Nuclear Gene Variation and Molecular Dating of the Cichlid Species Flock of Lake Malawi

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The cichlid fishes of Lake Malawi are famously diverse. However, evolutionary studies have been difficult because of their recent and uncertain phylogenetic history. Portions of 12 nuclear loci were sequenced in nine rock-dwelling species (mbuna) and three representatives of pelagic nonmbuna species. In contrast to the pattern of variation at mitochondrial genes, which do provide phylogenetic resolution at the level of mbuna versus nonmbuna, and among some genera, the nuclear loci were virtually devoid of phylogenetic signal. Only a small minority of variable positions were phylogenetically informative, and no phylogenetic branches are supported by more than one site. From the nuclear gene perspective the Malawian radiation appears to be a star phylogeny, as if the founding of the lake was accompanied by a partial bottleneck. The pattern is different from that found in Lake Victoria, in which nuclear loci share large amounts of ancestral variation. In the case of nuclear genes of Lake Malawi, the absence of phylogenetically informative variation suggests a relative absence of ancestral variation. Nuclear genes also differed from the mitochondria in having nearly twice the amount of divergence from *Oreochromis* (tilapia). An approximate splitting time between mbuna and nonmbuna lineages was estimated as 0.7 Myr. *Oreochromis* is estimated to have diverged from the cichlids in Lake Malawi and Lake Tanganyika about 18 MYA.

Introduction

The cichlid fishes (Teleostei: Cichlidae) of the East African Rift Valley lakes Malawi, Tanganyika, and Victoria and their satellites offer spectacular examples of rapid radiation (Fryer and Iles 1972; Echelle and Kornfield 1984). For Lake Malawi, over 600 species have been described (including formal and informal descriptions) (Konings 2001). The estimated age of the lake is 4–5 Myr, with periodical desiccations estimated to have occurred perhaps as recently as 570,000 years ago (Delvaux 1996). With the exception of a small number of tilappine species, all of the Malawi cichlids appear to form a monophyletic group (Moran, Kornfield, and Reinthal 1994; Kocher et al. 1995; Shaw et al. 2000; Sturmbauer et al. 2001; Seehausen et al. 2003; Terai et al. 2004) that makes up a large subset of the tribe Haplochromini (Eccles and Trewavas 1989; Salzburger et al. 2005).

All Malawi haplochromine types fall into one of the two widely used, albeit informal, taxa: the mbuna (from the local name for rock-dwelling types) and the nonmbuna. All the mbuna are similar small forms that dwell in rocky shallow regions of the lake. The nonmbuna include a variety of pelagic and benthic forms that live over sandy portions of the lake bottom. "Mbuna" is described by Eccles and Trewavas (1989, p. 10) as a local name first used in the literature by Fryer (Fryer 1959) (Fryer and Iles 1972) and which was adopted by aquarists and researchers. The mbuna include the genera numbered 2 through 10 of Trewavas (1935) in addition to Iodotropheus (Oliver and Loiselle 1972). Recently, one of the genera, *Pseudotropheus*, has been the subject of revision and discussion (Meyer and Foerster 1984; Konings 1988; Stauffer et al. 1997), so that depending on one's choice of taxonomy there may be 10 or 11 genera of mbuna.

A recurring and challenging quest in the study of Lake Malawi haplochromines has been the search for characters or genetic loci that can be used to help resolve the phylogenetic history of this radiation (Greenwood 1979; Moran and Kornfield 1993; Sueltmann and Mayer 1997; Albertson et al. 1999). Among mbuna, genetic variation has been showed to be shared, both among species and among genera, for several types of genetic markers, including allozymes (Kornfield 1978; McKaye et al. 1982, 1984), mitochondrial haplotype data (Moran and Kornfield 1993; Parker and Kornfield 1997), and microsatellite or short tandem repeat (STR) loci (Kornfield and Parker 1997) and nuclear DNA sequences (Hey et al. 2004). The pattern has largely been interpreted as caused by recent speciation and persistence of ancestral polymorphism (Moran and Kornfield 1993), as well as being caused by occasional gene exchange (Danley et al. 2000; Hey et al. 2004; Won et al. 2005).

