (Hadrys et al., 1992). The markers can also provide taxonomic insight. Random gene markers often discriminate among species and subspecies of tilapias with better resolution than morphometric traits.

Genetic diversity is the ultimate basis for the evolutionary ability of a species to respond to genetic and environmental changes. Our work ultimately seeks to determine whether the ecological versatility and resilience of exotic tilapia, especially *O. niloticus*, as exemplified by its dominance within the Lake Victoria region, is related to higher genetic variability of these species. Higher variability may be related in part to hybridization within the species assemblage of the Lake Victoria basin. To characterize population and species differences, we have used the Randomly Amplified Polymorphic DNA (RAPD) technique to assess genetically based differentiation within and between populations and among species of the Tilapiine taxa in the Lake Victoria region. We also attempted to identify species-specific genetic (RAPD) markers, for use in the assessment of levels of hybridization and introgression among the Lake Victoria Tilapiine species. Of special interest in this report is the question of introgression between introduced populations of *O. niloticus* and the endemic species *O. esculentus.*

Methods and Materials

SAMPLES: The study was conducted using material collected from the Lake Victoria Basin in East Africa. Fish from five species (*Oreochromis niloticus, O. esculentus,* and *O. leucostictus*, and *Tilapia zilli* and *Saratherodon galilaeus)* were collected from northern Lake Victoria (in the region around Jinja, Uganda, near the outlet to the Victoria Nile), from Lakes Albert and Edward, and from several satellite lakes of Lake Victoria. Satellite lakes are minor water bodies surrounding Lake Victoria, formed as backwaters derived through a series of drying and refilling which has characterized the geologic history of Lake Victoria (Kaufman, 1992). Samples were collected from eight satellite lakes: four (Lake Nabugabo, Lake Kayugi, Lake Kayanja, and Lake Manywa) make up parts of the Lake Nabugabo region at the northwest corner of Lake Victoria, south of the Katonga River, three (Lake Kijanebalola, Lake Kachira, and Lake Mburo) are located in the Koki lakes/marsh region between Lake Victoria and Lake Edward, south of the Katonga River, while a single satellite lake (Lake Kanyaboli) is part of the Yala River system, located on the east side of Lake Victoria, in Kenya between Mwanza Gulf and the Nzoia River. None of the satellite lakes are known to contain populations of Nile perch, with the exception of Lake Nabugabo, the closest of the satellite lakes to Lake Victoria. Each of the satellite lakes contained *O. esculentus* (Ngege), either as the only Tilapiine species or sympatric with *O. niloticus*, again with the exception of Lake Nabugabo, where *O. esculentus* had been extirpated. Lake Victoria's Tilapiine fauna no longer includes *O. esculentus*, while the species was never part of the fauna of Lakes Edward or Albert. *O. niloticus* was collected from the large lakes (Victoria, Albert and Edward) and from three satellite lakes (Mburo, Kachira and Nabugabo). *O. leucostictus* was obtained from Lakes Victoria, Nabugabo, Mburo and Kachira. Samples of the other two genera were more restricted; *Saratherodon galilaeus* was sampled only from Lake Albert and *T. zilli* only from Lakes Victoria and Nabugabo.

Fish were collected using small sized gill-nets and seine nets, or minnow traps for swampy and overgrown shorelines. In the field, muscle tissue was removed from individual specimens immediately after capture. All samples used in this study were from individuals that were readily classified in the field. Immature and/or uncertain morphs were avoided. About 0.5 cm^3 of muscle was removed from an individual and placed in 95% ethanol. After one hour, the tissue were transferred to a second vial with fresh ethanol (95%), where it remained until DNA was extracted. Population analysis was undertaken only if sample sizes for a species was ten individuals or more. Ten individuals from each locality were included in this analysis.

