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Intra- and Interspecific Mitochondrial DNA Sequence Variation within Two Species of Rock-Dwelling Cichlids (Teleostei: Cichlidae) from Lake Malawi, Africa

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Difficulties in interpreting the evolutionary significance of Lake Malawi cichlid morphologies led us to examine molecular techniques for resolving relationships among closely related species. Mitochondrial DNA (mtDNA) sequence variation in the first half of the control region (445 bp) was examined within and between two species of *Melanochromis*, a genus of rock-dwelling cichlids from Lake Malawi, Africa. The mean number of pairwise differences observed within *Melanochromis auratus* (Boulenger) and *Melanochromis heterochromis* Bowers and Stauffer mtDNA haplotypes was 2.0 (0.45%) and 5.0 (1.13%), respectively, and a mean of 4.9 (1.11%) pairwise differences between the two species was observed. Mean pairwise differences between *Melanochromis* species and *Pseudotropheus zebra* (Boulenger), another species of rock-dwelling cichlid and *Tramitichromis* cf. *liturus*, a sand-dwelling genus, were 11.2 (2.52%) and 21.9 (4.93%), respectively. Species divergence and radiation within the genus *Melanochromis* appears to have occurred rapidly and recently. Mitochondrial DNA sequence variation within this genus was sufficient for generating hypotheses concerning the evolutionary relationships within the genus and for examining generic-level relationships within and among the major cichlid lineages in Lake Malawi.

INTRODUCTION

The rock-dwelling cichlids (*mbuna*) of Lake Malawi constitute a major portion of the haplochromine species flock endemic to the lake. All of the *mbuna* are endemic to Lake Malawi and have probably evolved within the last one to two million years, some within the past 10,000 years (Ribbink *et al.*, 1983; Owen *et al.*, 1990). Rapid speciation among these fishes has resulted in the proliferation of reproductively isolated but morphologically similar species. In a major survey of the Lake Malawi *mbuna*, Ribbink *et al.* (1983) recognized 196 putative species and, recently, Konings (1990) identified over 200 described and undescribed species of *mbuna*. An understanding of the phylogenetic relationships within this complex assemblage of fishes is necessary to address questions concerning the processes responsible for such explosive evolution. The lack of discrete morphological characters suitable for cladistic analysis and confusion surrounding *mbuna* systematics and taxonomy, however, has led to difficulties in reconstructing species-level phylogenies.

Recent developments in molecular genetics have provided new techniques for examining the relationships among closely related taxa. Specifically, the discovery of extensive variation in mitochondrial DNA (mtDNA) has proven useful in the study of conspecific populations and recently diverged species (e.g., Avise *et al.*, 1987). Mitochondrial DNA studies of Lake Malawi cichlids have focused mainly on interlake comparisons (Meyer *et al.*, 1990) or on generic-level comparisons within lakes (Kornfield, 1991; Moran and Kornfield, 1993; Moran *et al.*, in press).

The objectives of the present study were to determine the extent and pattern of intra- and interspecific sequence variation in the mtDNA control region in two species of Lake Malawi cichlids and to evaluate the utility of mtDNA sequence variation for addressing phylogenetic questions at the generic and species level.

MATERIALS AND METHODS

We chose to sequence a 445-bp region located within the first half of the mtDNA control region, which has been shown to be a quickly evolving segment of the mtDNA genome (Greenberg *et al.*, 1983; Vigilant *et al.*, 1989). A total of 68 individuals of *M. auratus* from eight locations (Mitande Rocks, Chidunga Rocks, Mazinzi Reef, and Namalenje, Nakantenga, Maleri, Thumbi East, and Domwe islands) and 32 individuals

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of *M. heterochromis* from six locations (Zimbawe Rocks and Chinyamwezi, Chinyankwazi, Mumbo, Domwe, and Thumbi East islands) (Fig. 1) were collected using a monofilament net (7 m × 1 m × 1.5 cm) while SCUBA diving. Because of the strong lithophilic nature of *mbuna,* these fishes are rarely found over open water or along sandy beaches, and geographically isolated populations are often reproductively isolated. The possible exception is Domwe Island and Zimbawe Reef, which are close enough for the exchange of migrants.

