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DIVERGENCE WITH GENE FLOW IN THE ROCK-DWELLING CICHLIDS OF LAKE MALAWI

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Abstract.—Within the past two million years, more than 450 species of haplochromine cichlids have diverged from a single common ancestor in Lake Malawi. Several factors have been implicated in the diversification of this monophyletic clade, including changes in lake level and low levels of gene flow across limited geographic scales. The objectives of this study were to determine the effect of recent lake-level fluctuations on patterns of allelic diversity in the genus *Metriaclima,* to describe the patterns of population structure within this genus, and to identify barriers to migration. This was accomplished through an analysis of allele frequencies at four microsatellite loci. Twelve populations spanning four species within *Metriaclima* were surveyed. The effect of lake-level fluctuations can be seen in the reduced genetic diversity of the most recently colonized sites; however, genetic diversity is not depressed at the species level. Low levels of population structure exist among populations, yet some gene flow persists across long stretches of inhospitable habitat. No general barrier to migration was identified. The results of this study are interpreted with respect to several speciation models. Divergence via population bottlenecks is unlikely due to the large allelic diversity observed within each species. Genetic drift and microallopatric divergence are also rejected because some gene flow does occur between adjacent populations. However, the reduced levels of gene flow between populations does suggest that minor changes in the selective environment could cause the divergence of populations.

Key words.—Cichlid, divergence with gene flow, habitat fragmentation, Lake Malawi, mbuna, *Metriaclima,* population structure.

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Lake Malawi provides a unique natural laboratory to study the process of species formation (Fryer and Iles 1972). Since the origin of the lake basin two million years ago, more than 450 species of haplochromine cichlids have radiated from a single common ancestor (Meyer 1993). The diversity within this clade is particularly apparent with respect to trophic morphology, color patterns, and reproductive behavior (Fryer 1959a).

The source of this rapid diversification has been debated for decades. Early models focused on vicariant processes involving multiple invasions (Mayr 1942; Fryer 1977) or the isolation of populations into separate lake basins due to fluctuating lake levels (Trewavas 1947; Fryer and Iles 1972; Fryer 1977). Other discussions have focussed on the adaptive radiation of jaw morphologies (Liem and Osse 1975). Sexually dimorphic color patterns and high variance in male reproductive success suggest that sexual selection may also have played a role (Dominey 1984; McElroy and Kornfield 1990; McKaye et al. 1993; Taylor et al. 1998).

Reduced gene flow among populations facilitates divergence and the evolution of new species (Endler 1973; Lande 1982; Rice and Hostert 1993). Limited migration has long been suspected in Malawi cichlids, particularly in the rock-dwelling forms (locally known as ''mbuna''). These species often have restricted geographic distributions; many species are endemic to a single rock outcropping (Ribbink et al. 1983; Lewis et al. 1986; Konings 1990). Species relocated by fish collectors (a practice that is now illegal) tend to have limited distributions adjacent to the site of introduction (Ribbink et al. 1983; Hert 1990).

Molecular studies support the idea that population differentiation occurs over extremely short distances. Analyses of allozyme (McKaye et al. 1984) and mitochondrial DNA (mtDNA) haplotype frequencies (Moran and Kornfield 1995) have detected genetic differentiation between northern and southern populations separated by more than 200 km. Van Oppen et al. (1997) examined populations of four mbuna species using six microsatellite loci and detected significant levels of genetic differentiation among mbuna populations separated as little as 3 km in northern Lake Malawi. Arnegard et al. (1999) and Markert et al. (1999) detected significant levels of population structure in *Labeotropheus fuelleborni* and *Melanochromis auratus,* respectively, over a 43-km transect in Lake Malawi's southeastern arm.

Several factors are thought to contribute to population fragmentation in the mbuna. The mbuna are philopatric. They rarely leave the rocky habitats (Fryer and Iles 1972; Holzberg 1978), which are highly fragmented (Fryer 1959b) and the least prevalent substrate in the mosaic of Lake Malawi's shoreline (McKaye and Gray 1984). Furthermore, all Malawian cichlids maternally mouthbrood their young and thus lack a dispersing larval stage. The dynamic geologic history of the lake also contributes to population fragmentation. Geologic evidence suggests that Lake Malawi is prone to frequent changes in water level (Crossley et al. 1984; McKaye and Gray 1984; Scholz and Rosendahl 1988). At least four major desiccation/inundation cycles have occurred within the past 25,000 years. The most recent recession occurred between 1390 and 1860 A.D. when the lake surface was at least 121 m below its current level (Owen et al. 1990). A drop of this