MOLECULAR METHODS: The RAPD (Random Amplified Polymorphic DNA markers) technique detects DNA polymorphism by use of the Polymerase Chain Reaction (PCR). The techniques achieves PCR amplification of genetic regions which are flanked by small inverted copies of an arbitrary single primer sequence. In a vertebrate genome, a number of such flanked sequences will occur, randomly scattered through various chromosomes. A DNA fingerprint will be produced which can be used to compare individuals. The RAPD technique has increasingly been used in molecular ecology because it is relatively inexpensive and is adequately robust compared to other molecular techniques (Hadrys, 1992; Russell et al., 1992; Dawson et al., 1992). No prior DNA sequence information about the organisms is required. Distribution of amplification sites is assumed to be random. The RAPD technique, however, has some drawbacks. The technique is sensitive to reaction conditions and slight changes may lead to non-reproducibility of amplification products, requiring care in standardizing the reaction conditions. Use of duplicate reactions can control for reproducibility of the bands. In this study, like the case of Hadrys et al. (1992) and Williams et al. (1990), duplication of RAPD amplifications produced markers that could be judged as clearly reproducible and scorable. The technique also produces dominant markers, in contrast to other DNA markers such as the RFLP markers, which are codominant. This causes some problems in the interpretation of levels of variability. Even though not as sensitive as codominant DNA markers, use of RAPDs does allow estimation of allele frequency for population genetic analysis, and estimation of introgression.

DNA extraction amplification and gel electrophoresis. DNA was extracted from muscle using a standard phenol/chloroform extraction procedure. PCR reaction mixtures of 25 ul final volume contained about 50 ng of genomic DNA, 25 uM final concentration of each of the four nucleotides (dATP, dTTP, dCTP and dGTP), 1 ul of 200 Nm primer, reaction buffer and 0.1 unit of 5 uM Taq polymerase. A series of twenty different decamer (10 bp) oligonucleotide primers were obtained from Operon Technologies, Alameda, California (designated series M, according to G+C content) and evaluated for the production of scorable patterns in tilapia species. Eight of these primers were chosen for use in the study. Those used included primers OPM2, OPM7, OPM11, OPM12, OPM14, OPM15, OPM17, and OPM19.

PCR amplifications were performed in a Perkin-Elmer automated thermocycler, with the following amplification conditions: 2 min at 94°C as the initial step; 45 cycles of 30 seconds at 92°C, 1 min at 35°C, and 2 min at 75°C with a 5 min delay at 72°C at the end of the 45 cycles. Amplification products were separated by electrophoresis in 1.6% SYNERGEL agarose gels, stained with ethidium bromide, and viewed under ultraviolet light. Each set of PCR amplifications included positive and blank controls to ensure that the observed banding patterns were reproducible, repeatable, and uncontaminated before scoring. RAPD bands were scored against a series of standard DNA size markers, the 123 base pair ladder size standards. Given the size and position of an amplified band relative to the marker DNA, bands in different individuals were scored as '1' for present or '0' absent for each amplification product across all the 10 individuals sampled from each population.

Data analysis. The estimations and calculations of genetic variation followed the approach of Lynch and Milligan (1994), which provides estimates of gene diversity and the analysis of genetic structure, correcting for the bias due to dominance in RAPD data. Estimates of

hybridization and introgression were obtained by identifying allelic markers that were "species-specific", as defined by high relative allelic frequency differences (>0.8 between species samples from localities in which hybridization in unlikely). Frequencies of RAPD alleles characteristic of either "pure" *O. niloticus* from Lake Albert, or "pure" *O. esculentus* from Lake Kanyaboli were identified for all the locations, and proportions of alleles of one species which appear in a congener was estimated.

RESULTS

The eight primers generated a total of 167 scorable loci (bands) within the various populations of the five Tilapiine species. RAPD fragment patterns were examined for the presence of species-specific bands, bands that occurred among individuals of only one of the species. *O. esculentus* was found to contain the largest number of unique bands (13 of 140 total bands), followed by *O. niloticus* (11 of 115), *T. zilli* (7 of 52), and *O. leucostictus* (4 of 47). Surprisingly, *S. galilaeus* had no bands that occurred exclusively among the individuals sampled. In a similar manner, the three species of *Oreochromis* were examined for the presence of population-specific bands, those occurring exclusively among individuals of a particular population, and not shared between populations. *O. leucostictus* had the largest proportion of population specific bands (24.1% on average), followed by *O. niloticus* (average of 16.8% per population) and *O. esculentus* with the fewest (10.7% per population).