DNA Methods

A small sample of muscle was removed from the right side of each fish and fixed in 70% ethanol for transportation to the laboratory. Approximately 250 mg of muscle was digested in 500 μl extraction buffer (10 mM Tris, pH 8.0, 2 mM EDTA, in 10 mM NaCl, 1% SDS, 8 mg/ml dithiothreitol, and 0.4 mg/ml proteinase K) at 36°C for 12 h and then phenol extracted (Kocher *et al.,* 1989). The DNA was then ethanol precipitated by the addition of 2 vol 100% ethanol (4°C), resuspended into 250 μl TE (10 mM Tris, pH 8.0, and 1 mM EDTA), and stored at 4°C.

The region of mtDNA targeted for PCR was approximately 650 bases long and consisted of part of the proline tRNA gene and the first half of the control region. The forward primer (5′-AGCTCACGCCAGAGCGCCGGTCTTCTAAA−3′) is located in the threonine tRNA gene adjacent to the control region. The reverse primer (5′-CCTGAAGTAGGACCAGATC-3′) is located in a conserved sequence near the middle of the control region (Shields and Kocher, 1991). Amplifications were performed in a 50-μl reaction volume containing 67 mM Tris, pH 8.0, 2 mM $MgCl_2$, 9.8 mM β-mercaptoethanol, 0.1% Tween, 1 mM each dGTP, dATP, dCTP, and cTTP, 1.5 units *Thermus aquaticus* DNA polymerase (AmpliTaq, Perkin Elmer-Cetus), 1 mM each primer, and 1 μl template. Thirty cycles of amplifications, consisting of denaturation at 93°C for 30 s, annealing at 50°C for 60 s, and extension at 72°C for 120 s, were performed. Amplified products were purified using Centricon-100 columns (Amicon Division, W.R. Grace & Co.).

Sequencing from the forward primer proved unsatisfactory, so two overlapping regions were sequenced in the reverse direction. Amplification for sequencing was carried out using double-stranded template and the Taq DyeDeoxy Terminator Cycle Sequencing kit (Applied BioSystems, Inc.) for use with their Model 373A DNA Sequencing System. The two primers used included the reverse primer used in the initial amplification reaction (5′-CCTGAAGTAGGACCAGATC-3′) and another reverse primer with the sequence 5′-AGTTCTCATCGGTCTTAAAC-3′, located approximately 150 bases downstream from the other primer. These two primers provided reliable sequence information on 445 bases in all individuals. Ambiguous results from the two primers were resolved by repeated sequencing of both sections.

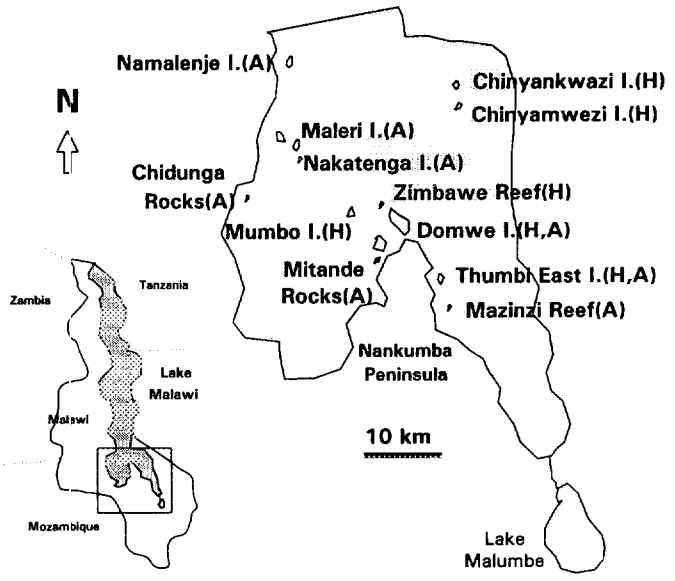

FIG. 1. Map of Lake Malawi showing sites where *M. auratus* (A) and *M. heterochromis* (H) were collected.

Phylogenetic Analysis of DNA Sequences

Relatively high levels of mtDNA sequence conservation among populations and between species permitted alignment of sequences by eye, and no insertions or deletions were observed. Population-level variation was evaluated by sequencing multiple individuals from several populations each of *M. auratus* and *M. heterochromis* (see Tables 1 and 2). Overall nucleotide diversity (π, Nei and Li, 1979) was calculated as a measure of polymorphism within each species. Intra- and interspecies level variation was estimated based on pairwise differences within and between species. Intergeneric sequence comparisons were also conducted using *Pseudotropheus zebra* Boulenger, another species of *mbuna* collected from Mitande Rocks, and *Tramitichromis* cf. *liturus,* a sand-dwelling species collected just north of the Nankumba Peninsula.