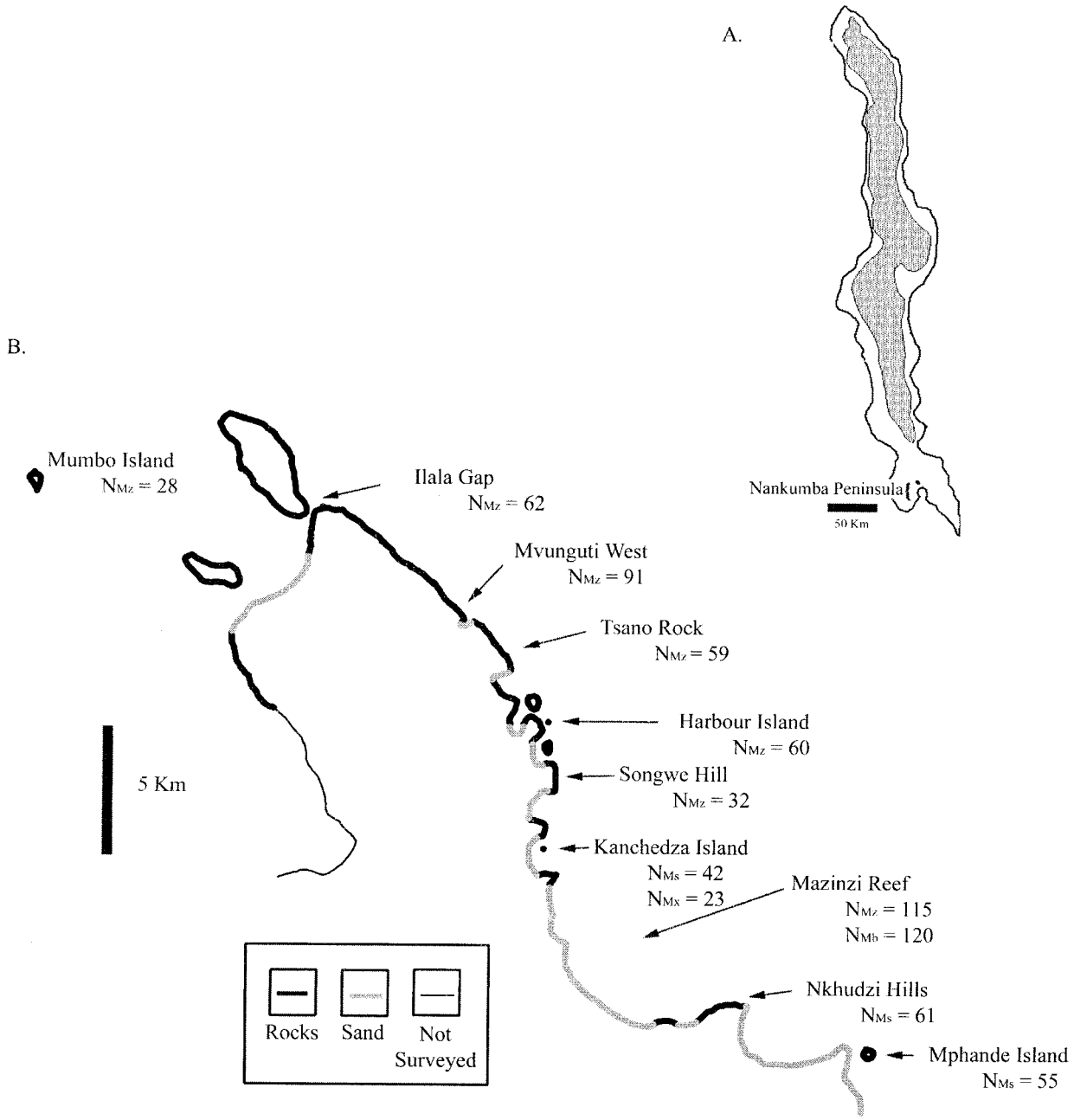

FIG. 1. Geographic distribution of collection sites and the number of individuals sampled (adapted from Markert et al. 1999). (A) The Nankumba Peninsula divides the southern aspect of Lake Malawi into two shallow basins. The white area represents that portion of the lake that was dry during a recent desiccation event that lowered the lake level 200 m below its current level (from Owen et al. 1990). (B) The geographic distribution of the 10 collection locations. The number of individuals sampled for each species at each location is given by N_{Mz}, N_{Ms}, N_{Mb}, N_{Mx}, for *Metriaclima zebra, M. sandaracinos, M. benetos,* and *M. xanostomachus,* respectively.

magnitude would have drained the entire southern portion of the lake, including both the southeastern and southwestern basins. Such temporal variability would result in the repeated extinction and creation of populations at a particular site.

The objective of this study was to describe the pattern of population structure within the rock-dwelling cichlids of Lake Malawi. Specifically, we wanted to determine the effect of recent lake-level fluctuations on patterns of genetic diversity, to measure gene flow at different geographic scales, and to identify migration barriers. These objectives were addressed by examining allele frequencies at microsatellite loci in populations of four *Metriaclima* species from the southeastern arm of Lake Malawi.

MATERIALS AND METHODS

Collection Sites

All of the collection sites were located on or adjacent to the Nankumba Peninsula in the southeastern basin of Lake Malawi (Fig. 1). The habitat at these sites is of extremely

TABLE 1. Population characteristics of four *Metriaclima* species from Lake Malawi. *N*, number of individuals sampled; habitat size, size of available habitat ($m^2/10^4$); depth, depth of the rock-sand interface (m); H_o, observed heterozygosity (standard deviation); n_e, effective number of alleles; UNH001–UNH231, number of alleles per locus per population; average, average number of alleles per locus per collection location.

Population	*N*	Habitat size	Depth	H_o	n_e	UNH001	UNH002	UNH050	UNH231	Average
M. zebra										
Mumbo	28	52.31	46	0.921 (0.0133)	10.94	14	16	19	18	16.75
Ilala Gap	62	212.85	36	0.949 (0.0043)	17.20	25	29	23	31	27.00
Mvunguti	91	212.85	27	0.949 (0.0044)	18.12	27	36	25	34	30.50
Tsano Rock	59	28.30	32	0.937 (0.0048)	14.32	26	28	19	31	26.00
Harbour Island	60	16.67	30	0.930 (0.0156)	14.31	23	28	22	27	25.00
Songwe Hill	32	3.08	4	0.908 (0.0133)	9.81	16	21	15	17	17.25
Mazinzi Reef	115	1.00	15	0.868 (0.0221)	8.05	23	21	18	23	21.25
All populations	447					38	44	30	45	39.25
M. sandaracinos										
Kanchedza Island	42	0.16	3	0.900 (0.0104)	9.25	14	17	17	20	17.00
Nkhudzi Hills	61	15.88	11	0.946 (0.0063)	16.51	25	27	28	26	26.50
Mphande Island	55	0.82	4	0.937 (0.0031)	13.95	23	20	22	22	21.75
All populations	158					32	32	33	32	32.25
M. xantomachus										
Kanchedza Island	23	0.16	3	0.910 (0.0152)	9.55	13	11	16	18	14.50
M. benetos										
Mazinzi Reef	120	1.00	15	0.836 (0.0151)	6.12	21	17	16	21	18.75

recent origin. Most sites are known to have been dry land within the past 500 years (Owen et al. 1990) and those less than 7 m deep are known to have been exposed as recently as 1900 A.D. (Crossley 1982). The collection sites consist of rocky habitats separated by a variety of intervening substrates. The northern sites (Mumbo Island, Ilala Gap, Mvunguti West, Tsano Rock, Harbour Island) are located in areas of large boulders that slope down to the sandy lake bottom (>30 m) (Fig. 1). The boulder habitat in this area is generally continuous except for a narrow (350 m), sandy beach between Mvunguti West and Tsano Rock, two sandy bays between Tsano Rock and Harbour Island, and a deep-water trench between Mumbo Island and Ilala Gap. The southern sites generally consist of smaller rocks and a shallow rock/sand interface (10–15 m). Substrates between the southern sites generally consist of alternating sandy and rocky coast and include long stretches of shallow sandy bottom (Songwe Hill to Mazinzi Reef and Kanchedza Island to Nkhudzi Hills). Detailed descriptions of the collection sites can be found in Arnegard et al. (1999) and Markert et al. (1999).

Habitat depth and size of the available habitat patch were estimated at each collection site. Habitat depth was measured as the depth of the rock-sand boundary. The size of the available habitat was calculated as the product of the depth of available rocky habitat (corrected for the slope of the habitat) and the shoreline length of interrupted rocky coast (following Arnegard et al. 1999).

Study Species

Metriaclima

Metriaclima, like most mbuna genera, diversified rapidly. As a result, this genus is exceptionally species rich. Although interesting from an evolutionary perspective, this rapid and extensive diversification can be problematic. The phylogenetic relationships among the mbuna and within *Metriaclima* are poorly understood (Albertson et al. 1999). In addition, the alpha-level taxonomy is currently incomplete. Stauffer et al. (1997) formally recognized 15 species, 10 of which were previously undescribed, in their description of this genus. They recognized, however, that at least nine currently suggested species in the genus *Pseudotropheus* and as many as 20 undescribed forms will be included in *Metriaclima* in the future.