To date the clearest phylogenetic portraits that have emerged come from studies on the mitochondrial genome, with studies done on restriction fragment length polymorphisms (Moran and Kornfield 1993; Moran, Kornfield, and Reinthal 1994), control region sequences (Bowers, Stauffer, and Kocher 1994; Meyer, Montero, and Spreinat 1996; Parker and Kornfield 1997; Nagl et al. 2000; Shaw et al. 2000; Sturmbauer et al. 2001; Salzburger et al. 2005), and sequences of the reduced form of nicotinamide adenine dinucleotide dehydrogenase subunit 2 (ND2) gene (Kocher et al. 1995; Shaw et al. 2000; Salzburger et al. 2005). The main points from these studies are that the mbuna are indeed closely related to one another, but that they do not quite form a monophyletic group, and that the genera *Rhamphochromis* and *Diplotaxodon* are basal groups within the Lake Malawi radiation. The mitochondrial data do not offer much resolution for resolving relationships within and among mbuna genera. However, success has been had on this front using large numbers of amplified fragment length polymorphisms (AFLPs) (Albertson et al. 1999).

Although a few studies on nuclear genes have included some representatives from Lake Malawi (Terai, Morikawa, and Okada 2002; Sugie et al. 2004; Terai et al. 2004; Sugawara et al. 2005), very little phylogenetic work

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has been done on Lake Malawi cichlids using nuclear DNA sequences. Here, we inquire of the patterns and levels of DNA sequence variation found among Malawi at nuclear genes. Our general goal was to see what the Malawi radiation looks like from the perspective of sequences from nuclear loci. In particular, we assess whether the pattern that has emerged from studies on the mtDNA also occurs in the nuclear genome.

Materials and Methods

Mitochondrial Sequences

There have been several studies on variation in the mitochondrial ND2 gene among African great lake cichlids (Kocher et al. 1995; Shaw et al. 2000; Salzburger et al. 2002, 2005; Duftner, Koblmuller, and Sturmbauer 2005; Schelly et al. 2005). For each represented species of mbuna or nonmbuna, one sequence was downloaded from GenBank (Supplementary Table 3, Supplementary Material online). The aligned partial sequences each had a length of 981 bp. For outgroups, we used sequences from the Lake Tanganyika species *Eretmodus cyanostictus* and from the tilapia *Oreochromis niloticus* (see below for discussion of outgroups).

Lake Malawi Species

Species of three genera of rock-dwelling (mbuna) cichlids, *Tropheops*, *Labeotropheus*, and *Melanochromis* were collected from the waters adjacent to the southern peninsula of Lake Malawi. These rock-dwelling cichlids include *Tropheops gracilior*, *Tropheops tropheops*, *Tropheops* sp. "broad mouth," *Labeotropheus fuelleborni*, *Labeotropheus trewavasae*, *Melanochromis auratus*, *Melanochromis vermivorus*, and *Melanochromis melanopterus*. Representatives of nonmbuna species, including *Lethrinops gossei*, *Rhamphochromis ferox*, and *Diplotaxodon greenwoodi*, were provided by J. Markert and M. Arnegard.

Outgroups and Dating

Lake Malawi is estimated to have last dried out somewhere between 0.57 and 1 MYA (Delvaux 1996). Because this date is recent and uncertain, we have used the origin of Lake Tanganyika together with a representative of the most basal cichlid lineage from that lake to root and date the radiation of Lake Malawi cichlids. The oldest parts of Lake Tanganyika arose 9–12 MYA (Cohen, Soreghan, and Scholz 1993), and Lake Tanganyika cichlids exhibit an ancient radiation, relative to those found in Lakes Malawi and Victoria. The most basal endemic group in Lake Tanganyika is the tribe Eretmodini (Kocher et al. 1995; Salzburger et al. 2002), and one representative of this small group, *E. cyanostictus*, was used as an outgroup. Given the uncertainties over the age and depth of the lake, we assumed that the age of this group falls somewhere between 5 and 10 MYA, with a median of 7.5 MYA for dating purposes.

For a more distant outgroup, we used a representative from the Tilapiini, a store-bought "red tilapia" which is of hybrid origin from both the Nile tilapia (*O. niloticus*) and the Mossambique tilapia (*Oreochromis mossambicus*). Both these species were included among 13 members of *Oreochromis* in an mtDNA study of tilapia-like cichlids (Klett and Meyer 2002). In that study, *Oreochromis* formed a well-supported monophyletic group (Klett and Meyer 2002).

Selected Loci

Twelve different genomic regions were used in the study. Three regions are from protein-coding genes, and nine are noncoding regions that flank STRs and which were accessible by the polymerase chain reaction (PCR) because of previous sequencing of the STR and its flanking regions in mbuna species.