The phylogenetic relationship among mtDNA haplotypes was estimated by the maximum parsimony criterion using outgroup rooting and the heuristic search algorithm of PAUP Version 3.0L (Swofford, 1991). A consensus tree was produced from equally parsimonious trees using the 50% majority rule. The bootstrap option of PAUP was used to demonstrate confidence in position of tree nodes, and 1000 replications were performed. Selection of appropriate outgroups for phylogenetic analysis of closely related *mbuna* is difficult because of the overall lack of understanding of cichlid evolutionary histories. Therefore, both *P. zebra* (another species of *mbuna*) and *T.* cf. *liturus* (a sand-

TABLE 1

Frequency Distribution of mtDNA Haplotypes among 68 Individuals of *M. auratus* from Eight Different Locations

	Haplotype					
Population	AUR1	AUR2	AUR3	AUR4	AUR5	AUR6
Domwe I.	1	0	0	0	0	0
Thumbi East I.	24	0	0	0	0	0
Namalenje I.	16	0	0	0	0	0
Mazinzi Reef	1	0	0	0	0	0
Chidunga Rocks	5	9	5	0	0	0
Nakantenga I.	0	0	0	5	0	0
Mitande Rocks	0	0	0	0	1	0
Maleri I.	0	0	0	0	0	1

dwelling cichlid) were used to root the trees. The sand-dwelling cichlids represent a lineage separate from, but closely related to, the *mbuna* (Fryer and Iles, 1972).

A phylogram generated using the neighbor-joining method of Saitou and Nei (1987) was identical to that obtained using maximum parsimony and is not presented.

RESULTS

Sequence Comparisons

Alignment of mtDNA sequences from *M. auratus* and *M. heterochromis* is shown in Figs. 2 and 3. Of the 445 sites analyzed, 6 (1.35%) were variable within *M. auratus* populations and 11 (2.50%) were variable within *M. heterochromis* populations. Within each species, the distribution of substitutions varied, with only one variable site shared between the two species. Among the 68 individuals of *M. auratus* examined, six unique haplotypes were identified (Table 1); another six unique haplotypes were found among the 42 individuals of *M. heterochromis* (Table 2). No mtDNA haplotypes were shared between species. When more than one individual was examined at a given location, we found little sequence divergence and 75% of the populations were fixed for a single haplotype. Five geographically distinct populations of *M. auratus* (see Fig. 1 and Table 1) shared a single haplotype; within *M. heterochromis*, three geographically proximate populations (see Fig. 1 and Table 2) shared a single haplotype. Only one population each of *M. auratus* (Chidunga Rocks) and *M. heterochromis* (Mumbo Island) was polymorphic, although multiple individuals were not examined at all locations. Nucleotide diversities (π) were 0.0008 and 0.0050 for *M. auratus* and *M heterochromis*, respectively.

A summary of pairwise differences within and between the *M. auratus* and *M. heterochromis* haplotypes is shown in Table 3. The number of transitional substitutions, based on pairwise comparisons, outnumbered transversional substitutions 6.5 to 1 in *M. auratus* and 14 to 1 in *M. heterochromis*. Such transitional bias in substitutions in mtDNA has been observed previously (Brown *et al.*, 1982, 1986; Wilson *et al.*, 1985), with apparent transition to transversion ratios generally decreasing with increased divergence times (Kraus and Miyamoto, 1991; Brown *et al.*, 1993). Among the six *M. auratus* haplotypes, the mean number of pairwise substitutions was 2.0 (0.45%), compared with 5.0 (1.13%) observed among *M. heterochromis* haplotypes (Table 4). Mean pairwise substitutions between *M. auratus* and *M. heterochromis* haplotypes were 4.9 (1.11%).

At the generic level, comparisons between *Melanochromis* species and *P. zebra* and *T.* cf. *liturus* revealed a total of 23 (5.2%) and 33 (7.4%) variable sites, respectively. The mean number of pairwise substitutions within the *mbuna* genera (*Melanochromis* spp. vs *P. zebra*) was 11.2 (2.52%) (Table 4). Comparisons between the *mbuna* (*Melanochromis* spp. and *P. zebra*) and *T.* cf. *liturus* (sand dweller) resulted in a mean of 21.9 (4.93%) pairwise substitutions.