The nomenclature used in this study largely follows that established by Stauffer et al. (1997), with one exception. Stauffer et al. (1997) recognized the *Metriaclima* population at Mumbo Island as a distinct species (*M. melabranchion*), while the current study includes this population within *M. zebra.* The *Metriaclima* population at Mumbo Island has generally been considered conspecific with *M. zebra* (Ribbink et al. 1983; Konings 1990). Furthermore, the sampled individuals generally lacked the markings (lateral body bars extending into the dorsal fin) that distinguish *M. melabranchion* from *M. zebra.*

Metriaclima zebra

Metriaclima zebra is one of the few mbuna species that is widely distributed throughout the rocky areas of the entire lake (Konings 1990). The absence of pigment in its pale blue dorsal fin and the presence of six to eight black vertical bars on its pale blue flank distinguish it from other members of the genus. Based on its distribution and its morphological features, Stauffer et al. (1997) consider *M. zebra* to be the most basal member of the genus.

A total of 447 individuals were sampled from seven collection sites (Table 1). The sites ranged from Mumbo Island in the north to Mazinzi Reef in the south (Fig. 1). The number of individuals sampled per collection site ranged from 28 (Mumbo Island) to 115 (Mazinzi Reef), with a mean sample size of approximately 56 individuals per collection site. The entire study area for *M. zebra* spanned 30.3 km.

Metriaclima sandaracinos

This species can be identified by its red/orange dorsal fin as well as six or seven black vertical bars on its pale blue

flank. Its distribution is limited to Kanchedza Island, Chirombo Bay, Nkhudzi Hills, and Mphande Island (Fig. 1) (Stauffer et al. 1997).

Metriaclima sandaracinos were sampled from three sites that ranged from Kanchedza Island in the north to Mphande Island in the south (Table 1). The number of individuals sampled ranged from 42 (Kanchedza Island) to 61 (Nkhudzi Hills). Mean sample size equaled approximately 53 individuals.

Metriaclima xanostomachus

Metriaclima xanstomachus is readily identified by its bright yellow gular region and dorsal fins. It is distributed throughout the Maleri Islands and at Kanchedza Island (Stauffer et al. 1997), where it is sympatric with *M. sandaracinos* (Fig. 1). Twenty-three individuals were collected from Kanchedza Island (Table 1).

Metriaclima benetos

Metriaclima benetos males closely resemble *M. zebra* males, but can be distinguished on the basis of male breeding color. Unlike *M. zebra* males, whose pale blue flanks are interrupted by six to eight black bars, *M. benetos* males are uniformly pale blue. This species is endemic to Mazinzi Reef (Stauffer et al. 1997), where it is sympatric with *M. zebra* (Fig. 1). Phylogenetic evidence suggests that *M. benetos* diverged from the ancestral *Metriaclima* prior to the divergence of *M. zebra* and *M. sandaracinos* (Albertson et al. 1999). A total of 120 individuals were sampled.

Collection Methods

Individual fish were collected using monofilament nets while SCUBA diving. For collections made outside of Lake Malawi National Park, fin clips of the right pectoral fin were collected and preserved in >90% ethanol. The remainder of each fish was preserved as voucher specimens in a 10% formalin solution. Fish collected from within Lake Malawi National Park were immediately released after clipping an unpaired fin (collection permit 684658).

Molecular Analysis

Four microsatellite loci were examined: UNH001 (Genbank accession number U17044), UNH002 (U17045), UNH050 (AF036714), UNH231 (G12382). Each locus consisted of perfect dinucleotide (CA) repeats. DNA extraction, locus amplification, and allele identification were performed as described by Markert et al. (1999). A number of these loci have been used to study population structure in other mbuna species (van Oppen et al. 1997; Arnegard et al. 1999; Markert et al. 1999). Previous studies indicate that these loci are not in linkage disequilibrium.

Statistical Analysis

Each locus was examined for evidence of Hardy-Weinberg equilibrium, the presence of null alleles, and linkage disequilibrium using GENEPOP 3.1 (Raymond and Rousset 1995). Significance thresholds were established for the tests of Hardy-Weinberg equilibrium and linkage disequilibrium through a Bonferroni correction for 12 comparisons. For example, the $\alpha = 0.05$ level after correcting for 12 comparisons is 0.0042 (0.05/12).

Overall estimates of population heterozygosity and its standard error were calculated using DISPAN (Ota 1993). A second estimate of allelic diversity, the effective number of alleles (n_e), was calculated as the inverse of the expected homozygosity (Hartl and Clark 1989). Wright's *F*-statistics were calculated to assess the level of population substructure using FSTAT (Goudet 1995). FSTAT follows the methods of Weir and Cockerham (1984) in calculating *F*-statistics. Standard deviations and *P*–values were calculated for the *F*-statistics by permuting the data for 5000 replicates.

Hedrick (1999) has suggested that, when examining highly variable loci, variation-dependent estimators of population differentiation, such as Wright's *F*-statistic (F_{ST}), may identify statistically significant differences in allele frequency distributions that lack biological meaning. To address this issue, we have calculated a second, variation-independent estimator of population subdivision, as suggested by Hedrick (1999). A multilocus estimate of the effective number of migrants (Nm) was calculated based on the frequency of private alleles (Slatkin 1985a; Barton and Slatkin 1986) using GENEPOP 3.1. Exact tests of allelic differentiation between adjacent populations were also performed using Genepop 3.1 to provide a third estimate of population subdivision. The significance levels for the exact tests were corrected for eight comparisons using a Bonferroni correction (see above).

The identification of migration barriers was carried out by regressing an estimate of per generation migration rates ($M = [1/F_{ST} - 1]/4$) on geographic distance (Slatkin 1993). Mantel tests performed by GENEPOP 3.1 were used to assess the significance of this relationship.

RESULTS

Locus Characteristics

Hardy-Weinberg equilibrium

Each population was tested for random union of gametes at each locus using an exact test (Guo and Thompson 1992). *Metriaclima sandaracinos, M. xanostomachus,* and *M. benetos* populations did not deviate from random union of gametes at any loci. Three of eight *M. zebra* populations deviated from Hardy-Weinberg expectations at UNH002 (Mvunguti West, Songwe Hill, and Mazinzi Reef), two deviated at UNH001 (Tsano Rock and Mazinzi Reef), and one deviated at UNH231 (Tsano Rock). UNH050 was at Hardy-Weinberg equilibrium in all populations.

Those populations that deviated from Hardy-Weinberg expectations were also examined for evidence of heterozygote deficiency. Only two populations were found to lack heterozygous individuals at the loci not in Hardy-Weinberg equilibrium (Mvunguti West and Songwe Hill at UNH002). A population may be deficient in heterozygotes for several reasons.

First, inbreeding resulting from limited population sizes may reduce the occurrence of heterozygotes. Second, population substructure within a collecting location may result

in an excess of homozygotes (Wahlund effect); however, efforts were made to mitigate the Wahlund effect by collecting samples within 100 m at each collection site. The observation of excess homozygotes may also suggest that a null allele may be present in the population.

