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USE OF DNA MICROSATELLITE LOCI TO IDENTIFY POPULATIONS AND SPECIES OF LAKE VICTORIA HAPLOCHROMINE CICHLIDS

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INTRODUCTION

Cichlid fish species in the three Eastern African Great Lakes, Lake Victoria, Lake Malawi, and Lake Tanganyika, form a remarkable and fascinating vertebrate species flocks, representing a unique example of vertebrate explosive speciation and adaptive radiation (Fryer & Iles, 1972). Each of the three lakes harbors hundreds of cichlid species (together with many fewer non-cichlid species). More interestingly, almost all of the cichlids (>99%) are endemic to a particular lake (Greenwood, 1991). This contrasts with the nearby river systems, which harbor fewer cichlid species and much lower lever of endemism, and to coexisting non-cichlids, which also show lower endemism (Greenwood, 1991). Most of the Eastern African cichlids, though apparently reproductively isolated under their nature conditions, share many morphological features, an attribute making morphological classification difficult. Moreover, phenotypic plasticity, which is common for some morphological characters in cichlids, can further confound the morphological classification. Therefore, to obtain a more dependable cichlid phylogeny, which is essential for understanding the fundamental principles underlying the spectacular speciation events of cichlid flock, one requires alternative, possibly more reliable, characters than are provided by the current morphological characters which have been used for classification.

In the past 20 years, biologists around the world have tried to apply genetic approaches to attack evolutionary and systematic questions of cichlid relationships. Unfortunately, such efforts have been severely hampered by the frequent finding that many of the conventional biochemical and molecular genetic markers revealed very low levels of intraspecific and interspecific variation in the Eastern African cichlids, especially in the haplochromine cichlids in Lake Victoria region (Sage et al., 1984; Meyer et al., 1990; Booton, 1995).

In our lab we have used several approaches to develop highly variable genetic markers for population genetic studies of cichlids (Fuerst et al., 1995; Booton, 1995; Black et al., 1995). One such approach involves the development of a series of marker loci, referred to as microsatellite loci, from an Eastern African cichlid species, *Astatoreochromis alluaudi* (Wu et al., 1996). Microsatellite loci consist of simple short tandemly repeated DNA sequences. Repeat units range from 1 to 5 base pairs (bp) (Beckmann and Weber, 1992). Such loci are often highly polymorphic, characterized by multiple alleles and very high heterozygosity. It is generally believed that allelic variation at these loci is primarily due to different numbers of the di-, tri- or tetranucleotide repeat units, whereas DNA sequences flanking the microsatellite repeats are generally conserved. This facilitates cross species application of typing for these markers. As highly polymorphic genetic markers, microsatellite loci have recently been used in genome mapping, linkage analysis, paternity exclusion, and forensic studies (O'Reilly and Wright, 1995; Queller et al., 1993). They have also been increasingly applied to a variety of population genetic studies (Brooker et al., 1994; McConnell et al., 1995; Paetkau and Strobeck, 1994; Roy et al., 1994). Of particular note, microsatellite markers have been successfully used to show a high level of polymorphism in a species where the conventional genetic markers revealed very little amount of variation (Hughes and Queller, 1993). It is reasonable to expect that this new type of genetic marker might be very useful for phylogenetic studies of recently diverged species, such as the haplochromine cichlids.

The goals of the present project are: (i) to study the patterns of intraspecific and interspecific variation at the microsatellite loci that we developed in *A. alluaudi*, (ii) to determine whether they are useful in differentiating *A. alluaudi* populations, and (iii) to determine whether they are informative in studies of the phylogenetics of the Lake Victoria haplochromine cichlids.

MATERIALS AND METHODS

Tissue Samples: All cichlid samples used in this study were collected in the Lake Victoria region between 1992 and 1995. Species sampled include: (i) *A. alluaudi* (3 populations: Jinja, northern Lake Victoria n = 14; Lake Kachira, n = 22; Lake Kyoga, n = 22), (ii) *Astatotilapia velifer* (2 populations: Lake Nabugabo, n = 41; Lake Kayugi, n = 29), (iii) *Paralabidochromis* "rock kribensis", (1 population: Jinja, n = 31), (iv) *Paralabidochromis* sp., (1 population: Lake Victoria, n = 16), *Yssichromis laparogramma*, (1 population: Lake Victoria, n = 10), and *Y. fusiformis*, (1 population: Lake Victoria, n = 30). White muscle tissue samples were collected in the field and stored in 95% ethanol until DNA extraction. After tissue collection, the entire fish were formalin

preserved, and was transported to the laboratories of L. K. in Boston for later morphological identification, or was photographed intact for identification and then discarded.

