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# Microsatellite variation demonstrates multiple paternity in lekking cichlid fishes from Lake Malawi, Africa

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## SUMMARY

The genotypes of two microsatellite loci were determined for the mouthbrooded progeny of 13 females, representing seven species of both rock- and sand-dwelling Lake Malawi cichlid fishes. Multiple paternity is clearly demonstrated for all but two broods. One brood contained the progeny of at least six males. Heterozygosity of these two microsatellite loci in a sample of 18 male *Copadichromis cyclicus* was 89% and 83%, respectively. Each locus displayed 16 alleles among the 18 males. The expected average exclusion probability using both loci, given a mother/offspring pair, is 0.968, suggesting that microsatellite variation will be useful for quantifying mate choice in these taxa. The low heterozygosity of a *Melanochromis auratus* brood is consistent with reduced levels of mtDNA polymorphism previously documented in this species. Thus microsatellite variation may provide insight into the genetic history of these populations.

## 1. INTRODUCTION

The great lakes of Africa harbour a rich assemblage of cichlid fishes. The third largest, Lake Malawi, holds a monophyletic radiation of more than 450 species which have arisen in the last 2 Ma (see, for example, Greenwood 1991). The shallow-water cichlids in this lake can be divided into two major ecological groups: the rock-dwelling species (Mbuna), and those which live over the sand. Male Mbuna are very brightly coloured, and the characteristic pigment pattern of a morphospecies varies among localities (Ribbink *et al.* 1983). Males of sand-dwelling species have less diverse coloration patterns, and typically breed over species-specific sandcastle bowers (McKaye 1991). Fishes in both ecological groups aggregate and defend territories, although the rock-dwellers do so on a more permanent basis (Hert 1990; Barlow 1991). Territories typically consist of a bower for the sand-dwellers and often a rocky cave for the Mbuna, neither of which offer significant resources to females and simply serve as mating sites (McKaye 1991). All of the endemic cichlids of Lake Malawi are female mouthbrooders (Fryer & Iles 1972).

One attractive explanation for the explosive speciation in this group is that sexual selection by female choice has led to the rapid divergence of microallopatrically isolated populations (Dominey 1984). The lek-like nature of the mating system of many

species may have further accelerated the speciation rate (Arnold 1983; McKaye 1991). Detailed field observations of several sand-dwelling species support hypotheses of a mating system characterized by unrestricted female mate choice (McKaye *et al.* 1990; McKaye 1991; Stauffer *et al.* 1995). Females of these taxa swim above large areas of the lek and choose to drop down into perhaps one third of the bowers over which they swim. They may actually begin a circular courtship sequence with a number of these males. The females freely leave some of the bowers without laying, and ultimately release eggs within just three to five of the dozens of bowers they survey (McKaye 1991). Direct observational data on Mbuna are more limited because the final stages of the mating sequence often occur out of sight within rocky caves (Holzberg 1978). Although behavioural data suggest multiple paternity is frequent in the sand-dwellers, in both groups fertilization takes place inside the mother's mouth. The possibility remains that females might exert some control over fertilization, or that sperm competition occurs within the female.

As an alternative to direct field observation, DNA fingerprinting techniques might be used to gain a better understanding of the mating events that take place among the Mbuna, as well as corroborating behavioural observations of mating in sand-dwellers. Analysis of hypervariable microsatellite sequences have proved useful in establishing parentage in many

systems (Tautz 1989; Queller *et al.* 1993). Microsatellites are tandem repeats of very short nucleotides (1–6 base pairs (b.p.) long) that are repeated over a stretch of DNA that is typically less than 200 b.p. (Rassman *et al.* 1991). These repetitive regions of nuclear DNA exhibit high variability due to length differences, yet are stably inherited. Less than one progeny in 10 000 is expected to exhibit a new mutation (Edwards *et al.* 1992).