Null alleles

Alleles that cannot be amplified due to mutations at the polymerase chain reaction priming site can affect estimates of both heterozygosity and levels of population subdivision. A previous study detected the presence of a true breeding null allele at UNH002 (van Oppen et al. 1997). Maximum-likelihood estimates of the frequency of null alleles were calculated using the EM algorithm of Dempster et al. (1977) in GENEPOP 3.1. This algorithm bases its null allele frequency estimates on the occurrence of apparent null homozygotes in the dataset. Null alleles were assumed to occur when only three of the four loci amplified after multiple attempts. The estimated frequencies suggest that null alleles are rare, with an estimated global average frequency (± 1 SD) of 0.039 (0.043). At this frequency they do not significantly alter the observed trends in allelic diversity. For example, the highest estimated null allele frequency was 0.16 at UNH001 in the *M. zebra* collection from Songwe Hill. The estimated frequency of the most common allele at this locus in this population differed from its calculated value by only 0.03 when corrected for the presence of the null allele. Estimated frequencies of the remaining alleles generally differed by less than 0.005

Linkage disequilibrium

The four loci used in this study were examined for evidence of linkage disequilibrium. Linked loci experience similar evolutionary processes and, as a result, will not provide independent estimates of heterozygosity and measures of population subdivision. Linkage disequilibrium was calculated separately for each population. Only two populations, Mazinzi Reef *M. zebra* and *M. benetos,* show disequilibrium between UNH001 and UNH002 ($P < 0.002$, $P < 0.001$, respectively, Fisher's exact test). These results, combined with evidence from an analysis of this pair of loci in other closely related species (Arnegard et al. 1999; Markert et al. 1999), suggest that these loci are not physically linked in the genome. The apparent linkage disequilibrium of these loci in the Mazinzi Reef populations is most likely due to genetic drift because of their limited population size or a recent founding event.

Allelic Diversity

Metriaclima zebra populations

Metriaclima zebra, like all other mbuna species surveyed to date, contains high levels of allelic diversity at all four microsatellite loci. The number of alleles at a locus ranged from 30 at UNH050 to 45 at UNH231, with an average of 39.25 alleles per locus (Table 1). The effective number of alleles (n_e) within populations ranged from 8.05 (Mazinzi Reef) to 18.12 (Mvunguti West). The corresponding heterozygosities were 0.868 (Mazinzi Reef) to 0.949 (Mvunguti West).

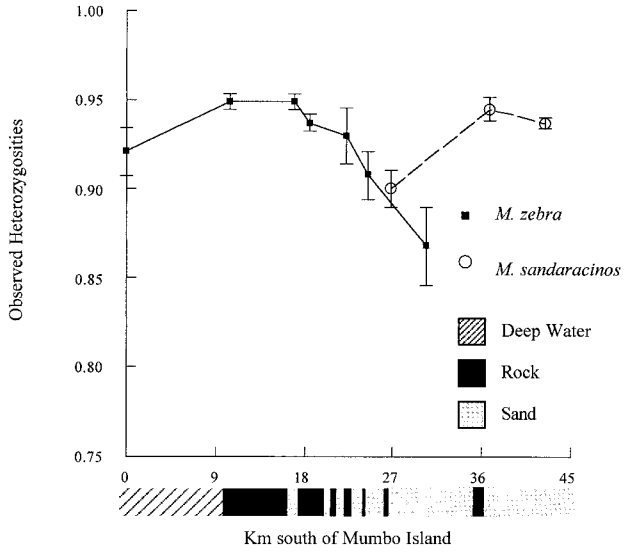

FIG. 2. The distributions of population heterozygosities for both *Metriaclima zebra* (filled square) and *M. sandaracinos* (open circles) are plotted against the distance from the northernmost collection site (Mumbo Island). The error bars indicate one standard error. The shaded area beneath the horizontal axis represents the nature of the substrate along the transect where the striped area indicates deep (>50 m) water, the black area represents rocks, and the gray area indicates shallow sand.

The geographic distribution of genetic diversity reveals some general patterns. Heterozygosities and n_e are largest at collecting sites surrounded by long stretches of rocky coasts, for example, between Ilala Gap and Mvunguti West (Fig. 2). South of these collecting sites diversity declines. The least diverse population occurs at Mazinzi Reef, which is a moderately sized rock reef nearly 3 km from the nearest shore in Madzidzi Bay. This population has the lowest heterozygosity (0.868) and the smallest effective number of alleles (8.05).

Differences in the observed allelic diversity could result from a number of factors. We investigated the relationship between two measures of allelic diversity (average heterozygosity and n_e) and (1) the number of individuals sampled; (2) the age of the collecting site estimated by its depth; and (3) the size of the population as estimated by the size of the available habitat patch. Average heterozygosity did not correlate with sample size or depth ($P = 0.48$, $P = 0.20$, respectively). Average heterozygosity tends to increase with the size of available habitat, but this trend was not statistically significant ($P = 0.09$). The effective number of alleles did not correlate with sample size ($P = 0.89$) or depth ($P = 0.31$), but an increase in the effective number of alleles was associated with an increase in available habitat at a collecting site ($P = 0.02$). Because no intermediate patch sizes were sampled, it is difficult to estimate the true relationship between patch size and allelic diversity.

Metriaclima sandaracinos populations

The *M. sandaracinos* samples were only slightly less diverse than the *M. zebra* collection. Thirty-two alleles were

observed for each locus, except UNH050, which had 33 (Table 1). Observed heterozygosities were high, ranging from 0.900 (Kanchedza Island) to 0.946 (Nkhudzi Hills). Values for n_e ranged from 9.25 (Kanchedza Island) to 16.51 (Nkhudzi Hills).

General geographic patterns are difficult to detect because only three populations of *M. sandaracinos* inhabit the study area. However, the site with the largest population, as estimated from the habitat size, is also the most genetically diverse (Nkhudzi Hills; Table 1).

Metriaclima xanstomachus

The single *M. xanstomachus* population sampled generally exhibited lower genetic diversity than either *M. zebra* or *M. sandaracinos* as estimated by the average number of alleles per locus (14.5), average heterozygosity (0.91), or the effective number of alleles (9.55). However, only the average number of alleles in *M. xanstomachus* was lower than the range found in either *M. zebra* or *M. sandaracinos.*

Metriaclima benetos

Despite being the most extensively sampled population in this study, *M. benetos* exhibited the most limited genetic diversity of all the species examined. Its heterozygosity (0.836) and effective number of alleles (6.12) was below the range found in any of the other species (Table 1). The number of alleles present was comparable to that found in the other three species, but the allelic distributions of each locus tended to be dominated by one or two alleles.