The noncoding loci were obtained using the inverse PCR protocol (Ochman et al. 1989) as previously described (Hey et al. 2004). Locus-specific primer sequences were developed to sequence the flanking region (Supplementary Table 2, Supplementary Material online). The GenBank accession numbers for these sequences are DQ231254–DQ231333.

Portions of three protein-coding loci involved in pigmentation in African cichlid fishes (Sugie et al. 2004) were also included: *Aim1* (Fukamachi, Shimada, and Shima 2001), endothelin receptor b1 (*ednrb1*) (Parichy et al. 2000), and *mitfb* (Lister et al. 1999; Lister, Close, and Raible 2001). For each locus, reported cDNA sequences from a Lake Malawi cichlid were mapped onto the genome of *Takifugu rubripes* to identify putative intron/exon boundaries. Primer pairs were designed so as to amplify both exon and intronic sequences. In the case of *Aim1*, the predicted gene region, based on the *Takifugu* genome assembly, contained a putative intron that turned out not to be present when the region was amplified from genomic DNA in the Malawi cichlids in this study. Consequently, the *Aim1* sequences reported here contain a single open reading frame and do not appear to contain intronic sequence. For both *ednrb1* and *mitfb*, amino acid sequences translated from the exon portion corroborated the reported exon/intron boundaries. The GenBank accession numbers for these loci are DQ239797–DQ239828.

Phylogenetic Analysis

Estimates of phylogenetic trees were generated under maximum parsimony using PAUP* v. 4.0 (Swofford 2002). For these analyses, all positions that were heterozygous within a species' sequence were coded as polymorphisms in the input file. Phylogenetic estimates were also generated using pairwise distances using the Tamura-Nei three-parameter method (Tamura and Nei 1993) as well as other methods. Distance tree estimates were generated under the minimum evolution criterion (Rzhetsky and Nei 1993) as implemented in the program MEGA 3.0 (Kumar, Tamura, and Nei 2004).

To test whether relative branch lengths are similar across loci, we used the relative ratio test of Muse and Gaut (1997) as implemented in the program HyPhy (Pond, Frost, and Muse 2005). These are likelihood ratio tests (LRTs) of the hypothesis that branch lengths for two trees generated for different data from a common set of taxa have the same relative lengths. Branch lengths were estimated for these