This study evaluates the utility of microsatellites for studies of parentage in a number of cichlid species from Lake Malawi. We were specifically interested in testing the hypothesis of multiple paternity of broods that can be inferred from behavioural observations of sand-dwelling cichlids (McKaye 1991) and also whether the Mbuna have a similar polyandrous mating system suggestive of unrestricted female choice. Finally, we wished to quantify paternal contributions to each brood, as a first step to evaluating the differential reproductive success of males due to sexual selection.

## 2. MATERIALS AND METHODS

While SCUBA diving in the southeast arm of Lake Malawi we captured fish from the two major habitat types, rocky outcroppings and intermittent sand. Thirteen brooding females representing seven species were collected (see table 1). The *Copadichromis cyclicos* samples were taken from Kanchedza Island, the *Pseudotropheus* species, *Melanochromis auratus* and *Protomelus* sp., at Manzinzi Reef. The undescribed species of *Copadichromis* was captured at Songwe Hill. Eighteen males of *C. cyclicos* were collected at Kanchedza Island so we could estimate allele frequencies in this population. All fry and tissue samples (muscle from above the lateral line) were stored on liquid nitrogen for transport to the laboratory. The remaining portion of each adult specimen was fixed in 10% formalin and preserved in 70% ethanol for long-term storage of the vouchers.

Microsatellite loci were isolated using the method of Pulido & Duyk (1994). Briefly, a primary genomic library was constructed from 300–600 b.p. fragments of AluI digested DNA. An enriched library was constructed by primer extension of uracil-substituted ssDNA with a CA<sub>10</sub> primer. This secondary library was plated and screened with the same CA<sub>10</sub> oligonucleotide. Of 69 positive clones sequenced, 94% contained an identifiable CA repeat and approximately 38 unique clones were identified. Primers flanking the dinucleotide repeat were designed for several clones using the program PrimerSelect [DNA\* Inc.]. The highest-scoring primer pairs were synthesized and tested against a multispecific panel of DNAs. Sequences for the two loci used here have been deposited in GenBank (accession numbers U17044–U17045). Primer sequences for locus UNH001 were 5'-gat taa ctc tgc ccc tgc ct-3' and 5'-ctg aag tgc taa aaa tag tgc t-3'. For locus UNH002 the primers were 5'-tta tcc caa ctt gca act cta ttt-3' and 5'-tcc att tcc tga tct aac gac aag-3'.

Before DNA extraction, all fry were inspected to assess developmental stage using the scheme of Anken *et al.* (1993). Subsequently, female tissue samples and fry were individually diced using a razor blade and suspended in approximately 1000 µl and 200 µl of extraction buffer (10 mM Tris, pH 8.0; 2 mM EDTA, pH 8.0; 10 mM NaCl; 1% SDS; 8 mg ml<sup>-1</sup> DTT and 0.4 mg ml<sup>-1</sup> of Proteinase K), respectively. The buffer and tissue were incubated for approximately 4 h at 37 °C, with periodic mixing.

Proteinaceous material was removed by three organic solvent extractions in one volume each of Tris saturated phenol, phenol/chloroform (1:1 by volume), and chloroform according to standard procedure (Maniatis *et al.* 1982). Sodium chloride was added to bring the total concentration to 0.1 M, and two volumes of cold absolute ethanol were added to precipitate the DNA. Samples were usually allowed to precipitate overnight at -20 °C before centrifugation to pellet the DNA. The supernatant was then decanted and the samples were dried. The maternal pellets were usually resuspended in 400 µl of sd H<sub>2</sub>O and the offspring pellets in 200 µl.