Population Differentiation

There is considerable uncertainty concerning the appropriate estimator of population differentiation for examining allele frequency distributions of highly polymorphic loci. Such loci have an extraordinary power to detect fine differences in allele frequencies between populations, but these differences may not reflect biologically meaningful differences in the populations (Hedrick 1999). In contrast, statistics that compare the sharing of alleles in different populations, such as Slatkin's (1985a) rare alleles method of estimating the effective number of migrants (*Nm*), may more accurately estimate population differentiation when using highly variable markers (Hedrick 1999). Estimates of population differentiation based on allele frequency distributions (F_{ST}, following Weir and Cockerham 1984) and shared alleles (*Nm*, following Slatkin 1985a) produce concordant results (Pearson $P = 0.015$) and are presented in Table 2.

Metriaclima zebra populations

Significant levels of structure were detected among the *M. zebra* populations (overall $F_{ST} = 0.041 \pm 0.008$, $Nm = 3.83$). Pairwise comparisons also indicate that gene flow is limited between adjacent populations (Table 2). All comparisons of adjacent populations yielded small but significant F_{ST}-values and low estimates of the effective number of migrants (*Nm*) between populations. Population differentiation is also supported by Fisher's exact test of allele frequency homogeneity. All pairwise comparisons of adjacent populations revealed

TABLE 2. Results of allele frequency distribution comparisons of adjacent populations of *Metriaclima* in Lake Malawi. Depth is the maximum depth separating populations; substrate indicates the nature of the intervening substrate between the two populations; exact is the probability that the two populations have homogeneous allele frequency distributions at all four loci (unless otherwise indicated) after a Bonferroni correction for eight comparisons. F_{ST} is calculated following Weir and Cockerham (1984); all F_{ST}-values are significantly greater than zero at $P < 0.005$. *Nm* is the estimated number of migrants estimated using Slatkin (1985a); *M* was calculated following Slatkin (1993). An asterisk indicates the value at UNH001.

	Comparison	Distance (km)	Depth (m)	Substrate	Exact	F_{ST} (SD)	*Nm*	*M*
M. zebra	Mumbo Island—Ilala Gap	10.4	> 50	deep sand (50 m)	$P < 0.05$	0.0240 (0.005)	3.04	10.16
M. zebra	Ilala Gap—Mvunguti West	6.6	~ 36	large rocks	$P < 0.005$	0.0105 (0.002)	3.45	23.56
M. zebra	Mvunguti West—Tsano Rock	1.5	~ 36	large rocks, small beach	$P < 0.05$	0.0119 (0.002)	4.63	20.75
M. zebra	Tsano Rock—Harbour Island	3.7	~ 30	rocky coast, deep sand channel	$P < 0.05$	0.0250 (0.006)	2.71	9.75
M. zebra	Harbour Island—Songwe Hill	2.2	~ 30	shallow alternating sand and rock	$P < 0.001$	0.0508 (0.007)	2.38	4.67
M. zebra	Songwe Hill—Mazinzi Reef	5.9	~ 15	open shallow sand	$P < 0.001$	0.0869 (0.010)	1.18	2.63
M. sandaracinos	Kanchedza Island—Nkhudzi Hills	10.0	~ 11	open shallow sand	$P < 0.001$	0.0271 (0.002)	4.68	11.27
M. sandaracinos	Nkhudzi Hills—Mphande Island	5.6	~ 11	shallow alternating sand and rock	$P < 0.005$*	0.0048 (0.003)	11.42	51.833

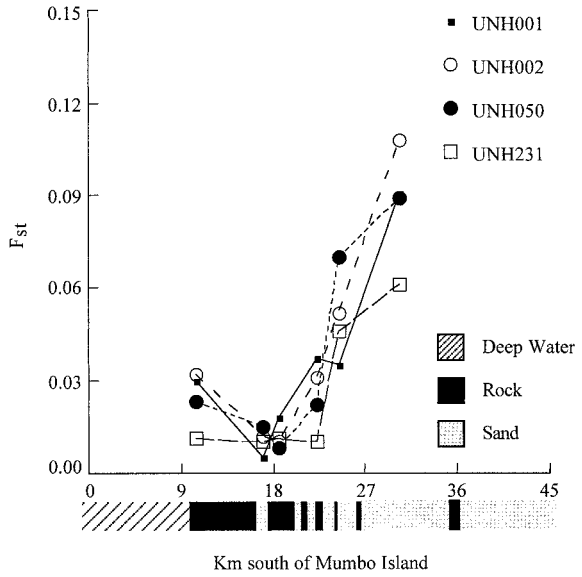

FIG. 3. A plot of pairwise *M. zebra* F_{ST}-values between adjacent sites versus the distance from the northernmost (Mumbo Island) collection site. F_{ST}-values for a given comparison are plotted above the more southern of the two populations for each of the loci examined. The closed squares represent locus UNH001, open circles UNH002, closed circles UNH050, opens squares UNH231. The shaded area beneath the horizontal axis represents the nature of the substrate along the transect where the striped area indicates deep (>50 m) water, the stippled area represents rocks, and the gray area indicates shallow sand.

heterogeneity in allele frequency data, at all four loci, at the $P < 0.05$ level (Table 2).

Estimates of F_{ST} and *Nm* suggest that genetic differentiation is lowest among samples collected along nearly continuous rocky coasts (Fig. 3). The lowest estimated F_{ST} (four-locus $F_{ST} = 0.0105 \pm 0.002$, estimate ± SD) occurred between two populations (Ilala Gap and Mvunguti West) separated by 6.6 km of continuous rocky coast. Similar F_{ST}-estimates were calculated for populations separated by 1.5 km of rocky coast and a narrow (350 m), shallow beach (Mvunguti West and Tsano Rock, $F_{ST} = 0.0119 \pm 0.002$). Although the F_{ST}-estimate suggests that this narrow beach may limit migration, the estimated *Nm* (4.63) between these populations is the largest of all *M. zebra* comparisons.

Population comparisons across deep water (Mumbo Island to Ilala Gap) yielded a larger four-locus F_{ST}-value (0.0240 ± 0.005) and a smaller *Nm* (3.04). There is a minor increase in population subdivision when the intervening substrate consists of alternating rocky and sandy coasts (Tsano Rock to Harbour Island, four-locus $F_{ST} = 0.0250 \pm 0.006$, $Nm = 2.71$). Estimates of population substructure dramatically increase when the intervening substrate is dominated by sand. Songwe Hill is separated from Harbour Island by 2.2 km, nearly half of which is sand. The four- locus F_{ST} (0.0508 ± 0.007) for this comparison is nearly five times greater than populations separated by a similar distance of rocky coast (Mvunguti West to Tsano Rock). The greatest level of population subdivision occurs between the Songwe Hill population and the Mazinzi Reef population (four-locus $F_{ST} = 0.0869 \pm 0.01$, $Nm = 1.18$). These populations are separated by 5.9 km of uninterrupted sand, highlighting the important role this barrier plays in isolating *M. zebra* populations.