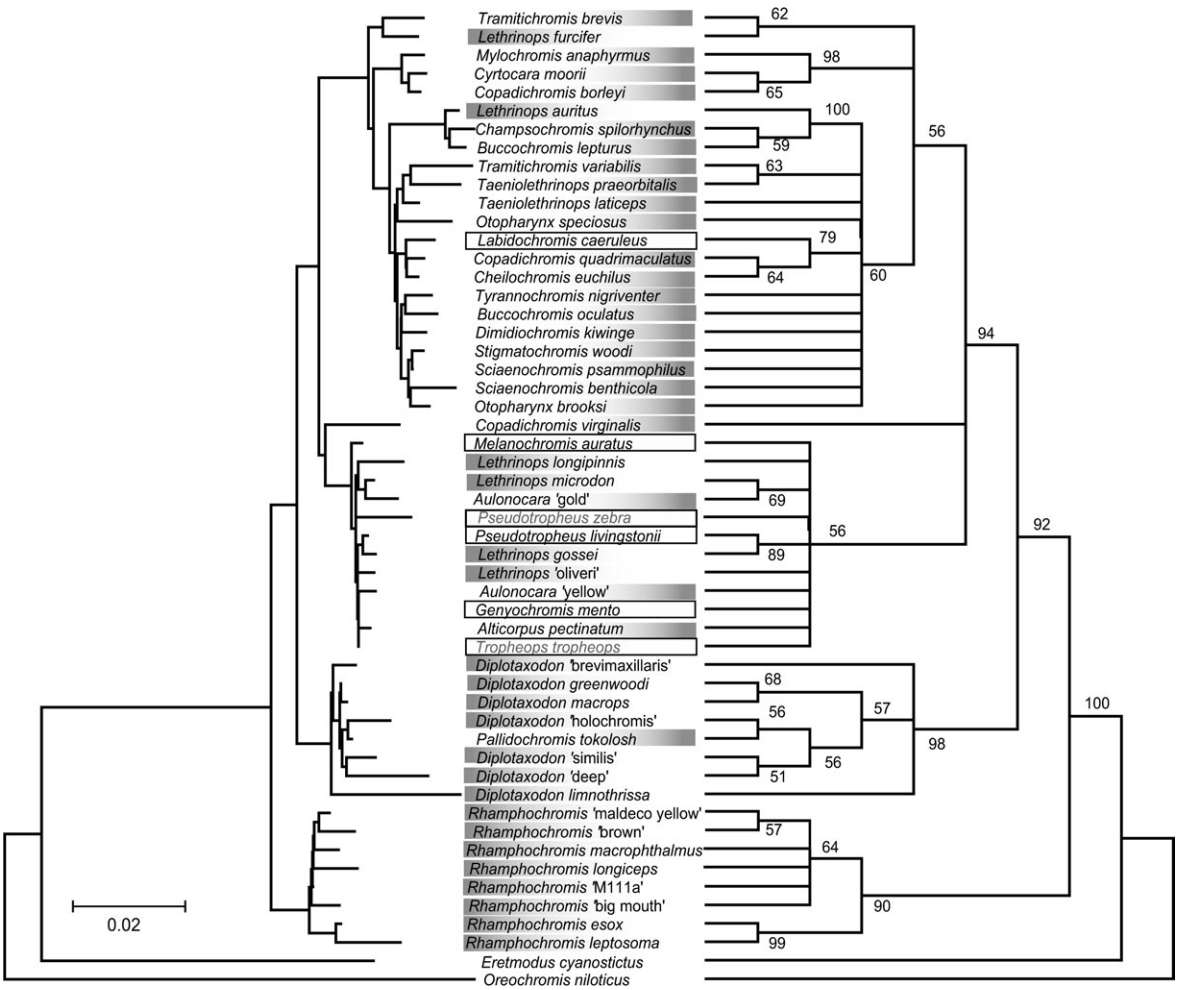

FIG. 1.—Phylogenetic tree estimates for ND2. On the left is shown the estimated minimum evolution tree (Rzhetsky and Nei 1993) based on distances calculated using the Tamura-Nei three-parameter model (Tamura and Nei 1993). On the right is shown a majority rule consensus of 1,000 bootstrapped trees constructed the same way. Mbuna species are shown in boxes. Representatives from genera that are also represented in the nuclear gene studies are shown with shading from dark to light (left to right). Representatives from other genera are shown with shading from light to dark.

tests under the Hasegawa-Kishino-Yano (HKY) mutation model (Hasegawa, Kishino, and Yano 1985).

Results

Mitochondrial DNA

In order to establish a useful benchmark for contrasting nuclear gene variation with mitochondrial variation, a single database sequence for ND2 was used for each of as many species of Malawi cichlids as were available. The ND2 gene was selected because it has been sequenced in a large number of species and because the amount of homoplasy evident in the pattern of variation is far less than appears in sequences from the control region of the mitochondria. Figure 1 shows a distance tree, constructed under the minimum evolution criterion (Rzhetsky and Nei 1993) as implemented in the program MEGA 3.0 (Kumar, Tamura, and Nei 2004), together with a majority rule consensus tree of 1,000 bootstrap replicates. In general, there seems to be good resolution for the deeper nodes of the tree. Very similar trees were found using maximum parsimony, and the parsimony bootstrap consensus tree had all of the same deeper branches with similar levels of support as the minimum evolution bootstrap consensus in figure 1. Additional control region data and ND2 data (Shaw et al. 2000) revealed the main exceptions from mbuna monophyly that are shown in figure 1. Most mbuna (shown in boxed text in figure 1) cluster together as well as with sequences from the genus *Aulonocara* and *Lethrinops*, while one mbuna sequence, *Labidochromis caeruleus* (as well as other *Lethrinops* sequences), cluster with other nonmbuna sequences.

Previous phylogenetic studies using mitochondrial DNA suggested that sequences from the genus *Rhamphochromis* were basal in the mitochondrial gene tree from Malawi (Meyer 1993; Moran, Kornfield, and Reinthal 1994; Meyer, Montero, and Spreinat 1996), or that the genera *Rhamphochromis* and *Diplotaxodon* were each others' sister groups, and that together they formed a group that is sister to the remainder of Malawi cichlids (Shaw et al. 2000; Salzburger et al. 2005). A nearly basal position for *Rhamphochromis* was also suggested by AFLP markers (Seehausen et al. 2003), although the most basal taxon (among Malawi cichlids) in that study was *Copadichromis virginalis*.