Optimal polymerase chain reaction (PCR) conditions were determined empirically. A 1/20 dilution (1 µl) of the resuspended DNA extracts was used in a 25 µl PCR reaction containing 2.5 µl 10 X 'Thermo' buffer (Promega), 2 mM MgCl<sub>2</sub>, 1.5 mM each dNTP, and 1.5 units *Taq* DNA polymerase. PCR was performed in a Perkin Elmer cyclor set for 35 cycles of denaturation at 94 °C for 15 s, annealing at 56 °C for 1 min and extension at 72 °C for 1 min, followed by a final 7 min extension at 72 °C. Amplified DNA samples (1 µl) were mixed with 0.5 µl of Applied Biosystems, Inc. (ABI) 500 Rox standard, and 2 µl of formamide loading buffer. The samples were then denatured for 2 min at 92 °C, quenched and loaded on a 6% denaturing polyacrylamide gel in an ABI 373A (Applied Biosystems, Foster City, California) sequencing system. Base size information for each lane was recorded by ABI's GeneScan Collection software and later analyzed using ABI's GeneScan Analysis software. The spacing of bands in the CA-repeat ladders averaged slightly less than 2 b.p. on the size standard. We therefore report allele sizes as scored by the ABI Genescan software using the local Southern method, rounded to the nearest integer. We estimate that the observed size range of alleles for locus UNH001 (152–222 b.p.) corresponds to 11–46 copies of the repeat plus 130 b.p. of flanking DNA. For locus UNH002, alleles of length 183–221 correspond to approximately 23–43 repeats.

## 3. RESULTS

The allele frequencies observed in 18 *Copadichromis cyclicos* males for UNH001 and UNH002 are given in figure 1. The expected heterozygosity for each locus was extremely high: 91.5% for UNH001 and 90.5% for UNH002. Actual heterozygosities were not significantly lower: 89% for UNH001 and 83% for UNH002. Average exclusion probabilities (Chakraborty *et al.* 1988) for this population were 0.829 for UNH001 and 0.812 for UNH002. The expected average exclusion probability using both loci, given a mother/offspring pair is 0.968.

Genotypes for one microsatellite locus (UNH001) were generated for 13 females and their 203 offspring (see table 1). Progeny ranged from developmental stages 10–25 (Anken *et al.* 1993). The offspring from the first *Pseudotropheus* cf. *gracilor* brood were the youngest included in this study, classified at developmental stage 10 (optic vesicles, approximately two days old). All but three of the progeny in the brood displayed one maternal and one non-maternal allele, implying that our procedure correctly identified the offspring, and not the maternal genotype of each embryo. Instances of identical maternal and offspring genotypes are probably due to the presence of the same allele in both the mother and father, rather than the

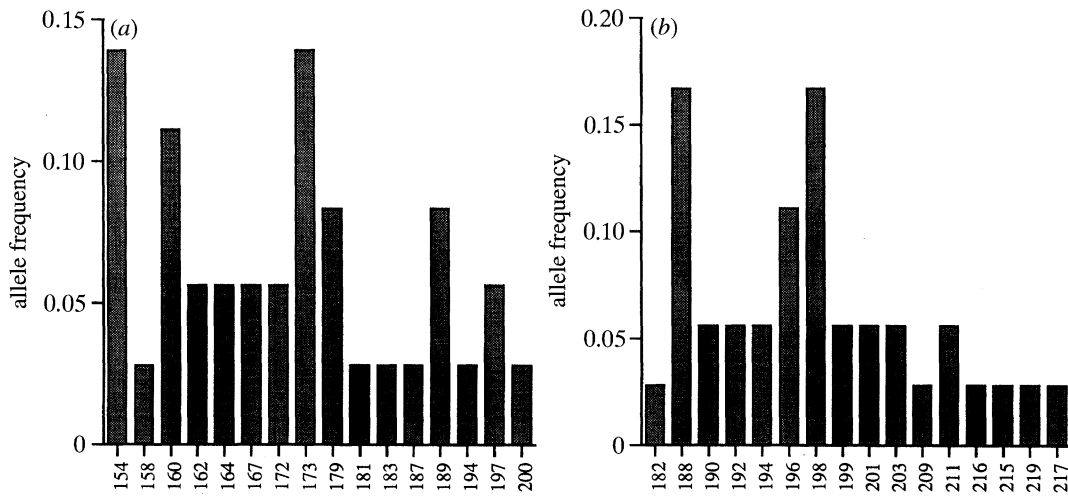


Figure 1. Allele frequencies for two microsatellite loci (a) UNH001, and (b) UNH002, in a sample of 18 male *C. cyclicus* from Kanchedza Island. Allele designations represent the length in base pairs of the amplified DNA.