Metriaclima sandaracinos populations

Metriaclima sandaracinos populations show low, but statistically significant, levels of population substructure (overall four-locus $F_{ST} = 0.017 \pm 0.003$, $Nm = 9.46$). Estimates of pairwise F_{ST}-values suggest that genetic structure exists between all adjacent populations (Table 2). The Kanchedza Island to Nkhudzi Hills population comparison yielded a larger four-locus F_{ST} (0.0271 ± 0.002) than the Nkhudzi Hills to Mphande Island comparison (0.0048 ± 0.003). This suggests that greater migration occurs between the latter two sites.

This observation is further supported by Fisher's exact tests for homogeneity of allele frequencies. The Kanchedza Island to Nkhudzi Hills comparison indicated significant heterogeneity at all four loci ($P < 0.001$). The Nkhudzi Hills to Mphande Island comparison indicated that allele frequencies differ at UNH001 ($P < 0.005$), whereas the allele frequency distributions of the remaining loci are statistically homogeneous.

This observation is consistent with the estimated number of migrants between adjacent *M. sandaracinos* populations. Migration appears to occur more frequently between Nkhudzi Hills and Mphande Island ($Nm = 11.42$) than between Kanchedza Island and Nkhudzi Hills ($Nm = 4.68$).

Barriers to Migration

The relationship between population substructure and geographic distance was examined in two ways. Following Slatkin (1993), the estimated number of individuals moving between populations each generation (*M*) was plotted against geographic distance on a log/log scale. Mantel tests were performed to test for a relationship between F_{ST} and geographic distance. Geographic distance explained very little of the variation in *M* (*M. zebra* $R^2_{adj} = 0.098$), and the Mantel tests failed to detect a significant relationship between geographic distance and the level of population structure in *M. zebra* ($P = 0.157$). The relationship between distance and the level of population structure was stronger in *M. sandaracinos* ($R^2_{adj} = 0.816$), but the Mantel test failed to detect a significant relationship between geographic distance and the level of population structure ($P = 0.167$). The results for *M. sandaracinos* are questionable due to the small number of comparisons.

Estimators of population subdivision suggests that the nature of the intervening substrate plays an important role in isolating populations. To test the significance of this relationship, Mantel tests were performed using a matrix of F_{ST}-values from all possible pairwise *M. zebra* population comparisons and a matrix of indicator values describing the nature of the intervening substrate. The indicator variable ranged from 1 (indicating continuous rock between collection sites) to 7 (indicating continuous sand between collection sites). Three of the authors (J. A. Markert, M. E. Arnegard, T. D. Kocher) separately generated substrate matrices based on their knowledge of the region prior to a discussion of the data. A Mantel test was performed separately for each of the

substrate matrices. Each Mantel test revealed a significant relationship between the level of population structure between populations and the nature of the intervening substrate (P = 0.04, 0.008, 0.02). Tests were not performed on *M. sandaracinos* due to the limited number of populations sampled and the similarity of substrates between all three populations.

Discussion

Patterns of Allelic Diversity

Metriaclima zebra

Metriaclima zebra collections with the highest diversity were made along the continuous rocky coast of the Nankumba Peninsula (Ilala Gap, Mvunguti West, Tsano Rock) (Fig. 2). These populations are generally the largest, thought to be the oldest based on the depth of suitable habitat, and occur in areas of high migration. In contrast, populations with the lowest allelic diversity generally occur at sites in the southern end of the transect. These sites are smaller, more recently available, and separated from other sites by inhospitable habitat. It is clear that the analysis of *M. zebra* diversity cannot decipher the influences of mutation rate, population size, and migration. However, the lack of a correlation between the estimated age of a collection site and the allelic diversity of that site's sample suggests that a population's genetic diversity is not greatly influenced by the accumulation of new mutations in persistent populations relative to historical influences, that is, its source population.

Metriaclima sandaracinos

Samples collected from Nkhudzi Hills, presumably the oldest location based on depth, were the most genetically diverse at the four microsatellite loci examined. The two remaining collections differed in their allelic diversity. The southern samples (Mphande Island) are nearly as diverse as the Nkhudzi Hills samples (Ht = 0.937, n_e = 13.95), whereas the northern samples (Kanchedza Island) are some of the least diverse in the entire study (Ht = 0.900, n_e = 9.25).

There may be several reasons for the difference in diversity of these two populations. First, the considerable genetic diversity of the southern (Mphande Island) collection may be the result of a recent colonization event by a large and diverse founding population. Second, more migration may (have) occur(red) between Mphande Island and Nkhudzi Hills than between Nkhudzi Hills and Kanchedza Island. This hypothesis is supported by both the data (Nm = 11.42 vs. 4.68, respectively) and the geographic arrangement of the sites (Mphande Island is 4 km closer to Nkhudzi Hills than Kanchedza Island). Finally, Kanchedza Island may support smaller numbers of individuals than would be expected by its patch size. As discussed above, *M. sandaracinos* co-occurs with a closely related species, *M. xanstomachus,* at Kanchedza Island. These species occupy similar ecological niches and the resulting competition may limit the population sizes of both species, allowing for the stochastic loss of genetic diversity.

Metriaclima xanstomachus

The reduced genetic diversity in this species may be due to a number of factors. First, Kanchedza Island, the only known site for this species in the southeastern arm of the lake, is one of the smallest habitat patches in the study area. Second, because the total population is extremely limited in number, only 23 animals were collected. Any estimates derived from such a limited sample of individuals should be considered preliminary. To fully address the genetic diversity of this species, other populations need to be studied.

Metriaclima benetos

The genetic diversity of *M. benetos* appears to be limited despite the large number of individuals that were sampled (N = 120, the largest sample in the study). Furthermore, the single location from which individuals were sampled (Mazinzi Reef) represents the only known area in which this species lives (Stauffer et al. 1997). The allelic diversity of the entire species should be well represented by this sample. The limited diversity in this species is most likely due to a genetically reduced founder population and/or a population bottleneck that has occurred during the dynamic history of the southeastern arm of the lake. However, the possibility that this species was derived from a genetically depauperate ancestral species cannot be ruled out.

Scale of Population Structure

The analysis of allele frequency data indicates that gene flow is restricted over limited geographical scales in both *M. zebra* and *M. sandaracinos.* Low but statistically significant levels of population structure were detected over the entire study area for both species (*M. zebra* F_{ST} = 0.041, Nm = 3.83; *M. sandaracinos* F_{st} = 0.017, Nm = 9.46). Pairwise comparisons suggest that gene flow is limited among all adjacent *M. zebra* populations, even when separated by less than 2 km of nearly continuous rocky coast (e.g., the populations of *M. zebra* at Mvunguti West and Tsano Rock). Pairwise comparisons of *M. sandaracinos* populations suggest that gene flow is limited between Kanchedza Island and Nkhudzi Hills, while gene flow is greater between Nkhudzi Hills and Mphande Island.

Significant levels of population structure were detected in *Labeotropheus fuelleborni* and *Melanochromis auratus* over a similar geographic area using the same loci (F_{ST} = 0.063, 0.151, respectively) (Arnegard et al. 1999; Markert et al. 1999). Van Oppen et al. (1997) detected structure over limited geographic scales among populations of four mbuna species, including *M. zebra* and *M. callainos,* in the Nkhata Bay area. These results suggest that structured populations are a general feature of the mbuna. The biological importance of this finding is discussed below.