Table 1
Patterns of Variation Among Malawi cichlids

Locus	Length	Number of Variable Sites[a]	OD1[b]	OD2[c]	Sites Fixed Between mbuna Genera	Sites Shared Among mbuna Genera	Sites Fixed Between mbuna and Nonmbuna	Sites Shared Among mbuna and Nonmbuna
AIM1	263	2	0.024	NA	0	1	0	0
DXTUCA3	419	2	0.0103	0.0320	0	0	0	0
EDNRB1	773	8	0.0123	NA	0	1	0	0
MITFB	723	6	0.0230	0.0407	0	0	0	0
Ppun7	556	4	0.005	NA	0	0	0	0
PZMSAT2	642	3	NA	NA	0	0	0	0
U14396	442	2	0.0326	0.0653	0	0	0	0
U66814	633	4	0.0143	0.0319	0	2	0	0
U66815	200	3	0.0068	0.0303	0	0	0	0
UNH001	357	2	0.0159	0.0728	0	1	0	0
UNH130	374	7	0.0117	0.0399	0	0	0	2[d]
UNH143-II	594	5	0.0174	NA	0	1[e]	0	0
Sum	5976	48	0.0186	0.0432	0	6	0	2

[a] Variable sites are the number of base positions in which more than one base was observed among the Malawi sequences.

[b] OD1 is the mean number of differences, per base pair, between Malawi sequences and the outgroup *Eretmodus*. ''NA'' indicates that sequence could not be obtained from *Eretmodus*.

[c] OD2 is the mean number of differences, per base pair, between Malawi sequences and the outgroup *Oreochromis*. ''NA'' indicates that the sequence could not be obtained from *Oreochromis*.

[d] One of the shared sites is an indel at position 142.

[e] This shared position is an indel at position 523.

The ND2 trees do not show the pattern, reported on the basis of both control region and ND2, in which *Rhamphochromis* and *Diplotaxodon* together formed a sister group to the other Malawi cichlids. Rather, the ND2 data clearly suggest a position for *Rhamphochromis* as a basal group, with *Diplotaxodon* (including a *Pallidochromis* sequence) forming the next basal group. This pattern did not change when trees where generated using other outgroup sequences from Lake Tanganyika (not shown). The most parsimonious trees had a length of 505 steps, whereas those that were constrained for monophyly of the *Rhamphochromis*, *Diplotaxodon*, and *Palidochromis* lineages had a minimum length of 528 steps. The difference was highly significant, with a Wilcoxon signed ranks test statistic of 4.27 ($P < 0.0001$) (Templeton 1983) and the "winning-sites" test (Prager and Wilson 1988) ($P < 0.0001$) as implemented in PAUP* 4.0.

If we assume that the divergence between *Eretmodus* and the Malawi cichlids began 7.5 MYA, then the estimated rate of divergence at ND2 is 0.008 changes per site per million years. Assuming a linearized tree, this rate places the base of the Malawi clade at just over 2 MYA, a date that is somewhat older than what might be expected if the radiation began within the past million years. A similar calculation for the base of the tree places the divergence of *Oreochromis* at 9.4 MYA, which is in accord with a previous estimate of this date based on ND2 data (Kocher et al. 1995). These rates and estimated time points change very little when alternative distance measures that correct for multiple hits in different ways are used.

It is possible that the 7.5 MYA figure is too large by a factor of two or more. If so, then the actual base of the Malawi clade for mitochondria would be closer to 1 MYA. Probably, a more likely explanation is that the current mtDNA tree includes considerable depth that has persisted and that was already present in the founding population of the Malawi clade.

Nuclear Genes

We examined DNA sequence variation across nearly 6 kbp from 12 loci (table 1), with the large majority of the sequence being noncoding (either intron or intergenic). A total of 48 variable base positions were found among eight mbuna and three nonmbuna cichlids from Lake Malawi. The variable base positions are shown in figure 2, together with two variable indel positions that were not associated with repeats (and which could be unambiguously aligned). Most individuals were heterozygous at one or more positions (as indicated by ambiguity codes in figure 2), and in many cases the heterozygous bases were also represented as differences between other sequences in the panel.