Table 1. Analysis of paternal contributions in 16 families using microsatellite loci UNH001 and UNH002

(Some progeny had genotypes identical to that of their mother: in these cases the paternal contribution remains ambiguous.)

species/locus scored	progeny	maternal alleles	paternal alleles	ambiguous paternity
<b>UNH001</b>				
rock dwellers				
<i>Pseudotropheus</i> 'mazinzi blue'	15	214 222	177(4) 188(9) 206(2)	
<i>P.</i> 'mazinzi blue'	15	171 188	188(4) 210(3) 212(1) 222(5)	2
<i>P.</i> 'mazinzi blue'	14	184 222	180(6) 182(1) 214(7)	
<i>P. cf. gracilor</i>	16	173 195	166(6) 173(2) 179(3) 188(5)	
<i>P. cf. gracilor</i>	13	166 166	179(6) 189(1)	6
<i>P. cf. gracilor</i>	7	174 195	166(1) 179(2) 181(4)	
<i>P. cf. tropheops</i>	18	166 173	166(1) 188(10) 194(7)	
<i>Melanochromis auratus</i>	22	188 188	188(22)	
sand dwellers				
<i>Copadichromis cyclicus</i>	5	167 192	155(1) 172(1) 182(1) 190(1) 216(1)	
<i>C. cyclicus</i>	36	162 176	156(5) 157(4) 158(5) 159(1) 166(2) 172(4) 177(5) 183(4) 184(2) 185(1) 189(1) 190(1)	1
<i>C. cyclicus</i>	3	194 198	152(1) 180(1) 200(1)	
<i>Copadichromis</i> sp.	16	162 179	152(8) 154(6) 156(1) 173(1)	
<i>Protomelus</i> sp.	20	187 194	194(12) 212(7)	1
<b>UNH002</b>				
rock dwellers				
<i>P.</i> 'mazinzi blue'	13	199 211	199(2) 221(4)	7
sand dwellers				
<i>C. cyclicus</i>	5	195 200	183(2) 187(1) 207(1) 221(1)	
<i>C. cyclicus</i>	35	188 201	188(6) 203(1) 205(3) 207(8) 211(1) 213(1) 217(8) 221(6)	1
<i>Protomelus</i> sp.	29	199 213	198(29)	

masking of paternal alleles by maternal DNA in the egg membranes. In any case, masking of paternal alleles would result in a conservative estimate of the number of males that are contributing to a single brood.

Detailed analyses of the first locus (see table 1) confirmed that all of the fry examined had at least one allele which matched a maternal band. Two *Copadichromis cyclicus* families had an unusually small

number of progeny. *C. cyclicus* brood sizes are typically between 20–30 (Fryer & Iles 1972), however these two families contained only three and five fry respectively. There are two possible explanations: the mother may have expelled a large portion of the brood during capture, or one of the many paedophagous fishes known to occur in this area may have eaten some of the young. Both broods were still in early development (stage 13–15; mouth development and fins buds;

Table 2. *A comparison of paternal alleles for two loci in the largest Copadichromis cyclicos family*

(The total number of progeny is lower than that in table 1 due to missing data for some cells.)

	UNH001 alleles										
	156	157	158	159	172	177	183	184	185	189	190
UNH002 alleles	188	1								1	1
	201	1									
	203	1									
	205	1	1						1		
	207			1		1	2	1			
	211										
	213					1					
	217		4				1				
	221				3	1					
Number of unique genotypes per UNH001 allele	4	1	1	1	1	3	2	1	1	1	1

5.5–7.0 days) so it is very unlikely that the mother had already released some of the offspring voluntarily. All of the other brood sizes seemed to coincide with expected ranges for these fishes. (Fryer & Iles 1972).