Barriers to Migration

Although limited migration appears to be a general feature of mbuna biology, it is not clear that a single universal barrier to their migration exists. Previous studies have identified several environmental factors that limit migration including deep water, distance between populations (Arnegard et al. 1999;

Markert et al. 1999), and the nature of the intervening substrate (van Oppen et al. 1997; Arnegard et al. 1999; Markert et al. 1999).

Deep water

Previous examinations of the mbuna species *L. fuelleborni* and *M. auratu*s have concluded that migration is primarily limited by their inability to cross deep water (Arnegard et al. 1999; Markert et al. 1999). These species tend to remain closely associated with the substrate and appear to be physiologically incapable of compensating for the change in pressure associated with the rapid change in depth (Hill and Ribbink 1978; Marsh and Ribbink 1981; Ribbink et al. 1983). In the current study, the effect of depth was less pronounced than other physical barriers (i.e., sand). *Metriaclima zebra* may be more likely to cross areas of deep water because their feeding mode is not closely tied to the substrate. *Metriaclima zebra* males feed much higher (>5m, pers. obs.) in the water column than other mbuna, and the analysis of gut contents suggest that their diet includes planktonic diatoms (Reinthal 1990). *Metriaclima zebra* may occasionally traverse deep water trenches while feeding high in the water column and so avoid having to compensate for changes in depth.

Distance

Arnegard et al. (1999) and Markert et al. (1999) also detected a significant relationship between the level of population structure and the distance separating populations across the same geographic area. Although distance most likely plays some role in disrupting migration between populations of *Metriaclima* (e.g., Ilala Gap and Mvunguti West *M. zebra* populations), a significant relationship between distance and isolation was not detected in either *M. zebra* or *M. sandaracinos.* Isolation by distance might not be detected if genetic equilibrium has not been established at these recently colonized sites. Other factors may have contributed to the observed lack of association between the level of population structure and distance.

Substrate

It appears that the nature of the intervening substrate plays an important role in limiting *M. zebra* migration. F_{ST}-values dramatically increase as the amount of sand separating populations increased (Fig. 3). The number of migrants crossing long stretches of open sand were estimated to be only one migrant per generation (Table 2), which approaches the limit that would allow populations to diverge by drift (Wright 1931; Slatkin 1985b). It is unclear why this species appears to disperse across deep water, but not across shallow sandy areas.

Sand does not appear to be a general migration barrier within *Metriaclima,* however. The greatest differentiation between adjacent populations of *M. zebra* occurred over 5.9 km of nearly continuous sand (Songwe Hill to Mazinzi Reef, four- locus $F_{ST} = 0.0869$, $Nm = 1.18$). A similar comparison among *M. sandaracinos* samples that are separated by 5.6 km of nearly continuous sand showed no consistent differentiation across loci and had the largest estimated number of migrants in the entire study ($Nm = 11.42$). Although the observed similarity of the two *M. sandaracinos* samples may have resulted from number of different processes (e.g., recent colonization of one or both sites by a diverse founding population, or high levels of ongoing migration), the data suggest that a large number of individuals crossed (or currently cross) sandy barriers that nearly extinguish migration in *M. zebra.* The ability of *M. sandaracinos* to cross open sand may be related to their habitat preference; they were generally found in sediment-rich areas near the sand-rock interface.

Although movement across sand by *M. sandaracinos* may be consistent with their habitat preference, it highlights two surprising features. First, it emphasizes the rapid diversification of behavioral characteristics within a group of fish with extremely limited morphological diversity. Rapid divergence of other behavioral characteristics within this genus (e.g., mating behavior, habitat choice; Ribbink et al. 1983; P. D. Danley and T. D. Kocher, unpubl. ms.) suggests that selection on behavioral, rather than morphological, characters is the primary force splitting species within this genera. Second, it identifies a paradox between migration rates and species distribution: the species with the highest migration rate (*M. sandaracinos*) is also the more geographically restricted. The observed distribution of *M. sandaracinos* may represent only a limited portion of a historically larger distribution, some populations of which may have been driven to extinction either due to changes in the habitat and/or competition.

Inferences Concerning Mbuna Diversification

Population bottlenecks/founder effects

The geologic history of Lake Malawi suggests that population bottlenecks and/or founder-flush processes could have played an important role in the diversification of Malawian cichlids (Owen et al. 1990). Geologic studies of the lake basin indicate that Lake Malawi, like other East African great lakes (Johnson et al. 1996), experiences frequent desiccation/inundation cycles that can change the lake level by more than 100 m (Owen et al. 1990). Changes in lake level impact the rocky habitats in particular, and populations that exist in these areas are prone to frequent extinction and recolonization. The low migration rate of the mbuna suggests that recently available habitats would be colonized by a limited number of founders that could then rapidly repopulate an area.

In contrast, genetic studies of mbuna suggest that species divergence rarely occurs via founder events or population bottlenecks. Although reduced genetic diversity has been observed in recently colonized and/or isolated population (e.g., Mazinzi Reef *M. zebra* and *M. benetos* populations), most species maintain high levels of genetic diversity at microsatellite loci (van Oppen et al. 1997; Arnegard et al. 1999; Markert et al. 1999; Table 1). The analysis of mitochondrial genes also fails to detect the widespread effects of founder events/population bottlenecks. Moran and Kornfield (1995) examined mtDNA haplotype diversity in four *Metriaclima* species. Three of the four species examined showed no reduction in genetic diversity. Furthermore, the retention of ancestral mtDNA polymorphisms (Moran and Kornfield 1995) strongly suggests that population bottlenecks/founder-

flush processes have not played a significant role in the diversification of the mbuna.

The observed genetic diversity of mbuna populations is interesting given the temporal instability of mbuna habitats. Although genetic diversity is expected to decrease because of frequent sampling events associated with multiple extinction/recolonization cycles, allelic diversity is not depressed in most mbuna species.

Genetic diversity within these species may be maintained by several means. Large, genetically diverse populations may have survived low-lake-level periods by migrating to sites currently located in deep water. Migrants from several of these deep-water refugia may have colonized the sites sampled in this study (Owen et al. 1990; Arnegard et al. 1999). Mutation may have also played a role in generating the large genetic diversity of our samples. However, mutation is expected to have played a minor role given the recent availability of the collection sites.

Founder effect speciation continues to generate considerable controversy. Theoretical predictions are often contradictory (Barton and Charlesworth 1984; Goodnight 1988; Wagner et al. 1994; Cheverud and Routman 1996; Barton 1998) as are conclusions from empirical studies (Powell 1978; Dodd and Powell 1985; Ringo et al. 1985; Moya et al. 1995; Bryant and Meffert 1996; Galiana et al. 1996). The results from the genetic analysis of mbuna populations, however, are clear. Founder effect speciation, if it occurs, is rare and likely has not played an important role in the diversification of the mbuna.