TABLE 2

Frequency Distribution of mtDNA Haplotypes among 42 Individuals of *M. heterochromis* from Six Different Locations

	Haplotype					
Population	HET1	HET2	HET3	HET4	HET5	HET6
Domwe I.	5	0	0	0	0	0
Zimbawe Reef	5	0	0	0	0	0
Mumbo I.	1	13	1	0	0	0
Thumbi East I.	0	0	0	15	0	0
Chinyankwazi I.	0	0	0	0	1	0
Chinyamwezi I.	0	0	0	0	0	1

Phylogenetic Relationships

A total of 13 equally parsimonious trees was found, and the 50% rule consensus tree is shown in Fig. 4, with results from 1000 bootstrap replications. Although the limited number of substitutions contributed to low bootstrap values, the branch separating the outgroups (PZEB and TLIT) and the *Melanochromis* species was highly supported (86%). The 13 trees differed primarily with respect to the relationship among *M. heterochromis* mtDNA haplotypes. The six *M. auratus* haplotypes, which differed by no more than three substitutions, formed an unresolved clade contained within the group of *M. heterochromis* haplotypes.

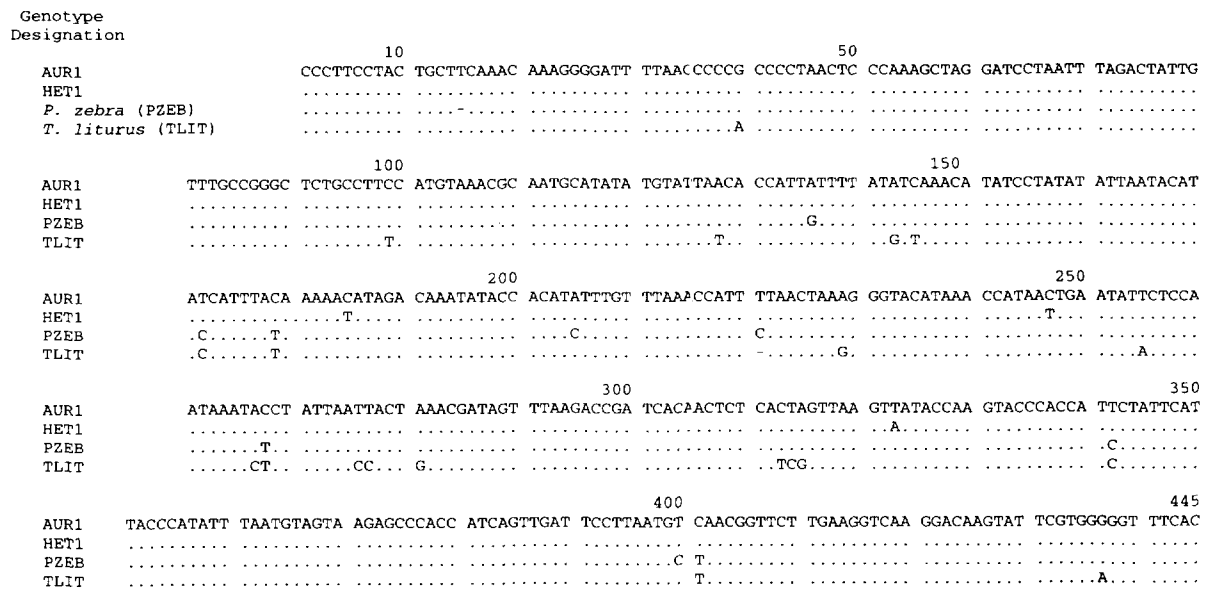

FIG. 2. Aligned control region sequences for AUR1 (*M. auratus*), HET1 (*M. heterochromis*), PZEB (*P. zebra*), and TLIT (*T.* cf. *liturus*) haplotypes. Dots indicate identity with AUR1 sequence and dashes indicate gaps.

DISCUSSION

Traditionally, discrimination of cichlid species has been based on morphology. Morphologically similar species were further distinguished on the basis of male breeding coloration or behavioral characteristics (Greenwood, 1965; Fryer and Iles, 1972; Lewis, 1982; Ribbink *et al.*, 1983; Stauffer, 1991). Recently, multivariate statistical analyses of morphometric and meristic data have been used to identify slight changes in overall shape associated with color pattern divergence in closely related species (Stauffer and Boltz, 1989; Stauffer and Hert, 1992). Subtle variation in color among conspecific allopatric populations, however, may not always be associated with a divergence in morphometry and meristics, and several geographically isolated color variants have been designated conspecific (Eccles and Lewis, 1978; Ribbink *et al.*, 1983; Bowers and Stauffer, 1993). Furthermore, plasticity in morphology and color polymorphisms exhibited by many cichlid species (Ribbink *et al.* 1983) may result in apparently morphologically dissimilar populations that are more closely related genetically to each other than to morphologically similar populations. These observations underscore the difficulties associated with cichlid species discrimination and the obstacles confronted in the phylogenetic reconstruction of this complex species flock.