An interesting pattern of paternal contribution was revealed upon examination of allelic frequencies (see table 1). For the seven species analysed, paternal allele counts ranged from a minimum of one to a maximum of 12 based on only the first locus. Accordingly, the number of males contributing to a single brood was calculated to be between one and six. It appeared, based on this first locus, that two alleles occurred at a higher rate within the broods. It is assumed, given random assortment, that these two alleles were contributed by the same male, which suggests that a single male was responsible for a disproportionate number of fertilizations within a brood. When analyses include the second locus (UNH002), however, a slightly different picture emerges.

We scored the number of paternal alleles identified for locus 2 in the subset of progeny containing a particular paternal allele at locus 1 (see table 2) for several of the larger families. The presence of more than two alleles at locus 2 indicates that more than one father contributed a particular allele for the first locus. For the highly heterozygous *C. cyclicos* families, the true number of fathers may be 20% greater than estimates based on a single locus. For species with fewer alleles, our estimates may be biased downward even more. Based on the additional paternal detail provided for locus 2 analyses, it appears that these species show high levels of polyandry with males contributing in more equal proportions than revealed by the first locus alone.

#### 4. DISCUSSION

Our results demonstrate the high levels of variability that microsatellites offer for studies of mate choice in these cichlids. The large exclusion probability obtained with just two loci is extremely encouraging. It suggests that 52% of the progeny from a lek of 20 males could be assigned to a particular father by exclusion. The scoring of two additional loci with similar levels of variability may allow assignment of 90% of the

progeny from a lek of 100 males. Likelihood methods for assigning paternal contributions may be useful even in very large leks.

Polyandry of cichlid females has been quantified through behavioural observations of several sand-dwellers, including species of *Copadichromis*, *Cyrtocara*, *Tramitichromis*, and *Lethrinops* (McKaye 1991; Stauffer *et al.* 1995). The results of our microsatellite DNA analyses not only confirm that several males contribute to each sand-dwelling brood, but demonstrate higher than suspected levels of multiple paternity. During field observations, the number of mating encounters may be underestimated simply because females are difficult to track and may be lost before leaving the breeding arenas (McKaye 1991). The proportions of progeny assigned to different males in our study are similar to those postulated from observations of behaviour. Because of their secretive spawning among rocks, few behavioural data are available to predict the mating patterns of Mbuna. Our results indicate that multiple paternity is common in these species as well, with females typically mating with at least two or three males. If heterozygosity of these species is lower than that of the sand-dwellers, the actual number of males contributing to each brood may be larger.

A number of factors could explain why multiple paternity has evolved in these species. We suspect disturbance by egg predators and/or sneaky males may be both a proximate, and the ultimate cause of polyandry. Predation pressure is intense throughout Lake Malawi, particularly for nutrient-rich eggs (Fernald & Hirata 1977; Holzberg 1978; McKaye 1984; Taborsky 1994). A number of paedophages have evolved in the lake (Stauffer & McKaye 1986), and these frequently have morphologies adapted to specific feeding modes (McKaye & Kocher 1983). Predators frequently enter the bowers of sand-dwelling species during spawning, causing a premature break in the mating activities of a pair. In addition, occasionally the mating male will consume an egg which may result in female departure (J. R. Stauffer Jr, personal observation). Matings are also interrupted by the arrival of sneaky males (McKaye 1984). Sneaky males are common throughout Lake Malawi, especially among sand-dwelling species (Taborsky 1994), and these dull-

coloured males dart into the bower at the moment of egg release to attempt fertilization. Females typically break off spawning upon the arrival of sneaky males. Circling pairs attract predators, and the longer a female stays in a particular breeding territory, the more likely it is that the mating bout will be interrupted. This selection pressure may have acted to reduce the length of each mating encounter.