DNA extraction and microsatellite analysis: DNA was either phenol-chloroform extracted (Kocher et al., 1989), or NaOH extracted (Zhang and Tiersch, 1994) from the ethanol-preserved muscle tissue. Nine microsatellite loci developed from a partial *A. alluaudi* genomic library (Wu et al., 1996) have been chosen for the current study. All PCR primers were designed using the program Oligo. Microsatellite PCR amplifications were performed in 5 or 10 µl of a mixture containing 25-30 ng of DNA template, 3 pmole of each primer, 1 nmole of dNTPs, 12.5 nmole of $MgCl_2$, and 0.5 to 1 unit of BRL *Taq* DNA polymerase. One of each pair of the primers was 5'-end labeled with γ^{32}p-dATP. PCR products were resolved in 6% or 8% polyacrylamide sequencing gel using the known sequence of the M13 bacteriophage as a size marker. Table 1 shows the core repeat sequence, the primer sequences, and the PCR conditions for the nine marker loci.

Table 1. Microsatellite core sequences, primer sequences and PCR conditions*

locus	core sequence	primer sequences	Tm(°C)	cycles
OSU09d	$(TG)_{20}(CGT)_{14}$	CCTCTGTAGTGATGTTTAATCTCTGT	60	28
		TGACACTGCACTTACTTGGCT		
OSU12t	$(NGC)_{13}$	TCAAACACCCACAGCCTTCA	60	22
		CGGTGATTGCTGTTGATACTGA		
OSU13d	$(GT)_{25}$	TAAGCTGATAGGAACCCAAC	58	30
		ACTCCTATTTTGTTATTTTTGTGA		
OSU16d	$(GT)_{10}$	GGCGAATGGTGGGTCAAG	58	32
		ATGTTGCTTGCCGCTGC		
OSU19d	$(GT)_{47}$	CAGTGCTTTGGTGGTGCT	55	30
		CATGACGTCTTTCAATAAGGAT		
OSU19t	$(CA)_{11}(ANC)_{12}$	TGAAGGACAAAGCAGGACTG	60	28
		TGCCCGAACCTTTTTATTTA		
OSU20d	$(GT)_{47}$	GAATGTGGATTTGCAGCTTG	60	30
		CATGCTTACAAAGAACAGGGTTAC		
OSU21d	$(GT)_6GC(GT)_4$	GCCGCTCAGAGTTTGGTG	60	22
		AGGCATGTGTCAGTTCATCCT		
OSU22d	$(GT)_{41}$	TGAAATCAAATACTAGAGCAAATA	55	32
		GGAGTTTAAAAATGATGCGT		

*PCR conditions are defined by PCR cycle numbers and annealing temperature (Tm).

RESULTS AND DISCUSSION

Genetic variability of microsatellite markers among *A. alluaudi* populations: Of the nine microsatellite markers analyzed, only one (OSU12t) was found to be monomorphic, in regard to the repeat motif. The remaining eight markers are polymorphic. The average observed number of alleles at polymorphic loci ranged from 7.9 (Lake Kachira population) to 12.3 (Lake Kyoga population), and the average observed heterozygosity from 0.42 (Lake Kachira population) to 0.60 (Jinja population) (Table 2). Loci OSU16d and OSU21d, which consist of shorter repeat motifs than the other six polymorphic loci, have a smaller number of observed alleles and lower observed heterozygosity. This is consistent with suggestions that the longer the average repeat motif, the more variable the locus will be (Weber, 1990).