Polymorphism: Levels of variability were estimated in two ways. First, the proportion of polymorphic bands within a population was estimated (Table 1). Among the *Oreochromis* species, *O. leucostictus* was most variable, having an average of 66.5% of its bands polymorphic in a population, followed by *O. niloticus* (59.0% of bands polymorphic per population), with *O. esculentus* being least variable (only 54.4% of bands per population being polymorphic). Populations of *Saratherodon galilaeus* and *Tilapia zilli* were found to be quite variable, showing 70.0% and 65.0% of bands polymorphic per population. There was considerable variation in the levels of polymorphism seen in different populations of *O. niloticus*, ranging from a high of 81% in the Lake Kachira population to a low of only 36% in the Lake Albert sample. However, the highest levels of variation were seen in Lake Kachira and Lake Nabugabo (75% polymorphic loci), two populations invaded by the Nile tilapia and in which it has become dominant, severely impacting the endemic *O. esculentus* populations. In contrast, a native habitat for the Nile tilapia, Lake Albert, has the lowest levels of polymorphism, and may represent relatively unhybridized stocks of *O. niloticus*. In the endemic species *O. esculentus*, results were also heterogeneous, with a high of 78% polymorphic loci in Lake Mburo down to a low of only 33% in Lake Kijanebalola. Relatively lower levels of polymorphism occurred in *O. esculentus* populations that were geographically more isolated from other tilapiine species, especially the Lake Kayanja sample (43%), or in places in which *O. esculentus* continues to be the predominant species when compared to the exotic *O. niloticus*, as in Lakes Kanyaboli (40%) and Kijanebalola (33%). Populations from other lakes in which other tilapiines are more dominant showed more polymorphism (average 67%) . In general, *O. leucostictus* populations were more homogeneous than other *Oreochromis* species, ranging from 78% in Lake Kachira to 54% in Lake Nabugabo.

Gene diversity (Heterozygosity): Gene diversity estimates showed patterns generally similar to those exhibited by levels of band polymorphism. *O. esculentus* had the lowest average gene diversity, significantly lower than that seen in the other species (Table 2). The other species were not significantly different from each other. Considering population gene diversity within a species, the populations of *O. niloticus* from Lake Kachira (H = 0.27) and Lake Nabugabo (H = 0.29) had the highest levels of gene diversity. In *O. niloticus*, the Lake Edward population had the lowest diversity (H = 0.14). Populations of the Nile tilapia from Lakes Albert (H = 0.17) and Victoria (H = 0.20) had intermediate levels. Lake Kachira held the most variable population of *O. leucostictus* (H = 0.25), followed by Lake Victoria (H = 0.22) and Lake Nabugabo (H = 0.18). The Lake Mburo population (H = 0.15) was lowest of the four populations compared. As with

polymorphism, populations of *O. esculentus* that appear to have interacted less with *O. niloticus* (Kayanja; H = 0.11), or where *O. esculentus* has continued to be dominant when they co-exit, such as in Lake Kanyaboli (H = 0.09), had lower gene diversity levels compared to populations from lakes where *O. niloticus* is dominant such as Lake Mburo (H = 0.22). Lakes Kachira, Kayugi and Manywa have gene diversity levels of 0.16, 0.17 and 0.17, respectively. Populations of *T. zilli* were nearly equal in gene diversity levels (Nabugabo: H = 0.21; Lake Victoria: H = 0.23), relatively high compared to most populations in the other species. The Lake Albert sample of *S. galilaeus* had a heterozygosity (H = 0.20) close to that of *O. niloticus*.