Predation pressure may be less intense within the often concealed breeding sites of the Mbuna than on the open bowers of sand-dwellers (G. F. Turner, personal communication). Although both predation and the number of sneaky matings needs to be quantified in these species, the multiple paternity revealed by our microsatellite analysis invites alternative explanations (Parker & Kornfield 1995). The concept of bet-hedging appears in many different forms and under different names throughout the literature with a common underlying theme of evolutionary tradeoffs between expected fitness and the variance of fitness (Seger & Brockmann 1987). Mating strategies may have evolved to minimize the variance in fitness which might arise from imperfect mate choice. This seems plausible given that the females in lekking species have only a brief association with males before mating and therefore the possibility of errors in discriminating between suitable and unsuitable mates increases (Watson 1991; Wiley 1991). In lekking cichlids bet-hedging could take several possible forms: mating to optimize genetic diversity, to account for various female preferences in the population, or to hedge against imperfect selection criteria due to variation among males in the heritability of preferred traits.

Mating to optimize genetic diversity seems unlikely in the cichlid populations of Lake Malawi because this form of bet-hedging is thought to arise in unpredictable environments where genetic diversity is the best guarantee for at least some offspring survival (Seger & Brockmann 1987; Westneat *et al.* 1990). Lake Malawi, however, is considered to be a relatively stable environment on a short timescale due primarily to its tropical location, although there is evidence of dramatic lake level changes on a scale of hundreds to thousands of years (Scholtz & Rosendahl 1988; Owen *et al.* 1990) which might alter selective forces. Females may also have evolved strategies in which mating with several different 'types' of males ensures that at least some of her male offspring will be considered attractive no matter what female preference criteria are applied. Several studies have evaluated female mate choice among Old and New World cichlids and identified male characters such as bower size, egg-spot numbers and male body size that lead to non-random mate selections (Noonan 1983; Keenleyside *et al.* 1985; McKaye *et al.* 1990; Hert 1991). Although female choice experiments provide evidence for consistent preferences throughout a population (Noonan 1983; Keenleyside *et al.* 1985; McKaye *et al.* 1990; Hert 1991), additional research in this area is needed.

Varying heritability of preferred phenotypic characteristics can also present a problem in optimal mate selection, and can lead to different fitness levels among

offspring even when females apply the same preferences. Under this hypothesis females might select only those males displaying the preferred characters, but might mate with several from that group to hedge the heritability bet (Watson 1991). A more complete understanding of the genetics of preferred characteristics in these organisms will be required to evaluate fully the possibility of unequal heritability of traits among males.

Whether the display of polyandry is due to a bet-hedging strategy, predation avoidance or some unexplored force, it appears to be common throughout the rock- and sand-dwelling groups. Table 1 shows two interesting exceptions in which families appear to have a single father. The *Protomelus* brood has only two paternal bands at the first locus and is completely homozygous at the second, suggesting a single male contribution. This species is a paedophage, and it could be that females mate with only one male because searching for additional mates might result in heavy predation on the eggs already being carried. Alternatively, this species might have reduced heterozygosity, and we may have been unable to detect multiple paternal contributions.

The second aberration, the *M. auratus* brood, is even more engaging. Each individual in this brood is a homozygote for the same allele, suggesting a greatly reduced level of heterozygosity in this population. Our previous work has demonstrated reduced mtDNA polymorphism in this species (Bowers *et al.* 1994). Although this result should be confirmed by examination of population samples, rather than families, our results suggest that microsatellite loci may be useful for examining population histories.

Multiple paternity seems to be the norm among these cichlid taxa and, like behavioural observations, suggests that females are normally free to exercise mate choice. This freedom is an underlying assumption in models of sexual selection which have been postulated as forces leading to the diverse fauna of Lake Malawi (Dominey 1984; McKaye 1991). Dominey (1984) argued specifically that 'runaway' sexual selection, based on the expression and heritability of female preferences and male characteristics, could have a role in this speciation. This is supported by field observations of the sand-dwellers in which bowers with specific characteristics (size and position within the lek) receive a greater percentage of the overall mating activity (McKaye *et al.* 1990; McKaye 1991). Furthermore, laboratory experiments supplement the field data with consistent female preferences being displayed for egg-spot numbers and male body size (Noonan 1983; Keenleyside *et al.* 1985; Hert 1991). Additional studies measuring the consistency of female mate choice will be necessary, particularly for the Mbuna, to evaluate the effect that multiple paternity might have on speciation rates.

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