Divergence by drift

Although population bottlenecks severe enough to generate reproductive isolation have not been detected, several lines of evidence suggest that mbuna populations may be particularly susceptible to the action of genetic drift. The apparent sedentary nature of the mbuna contributes to fine-scale geographic isolation that may create many isolated populations (van Oppen et al. 1997). In some cases, these populations can be relatively small (e.g., *M. zebra* at Songwe Hill). The high variance in male reproductive success (McElroy and Kornield 1990) may futher reduce the effective population size (Hartl and Clark 1989). Some researchers have argued, however, that the polygynandrous mating system of the mbuna may inflate the effective population size. Multiple matings by both males and females each season may maintain a population's allelic diversity above what would be expected based on a census alone (Parker and Kornfield 1996).

The microsatellite data suggest that stochastic sampling events, particularly associated with the founding of populations and migration between populations, can lead to statistically significant differences in microsatellite allele frequencies (van Oppen et al. 1997; Arnegard et al. 1999; Markert et al. 1999). However, the estimated number of migrants between populations in each of these studies exceeds the one migrant every other generation needed to prevent populations from diverging purely by drift (Wright 1931; Slatkin 1985b).

However, drift may play a significant secondary role in the diversification process. Lande's (1981) model of runaway sexual selection, which has often been discussed with reference to the diversification of East African cichlids (Dominey 1984; McElroy and Kornfield 1990; McKaye et al. 1993; Stauffer et al. 1997), predicts that random changes in either the males' sexually selected trait or the females' mating preference can drastically influence the divergence of populations. More recently, Kondrashov and Kondrashov (1999) have suggested that transient linkage disequilibrium resulting from drift can drive species divergence even in the absence of a reduction in gene flow.

Microallopatric speciation

Previous analyses of the mbuna (including *M. zebra*) have detected statistically significant differences in microsatellite allele frequencies over 3 km (van Oppen et al. 1997). These authors conclude that mbuna populations are isolated over extremely limited geographic scales and that such populations could diverge via drift or selection. Our studies (Arnegard et al. 1999; Markert et al. 1999; this paper) suggest that population divergence does not occur on microgeographic scales due solely to drift. In all three studies, migration between adjacent sites exceeds one migrant every other generation. Furthermore, the number of migrants moving between the two end points of the transect (43 km) were estimated to be less than one migrant per generation in only one of the species (*M. auratus*, $Nm = 0.32$, Markert et al. 1999). These migration estimates are too high for divergence by drift on microgeographic scales. The depressed migration rates of the mbuna, however, may facilitate the diversification of populations that experience low levels of divergent selection.

Divergence with limited gene flow

Sympatric speciation models have been used to explain the diversification of Lake Malawi's cichlids (McKaye et al. 1984; Turner and Burrows 1995; Dieckmann and Doebelli 1999; Kondrashov and Kondrashov 1999). Simulations have shown that sympatric speciation can result from a variety of selective forces including sexual selection (Turner and Burrows 1995) and divergent selection on ecological characters (Dieckmann and Doebelli 1999; Kondrashov and Kondrashov 1999). Furthermore, there is clear evidence that sympatric speciation has occurred in other cichlid systems, most notably the cichlids of Cameroon's crater lakes (Schliewen et al. 1994).

However, models that incorporate the highly fragmented nature of the mbuna, such as Rice and Hostert's (1993) divergence with gene flow model, may be more compatible with the Lake Malawi system. Under this model, reproductive isolation develops via pleiotropy when populations experience strong, divergent selection on multiple characters. As long as selection is strong relative to gene flow, diversification will occur in the presence of considerable migration. Experimental evidence supports this model (Rice 1984; Rice and Salt 1990) and has indicated that prezygotic isolation will develop when habitats are discrete and different, when selection is strong and multifarious, and when bridging populations (populations existing in intermediate habitats and exhibiting intermediate phenotypes) are not expected. This model also suggests that a positive feedback loop can develop

when strong selection initiates a divergence process that reduces gene flow between populations. As gene flow is reduced, traits that could not initially differentiate due to the homogenizing effect of gene flow will diverge. With each iteration of this process, populations are freed to adapt in response to incrementally lower selection pressures.

Several hypotheses can be generated through the application of this model. One such hypothesis concerns the variation in species richness among mbuna genera. Some genera contain as few as one or two species (e.g., *Genyochromis* and *Labeotropheus,* respectively), whereas others may have more than 30 (e.g., *Metriaclima*). Rice and Hostert's (1993) model predicts that diversification is dependent on two factors: the level of gene flow and the strength of selection (Rice and Hostert 1993, fig. 2). Because estimated migration rates are similar among the mbuna (van oppen 1997; Arnegard et al. 1999; Markert et al. 1999; this paper), it seems unlikely that the variation in species richness has resulted from differential rates of gene flow. However, little is known concerning the strength of selection in natural populations. It is possible that species-rich genera, such as *Metriaclima,* experience higher levels of selection than species-poor genera. In situ comparative studies of selection pressures are needed to test this hypothesis.

Rice and Hostert's (1993) model also recognized sexual selection's ability to drive speciation. Diversification is expected to follow a hierarchical pattern in which sexual selection predominates after divergent natural selection reduces the impact of interspecific competition. Phylogenetic analysis of the mbuna supports this notion. Albertson et al. (1999) investigated the phylogenetic relationships of eight mbuna species. They found an early divergence of genera that have been distinguished on the basis of trophic morphology. The subsequent divergence of species within genera corresponded with the diversification of male secondary sexual characteristics (e.g., male color pattern).

Variation in sexual selection pressures among mbuna species is poorly understood. Future research is needed to quantify the strength of female mating preferences in the field, identify the male characters under selection, determine if the targets of female preferences are different among populations, and assess the mutability of male and female traits under sexual selection. The potential for natural selection to drive diversification in the rock-dwelling cichlids of Lake Malawi has been underappreciated in recent years. However, there remain many aspects of sexual selection that need to be quantified to characterize the selective forces acting to differentiate the mbuna.

Conclusion

Several conclusions can be drawn from this study. First, the impact of recent lake-level fluctuations can clearly be seen in the patterns of allelic diversity among collection locations. The samples collected at the most recently available sites possess reduced allelic diversity compared to samples drawn from deeper, older locations. The reduction in genetic diversity at these locations suggests that stochastic events, including population bottlenecks and drift, influence the patterns of allelic diversity. However, it is unlikely that these factors alone have contributed significantly to the process of species divergence. Second, reduced gene flow appears to be a general feature of the mbuna. The reduced gene flow of this group has most likely had a strong influence on their diversification by allowing minor changes in the selective environment to drive the divergence of populations. However, it is unclear what role the reduction in gene flow has played in generating species diversity within the mbuna. The data suggest that the more species-rich genera experience stronger selective pressures rather than reduced levels of gene flow. Additional work is needed to determine if and how selection pressures vary across populations and species.

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