Table 1: Total number of bands (T), number of polymorphic bands (P), and proportion of polymorphic bands (Pm), in populations of five Tilapiine species.

species/	*O. niloticus*			*O. esculentus*			*O.leucostictus*			*S. galilaeus*			*T.zilli*		
Lake	**T**	**P**	**Pm**	**T**	**P**	**Pm**	**T**	**P**	**Pm**	**T**	**P**	**Pm**	**T**	**P**	**Pm**
Victoria	40	24	.60		-		50	35	.70		-		44	24	.55
Nabugabo	44	33	.75		-		50	27	.54		-		52	39	.75
Edward	51	26	.51		-			-			-			-	
Albert	11	4	.36		-			-		50	35	.70		-	
Mburo	43	21	.49	73	57	.78	39	25	.64		-			-	
Kachira	53	43	.81	57	38	.67	55	43	.78		-			-	
Kayugi		-		62	37	.60		-			-			-	
Manywa		-		37	22	.60		-			-			-	
Kayanja		-		44	19	.43		-			-			-	
Kanyaboli		-		60	24	.40		-			-			-	
Kijanebalola		-		48	16	.33		-			-			-	

Table 2. Average values of gene diversity (Hj) in populations of the five Lake Victoria region tilapiine species.

species	average # loci	H_j
O. niloticus	41.7	0.213
O. leucostictus	48.5	0.202
O. esculentus	54.4	0.143
S. galilaeus	50.0	0.196
T. zilli	40.5	0.222

Partitioning of genetic diversity within and between populations: Since four of the species, had been sampled for more than one population, it was possible to evaluate how total gene diversity is partitioned into within- and between-population components. This is the application of the F_{ST} analysis first expounded by Sewall Wright (1951). F_{ST} represents the proportion of the total gene diversity (heterozygosity) in the sample which is contributed by differences in allele frequency between populations. For the *Oreochromis species*, this proportion is very large, indicating that the species is highly structured and populations differentiated, probably exchanging few migrants with respect to RAPD markers. All *Oreochromis* species were highly subdivided

as measured by F_{ST}, with values of F_{ST}= 0.69, 0.74 and 0.80 for *O. niloticus*, *O. leucostictus* and *O. esculentus*, respectively. Thus, although *O. esculentus* populations had lower variability, on average, the species was more subdivided than the other two species of the genus *Oreochromis*.

Estimation of gene flow between tilapia species of the Lake Victoria basin: The extent of introgression between the introduced Nile tilapia and the endemic form *O. esculentus* was estimated using specific diagnostic marker alleles. We define diagnostic alleles as bands observed to have a high frequency difference between 'pure' populations of a pair of species. These pure populations are ones that had not been in contact with, or which were much less likely to have been in contact with, or that were geographically isolated from an exotic species. 'Fixed' alleles were alleles that had frequency 95% or greater in a particular population. In addition, populations that could be regarded as a possible source of the exotic species, or which was found to be basal to the other populations of the same species in a phylogenetic analysis, were used to assess the extent of gene flow among congeners.

Among the *O. niloticus* populations, alleles in both the Lake Edward and Lake Albert populations that were fixed and specific to *O. niloticus* were used to estimate the extent of gene introgression of *O. niloticus* alleles into *O. esculentus*. Lake Edward and Lake Albert *O. niloticus* populations are considered as a likely indigenous source of the Nile tilapia in the Lake Victoria basin. To examine gene flow in the direction from *O. esculentus* into *O. niloticus*, the *O. esculentus* population from Lake Kanyaboli was used. Lake Kanyaboli population 'fixed alleles' were used to estimate *O. esculentus* alleles represented in *O. niloticus* populations. In Lake Kanyaboli, *O. esculentus* is the most dominant species, although it is likely that here *O. esculentus* coexists with some *O. niloticus*. The estimates of introgression give a qualitative picture of possible hybridization in these lakes. Results are presented in Tables 3 and 4. Populations of a species from lakes that had mixed species showed relatively higher levels of alleles that were specific to the 'pure' populations of the congeneric species, than populations of species that were not co-existing or were most dominant. *O. niloticus* populations had a comparably lower proportion of *O. esculentus* alleles than the proportion of *O. niloticus* alleles contained in *O. esculentus*. Among the *O. esculentus* populations, Lake Mburo population had the highest percentage of *O. niloticus* diagnostic alleles and Lake Kanyaboli population had the smallest (Table 3). Among the *O. niloticus* populations, the Lake Nabugabo population, from which *O. esculentus* has been extirpated, had the highest share of *O. esculentus* bands, suggesting substantial hybridization in this restricted habitat, while Lake Victoria had only a very small proportion of *O. esculentus* specific alleles, suggesting that *O. niloticus* was able to out compete *O. esculentus* without substantial hybridization and backcrossing to the Nile tilapia invaders.