The biological species concept (Mayr, 1992), which requires the potential for interbreeding (reproductive compatibility), is difficult to apply with respect to Lake Malawi *mbuna*. Because of their lithophilic nature (Fryer and Iles, 1972; Ribbink *et al.*, 1983), *mbuna* rarely venture over open water or long expanses of

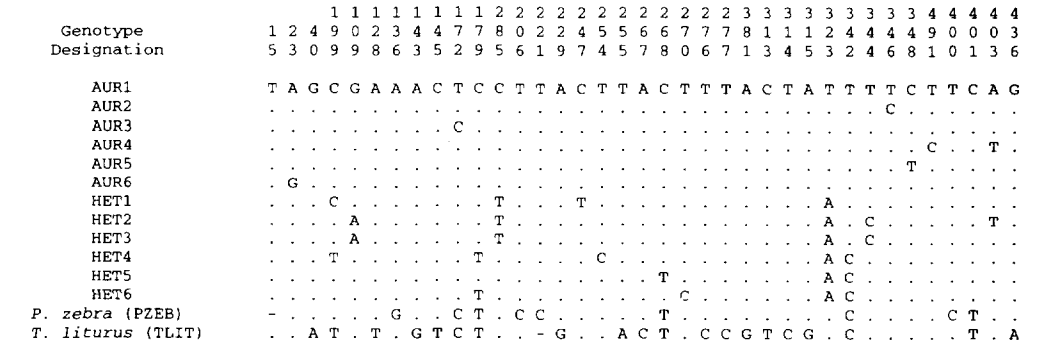

FIG. 3. Aligned DNA sequences showing variable nucleotide positions in the control region. See Tables 1 and 2 for genotype designations. Dots indicate identity with AUR1 sequence, dashes indicate gaps, and position numbers are shown across the top. These sequences have been submitted to the GenBank data library under Accession Nos. U01929–U01931 (*M. auratus*) and U01936–U01941 (*M. heterochromis*).

TABLE 3

Pairwise Differences among *M. heterochromis* (HET) and *M. auratus* (AUR) Haplotypes for the First Half of the Control Region (below the Diagonal) and the Ratio of Transitions to Transversions for Each Comparison (above the Diagonal)

Genotype	HET1	HET2	HET3	HET4	HET5	HET6	AUR1	AUR2	AUR3	AUR4	AUR5	AUR6	PZEB	TLIT
HET1	—	4/1	3/0	6/0	4/0	4/0	2/1	3/0	3/1	3/2	4/0	3/1	12/1	20/4
HET2	5	—	1/1	6/1	4/1	6/1	2/2	3/2	3/2	3/1	5/0	3/1	12/3	20/5
HET3	3	2	—	7/0	7/0	7/0	3/1	4/1	3/2	4/2	5/0	4/1	12/1	20/4
HET4	6	7	7	—	4/0	4/0	4/1	5/1	5/1	5/2	6/0	5/1	10/1	16/4
HET5	4	5	7	4	—	4/0	2/1	3/1	3/1	3/2	4/0	3/1	8/1	16/4
HET6	4	7	7	4	4	—	4/1	5/1	5/1	5/2	6/0	5/1	10/1	18/4
AUR1	3	4	4	5	3	5	—	1/0	1/0	1/1	2/0	1/1	10/0	18/3
AUR2	4	5	5	6	4	6	1	—	2/0	2/1	2/0	2/0	11/0	19/3
AUR3	4	5	5	6	4	6	1	2	—	2/1	2/0	2/0	9/0	17/3
AUR4	5	4	6	7	5	7	2	3	3	—	2/0	2/0	11/1	19/4
AUR5	4	5	5	6	4	6	1	2	2	3	—	2/0	11/0	19/3
AUR6	4	4	5	6	4	6	1	2	2	3	2	—	11/0	19/3
PZEB	13	14	13	11	9	11	10	11	9	12	11	11	—	17/3
TLIT	24	25	24	20	20	22	21	22	20	23	22	22	20	—