The Lake Kachira population, characterized by a smaller number of observed alleles and lower level of observed heterozygosity, appears to be less heterogeneous than the other two populations. An extreme example is seen at locus OSU09d, for which the Lake Kachira population has only three observed alleles and a very low level of heterozygosity (0.27), while the observed numbers of alleles for the Jinja and Lake Kyoga samples are 14 and 16, and heterozygosities are 0.83 and 0.91, respectively. These data are in concert with results from RAPD (randomly amplified polymorphic DNA) markers collected in our lab, which indicate that *A. alluaudi* individuals from the Lake Kachira population are more similar to one another than are individuals either from Jinja or from Lake Kyoga (Black et al., 1995).

Table 2. Observed number of alleles (N) and observed heterozygosity (Ho) at the eight polymorphic loci among *A. alluaudi* populations

locus	Jinja		Kachira		Kyoga		average	
	N	Ho	N	Ho	N	Ho	N	Ho
OSU09d	14	0.83	3	0.27	16	0.91	11.0	0.67
OSU13d	15	0.79	13	0.73	20	0.77	16.0	0.76
OSU16d	5	0.29	1	0.00	4	0.18	3.3	0.16
OSU19d	10	0.57	6	0.57	19	0.77	11.7	0.64
OSU19t	6	0.86	3	0.45	6	0.68	5.0	0.66
OSU20d	16	0.69	21	0.57	13	0.43	16.7	0.56
OSU21d	2	0.07	2	0.09	1	0.00	1.7	0.05
OSU22d	15	0.71	14	0.68	19	0.59	16.0	0.66
average	10.4	0.60	7.9	0.42	12.3	0.54	10.2	0.52

Several observations about the microsatellite data also indicate that the three *A. alluaudi* populations are genetically differentiated from one another. First, each of the three populations has a high percentage of unique alleles, ranging from 24.1% (Jinja) to 44.4% (Lake Kachira) (Table 3). Overall, about 1/3 (80/244 or 32.8%) of the observed alleles are unique to a particular population, strongly suggesting that the three populations are genetically differentiated. Second, although most of the observed alleles are shared by at least two of the three populations, the different populations usually have different allele frequencies for the shared alleles. Figure 1 shows such differences for locus OSU09d, at which allele No. 6, which is predominant in the Lake Kachira population, is absent in the Jinja population and has very low frequency in the Lake Kyoga population, whereas allele No. 10, which is the predominant allele in Lake Kyoga, is less frequent in the Jinja population and absent in Lake Kachira.

Table 3. Observed number (N) and percentage (%) of unique alleles at the eight polymorphic loci among *A. alluaudi* populations

locus	Jinja		Kachira		Kyoga	
	N	%	N	%	N	%
OSU09d	6	42.9	0	0.0	7	43.8
OSU13d	1	6.7	3	23.1	4	20.0
OSU16d	2	40.0	0	0.0	1	25.0
OSU19d	1	10.0	2	33.3	7	36.8
OSU19t	1	16.7	0	0.0	2	33.3
OSU20d	5	31.3	14	66.7	5	38.5
OSU21d	0	0.0	1	50.0	0	0.0
OSU22d	4	26.7	8	57.1	6	31.6
average*	2.5	24.1	3.5	44.4	4	32.7

*average % of unique alleles = (total number of unique alleles) / (total number of observed alleles)

Genetic variability of microsatellite markers among other Lake Victoria haplochromine cichlids: Seven of the nine markers (all except OSU13d and OSU22d) were found to amplify a microsatellite locus in every haplochromine cichlid species tested. Our preliminary studies on five species which are members of the central species flock of Lake Victoria haplochromine cichlids indicate several general findings. First, most of the microsatellite markers revealed a high level of intraspecific variability within each of the five species, characterized by multiple alleles at each locus. Table 4 shows the number of observed alleles and the number of unique alleles for the seven loci. As was seen in *A. alluaudi*, each of the other five haplochromine cichlids exhibited low variability at loci OSU16d and OSU21d. Locus OSU12t, which is monomorphic in *A. alluaudi*, is polymorphic in all the other five haplochromine cichlids, but has only a few observed alleles. Second, in contrast to the high levels of intraspecific variability, interspecific differentiation among the five species appears to be quite limited, based on the overall low percentage (0.9/12.4 or 7.3%) of unique alleles (Table 4). This supports the notion that the