Table 3. Proportion of *O. niloticus* diagnostic bands which appear in particular populations of *O. esculentus*.

LOCATION	% *O.n.* genes present
Lake Mburo	35.27
Lake Kayugi	14.39
Lake Kachira	14.11
Lake Manywa	13.73
Lake Kayanja	12.94
Lake Kanyaboli	6.72

Table 4. Proportion of *O. esculentus* diagnostic bands which appear in particular populations of *O. esculentus*.

LOCATION	% *O.e.* genes present
Lake Nabugabo	21.10
Lake Kachira	8.20
Lake Mburo	6.67
Lake Victoria	0.91

DISCUSSION

RAPD population analysis: The RAPD technique was reliable and simple to apply, proving to be cost effective and appropriate for population genetic structure analysis. Using eight arbitrary PCR primers, 167 random markers were generated for 20 populations. It was useful in studying large numbers populations as well as individuals. The technique revealed substantial nuclear genomic variation, which has been a difficulty in some previous work with Tilapiines. Most variation obtained with other techniques was sufficient only to discriminate among species (Kornfield et al., 1979; McAndrew and Majumdar, 1983; Abban, 1988). Capili (1990) and Seyoum and Kornfield (1992) discriminated subspecies of Nile tilapia, *O. niloticus*, using mitochondrial DNA markers. Franck et al. (1992) used satellite DNA which revealed genetic variability among Tilapiine species. The RAPD technique was used by Bardakci and Skibinski (1994) to differentiate species and subspecies of the Nile tilapia and three other species of the genus *Oreochromis* in aquaculture. This study represents the first application of RAPD technique to the assessment of variation within and between naturally occurring Tilapiine populations and species.

Polymorphism: The significant differences in polymorphism among both *O. niloticus* populations and *O. esculentus* populations appears to be due in part to hybridization when populations of these two species co-exist and exotics dominate. Where the species co-exist, populations were more polymorphic than populations that did not overlap. Low polymorphism exhibited by *O. niloticus* populations from the endemic geographic origins of this species, Lake Albert and Lake Edward, probably indicates the absence of significant gene exchange among Tilapiine species of these lakes. The low levels of heterozygosity exhibited by *O. esculentus* populations correlate with its highly specialized mode of ecology. This and its relegation to minor isolated water bodies, and severely reduced population sizes in lakes where other Tilapiine species were introduced, puts *O. esculentus* in danger of extinction. This may also account for the higher population subdivision of *O. esculentus* compared to *O. niloticus* and *O. leucostictus*. However, the latter two still show substantial subdivision, possibly because of small stock sizes that characterized introductions of these exotics into parts of the Lake Victoria region (Balirwa, 1992).

There is an imbalance in the proportion of *O. esculentus* alleles compared to *O. niloticus* alleles which have found their way into their congener. One explanation is that hybridization may be much greater into *O. esculentus* from *O. niloticus*, with only a small gene flow in the reverse direction. Males of *O. niloticus* probably hybridize freely with *O. esculentus* females, while stronger isolating mechanisms prevent *O. esculentus* males from hybridizing with *O. niloticus* females. This could occur by the dominance of *O. niloticus* both in number and behavior. A

much larger proportion of "*O. esculentus*" morphotypes would have some *O. niloticus* genetic ancestry. "*O. niloticus*" morphotypes with *O. esculentus* genes occur only when hybrid females backcrossed to *O. niloticus* males. It is possible that hybrid males would still be at a disadvantage to *O. niloticus* males, but might be acceptable to *O. esculentus* females, causing more hybrid gene flow into *O. esculentus*. Greater population sizes associated with *O. niloticus* would also skew the allele frequencies toward more *O. niloticus* introgression into *O. esculentus* than the converse.

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