Note. Comparisons with *P. zebra* (PZEB) and *T.* cf. *liturus* (TLIT) are also shown.

sand, effectively isolating populations that may potentially interbreed. This may result in little or no gene flow among populations that otherwise would be considered conspecific. In addition, demonstration of reproductive compatibility under laboratory conditions, often considered support of conspecificity, is not necessarily valid for cichlids, since premating reproductive isolating mechanisms have been shown to break down under artificial conditions (Holzberg, 1978; Stauffer and Hert, 1992). Furthermore, reproductive compatibility is considered a shared primitive feature, and grouping of populations into species on the basis of this trait is inconsistent with the study of the underlying processes of evolution (Rosen, 1979). Barring any unusual selection pressures, previously sympatric populations that have become geographically isolated should remain on the same phylogenetic trajectory for some time (Frost and Hillis, 1990) and thus be considered conspecific.

TABLE 4

mtDNA Sequence Variation Based on Pairwise Comparisons Between Haplotypes

Comparison	Mean ± SD	%
Intraspecific		
M. auratus (15)	2.0 ± 0.75	0.45
M. heterochromis (15)	5.0 ± 1.61	1.13
Interspecific/congeneric (36)		
M. auratus vs *M. heterochromis*	4.9 ± 1.11	1.11
Intergeneric—*mbuna* (12)		
Melanochromis vs *Pseudotropheus*	11.2 ± 1.48	2.52
Intergeneric—*mbuna* vs sand (13)		
Melanochromis and *Pseudotropheus* vs *T.* cf. *liturus*	21.9 ± 1.69	4.93

Note. The number of comparisons made is shown in parentheses.

Extent and Pattern of Sequence Divergence

Mitochondrial DNA sequence variation among conspecific populations of cichlids in Lake Malawi was low, but the extent and pattern of variation differed between the two species examined. Five allopatric populations of *M. auratus* shared a single haplotype, and this haplotype was fixed in two of the populations in

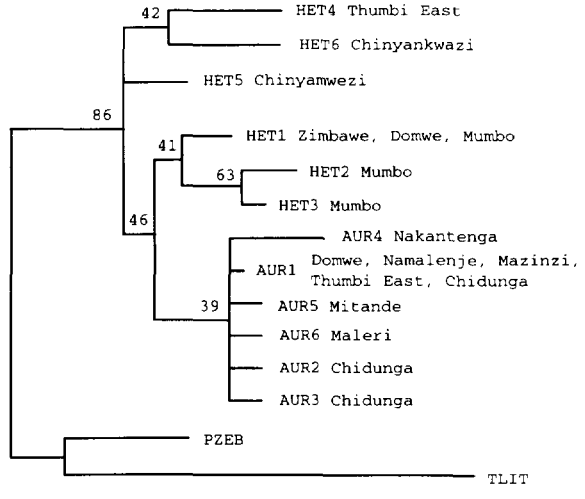

FIG. 4. Majority rule consensus phylogram of the 13 minimum length trees for *M. auratus* and *M. heterochromis* mtDNA haplotypes based on maximum parsimony using *P. zebra* and *T.* cf. *liturus* as outgroups. Bootstrap scores (percentages over 1000 replicates) are indicated for the main branches. The 13 trees summarized in the consensus diagram each had a length of 45 and a consistency index of 0.844. The geographic location for each haplotype is shown.

which multiple individuals were analyzed, supporting either the recency of population divergence or the genetic cohesiveness of these populations. The polymorphic population of *M. auratus* was from Chidunga Rocks (see Fig. 1), a rocky outcrop located offshore and effectively isolated from other suitable *mbuna* habitat.

Sequence divergence within *M. heterochromis* was greater than that observed within *M. auratus,* possibly because of differences in life histories between the two species, or because *M. heterochromis* may be an older lineage. Three populations of *M. heterochromis* (Mumbo and Domwe islands and Zimbawe Reef), located within 5 km of each other, shared a single haplotype, although the Mumbo Island population was polymorphic. A few individuals of *M. heterochromis* from Mumbo Island, possessing the shared haplotype, may possibly have colonized Zimbawe Reef and Domwe Island during the southern expansion of the lake, resulting in the present distribution of mtDNA haplotypes.