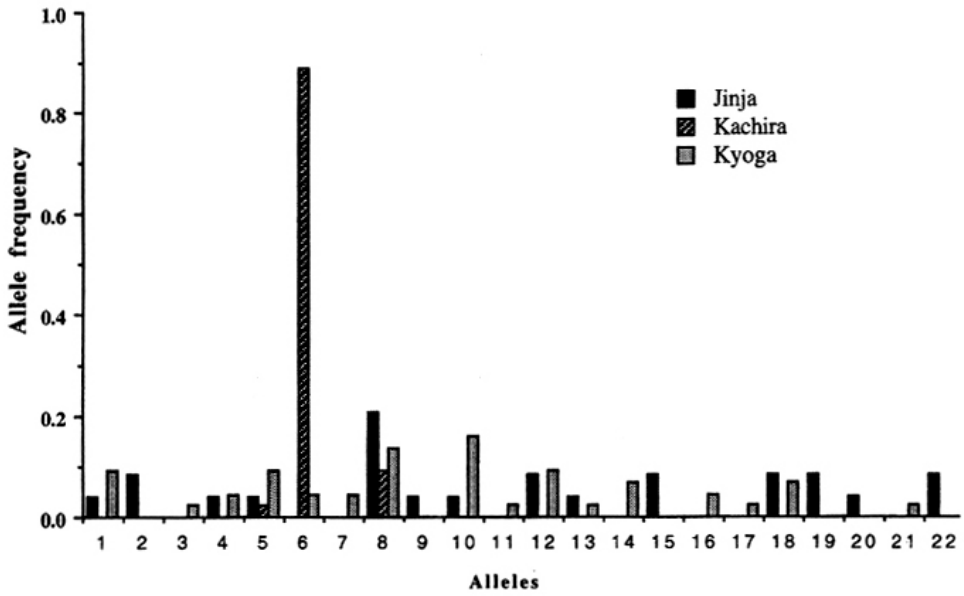

Figure 1. Histogram of allele frequencies at locus OSU09d in the three *A. alluaudi* populations

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Table 4. Observed number of alleles (and the number of unique alleles) at seven microsatellite loci among five species of Lake Victoria basin haplochromine cichlids

locus	*Astatotilapia velifer*	*Paralabidochromis* "rock kribensis"	*P.* sp.	*Yssichromis. laparogramma*	*Y. fusiformis*
OSU09d	7 (3)	3 (0)	4 (0)	4 (0)	6 (3)
OSU12t	4 (2)	2 (0)	2 (0)	2 (0)	2 (0)
OSU16d	27 (0)	20 (1)	15 (0)	12 (0)	23 (1)
OSU19d	28 (6)	16 (1)	14 (0)	10 (0)	20 (0)
OSU19t	25 (0)	24 (3)	16 (1)	11 (0)	25 (3)
OSU20d	28 (1)	22 (3)	16 (0)	13 (0)	23 (3)
OSU21d	3 (1)	2 (0)	2 (0)	2 (0)	2 0)
average	17.4 (1.9)	12.7 (1.1)	9.9 (0.14)	7.7 (0)	14.4 (1.4)

haplochromine flock within the Lake Victoria basin diverged very recently. Third, when measuring interspecific differences by using either Nei's unbiased genetic distance (Nei, 1978), or the Delta mu genetic distance for microsatellite loci (Goldstein et al., 1995), we found that the populations of *A. alluaudi* are more distantly related to any of the other haplochromine forms than the latter are separated from one another. This finding is consistent with both allozyme data (Sage et al., 1984) and mtDNA data (Meyer et al., 1990), both of which place *A. alluaudi* into an outgroup separated from other Lake Victoria haplochromine cichlids. Fourth, although microsatellite markers appear to be potentially informative for phylogenetic studies of haplochromine cichlids, a reliable haplochromine phylogeny based on microsatellite data may not be accurately inferred unless many more taxa are included and additional microsatellite loci are used. The original observations of Nei (1978) concerning the great importance of including in a study the largest number of loci possible to improve the accuracy of the estimated genetic distances are especially relevant to future studies of these recently evolved species.

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