The low level of mtDNA sequence divergence observed within these two species of *Melanochromis* may be a result of recent isolation of populations (e.g., less than 10,000 years) or possible population bottlenecks following colonization events. Lake levels have fluctuated substantially over the history of the lake, and rocky outcrops in the southern portion of the lake containing endemic fauna may be less than 200 years old (Owen *et al.,* 1990). In addition, Lake Malawi cichlids are predominantly mouth brooders and a new population can be established by a single female, resulting in a potential genetic bottleneck. The effective population size of *mbuna* species is difficult to estimate and may be quite variable. For example, estimates of *M. auratus* abundances at several locations in the southern portion of the lake ranged from 2 to 10 individuals per 50 m^2, and distribution varied depending on depth and substrate size (Ribbink *et al.,* 1983). Small effective population sizes may contribute to rapid mtDNA haplotype extinction and eventual monomorphism, and our results suggest exchange of mtDNA haplotypes has been infrequent among geographically isolated populations.

Intergeneric comparisons among rock-dwelling species revealed a mean sequence divergence of 2.52%, and between rock- and sand-dwelling species, the mean sequence divergence was 4.93%. These values are higher than those reported by Moran and Kornfield (1993), who analyzed mtDNA restriction fragment length polymorphisms in several genera of cichlids from lakes Victoria, Tanganyika, and Malawi. They found that within the *mbuna,* morphologically discrete genera differed by as little as 0.2%, and sequence divergence between rock- and sand-dwelling genera averaged 3.3% (Moran and Kornfield, 1993). Differences between these two studies may be because restriction fragment analysis may underestimate sequence divergences (i.e., Aquadro and Greenberg, 1983; Thomas and Beckenbach, 1989; Brown *et al.,* 1993), or because the control region may evolve more rapidly than the mtDNA genome as a whole. It is also possible that these differences simply reflect differences in the taxa examined.

Phylogenetic Analyses

Despite the limited sequence divergence, we found the species examined could be differentiated based on mtDNA haplotypes. The low bootstrap values obtained reflect, in part, the limited number of substitutions exhibited among species, and results may be improved by increasing the number of base pairs sequenced. The six *M. auratus* haplotypes formed a subclade within a larger assemblage made up of *M. heterochromis* and *M. auratus,* suggesting that *M. heterochromis* may be paraphyletic with respect to its mtDNA. Phylogenetic lineage sorting of mtDNA during speciation may have resulted in discordance between biological species boundaries and mtDNA haplotypes (Avise *et al.,* 1983; Moran and Kornfield, 1993). We are presently sequencing the D-loop region in all *Melanochromis* species, which will allow us to draw conclusions concerning the relationship between these two species and the remainder of the genus.

Retention of Ancestral Polymorphisms

One problem associated with phylogenetic analysis of recently diverged species that have undergone rapid trophic radiation is the retention of ancestral mtDNA polymorphisms (Neigel and Avise, 1986; Moritz *et al.,* 1987; Nei, 1987). Following a speciation event, the distribution of mtDNA haplotypes within a species may be polyphyletic prior to becoming monophyletic. The time to monophyly is affected by effective population size, population growth rate, variance in number of offspring, and mode of speciation (Moritz *et al.,* 1987; Avise *et al.,* 1984). Within lineages that have undergone rapid radiation, it is possible that not enough time has elapsed for character evolution, leaving very little evidence available for determining the sequential pattern of speciation events.

Recently, Moran and Kornfield (1993) have suggested the presence of retained ancestral polymorphisms within the Lake Malawi cichlid lineages. Based on restriction fragment length polymorphisms, they distinguished two major groupings of mtDNA haplotypes within the *mbuna,* with some species containing both lineages; thus, some species were polymorphic for divergent mtDNA lineages. They attributed their findings to incomplete mtDNA lineage sorting and the retention of an ancestral polymorphism. Although we cannot rule out this hypothesis, our results suggest that population-level mtDNA sequence variation may have been quite low or even nonexistent in some *mbuna* species. In populations for which multiple indi-

viduals were examined, 75% were fixed for a single mtDNA haplotype, and similar results were observed in populations of sand-dwelling species (unpublished). It is possible, however, that the riverine ancestral species that gave rise to the Lake Malawi species flock contained enough mtDNA polymorphism to confound phylogenies estimated using mtDNA sequence divergence. Additional sequencing and the development of nuclear markers will be necessary to resolve the question of retained ancestral polymorphisms.

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