Visual inspection of figure 2 reveals a fairly striking observation in that there are few variable positions that seem to offer phylogenetic signal. For example, on the basis of the mtDNA, it might be expected that some sites would reveal an apparently derived base (i.e., different from the outgroup) that is limited to the mbuna sequences. Not one site reveals a base that is fixed or even nearly fixed among the mbuna. Similarly, on the basis of the mtDNA, which suggests that *Rhamphochromis* is basal within Malawi cichlids, it might be expected that some sites would support a branch between the pair of *Rhamphochromis* and *Eretmodus* and the remaining mbuna and nonmbuna sequences. One such position is found (gene 10 in figure 2) offering weak nuclear gene support for this mtDNA pattern. In the majority rule consensus of all most parsimonious trees, there are only two branches: one consisting of the mbuna species *M. auratus*, *M. melanopterus*, *T. gracilior*, and *Tropheops* broad mouth and the other consisting of the mbuna species *L. fuelleborni* and *T. tropheops*. Not included in any groups are the other mbuna species, *L. trewavasae* and *M. vermivorus*, and all three of the nonmbuna, *L. gossei*, *R. ferox*, and *D. greenwoodi*. In effect, the nuclear sequence data offer

Locus #	1	2	3	4	5	6	7	8	9	10	11	12
Base	2	1	11233367	45666	15	35	24	335		22	111233	1445
Position	80	55	19323500	122889	144	208	82	8082	445	33	1134836	34792
	23	41	90197245	009895	864	080	50	1082	568	12	2792940	28923
Consensus	AG	GG	CCGCCTGT	AATCCG	AGC	ACA	AA	CTGA	CGC	TA	CCADATC	CCTCI
Melanochromis auratus	-C	--	-----C--	NNNNNN	---	---	--	----	-A-	A-	--CC---	-----
Melanochromis vermivorus	--	--	Y-------	------	---	G--	C-	A---	---	--	--MC---	-----
Melanochromis melanopterus	-S	--	-----C--	------	---	---	--	----	--Y	A-	-----C-	-----
Labeotropheus fuelleborni	-S	--	NNNNNNNN	NNNNNN	---	---	--	-C--	---	--	----G--	TT--D
Labeotropheus trewavasae	--	-C	-Y---C--	---AA-	G--	---	--	-C--	---	--	-------	YY---
Tropheops tropheops	--	R-	--------	-----A	---	---	--	-Y--	---	--	AG-----	--CTD
Tropheops gracilior	-C	R-	-----Y--	------	---	--R	--	AY--	---	A-	------T	-----
Tropheops 'Broadmouth'	NN	--	--K-YY--	------	---	---	--	----	M--	A-	-------	-----
Lethrinops gossei	T-	--	------R-	-RK---	-A-	-G-	--	---W	---	--	-------	NNNNN
Diplotaxodon greenwoodi	--	--	--------	G----D	---	---	-G	--A-	---	--	--CT---	-----
Rhamphochromis ferox	--	--	---T---C	NNNNNN	--T	NNN	--	----	---	-T	-------	-----
Eretmodus cyanostictus	--	--	------A-	------	---	NNN	--	-CA-	---	-T	---C---	-----

FIG. 2.—Variable base positions among Malawi cichlids. Ambiguity codes are shown to represent the two bases observed in heterozygous individuals. The outgroup *Eretmodus* is also shown for those positions that were variable among the Malawi cichlids. Only one phylogenetically informative indel that could be unambiguously aligned was observed. It is indicated as I and D in gene 12, the insertion form of the sequence was "TGTGT." The position highlighted by a circle was heterozygous for an indel that spanned the variable base position. A string of **N**'s for a given gene indicates cases where PCR primers did not amplify and sequence could not be obtained.

only very weak support for relatedness among some mbuna species.

Among the 48 variable positions found among Malawi cichlids, only 10 were parsimony informative (i.e., the less frequent base occurs at least twice), and all of these correspond to different partitions among the operational taxonomic units (OTUs). Six of these sites were found to be shared among mbuna genera, and two sites in the locus UNH130 were found to be shared between mbuna and nonmbuna. Surprisingly, none of the variable positions were found to be fixed in any type of comparisons (fig. 2). The absence of consistent patterns and fixed nucleotide differences among groups suggests that the nuclear loci are essentially devoid of phylogenetic signal among the species examined.

A DNA distance tree constructed for the concatenated nuclear gene data using the minimum evolution criterion as for ND2 (Rzhetsky and Nei 1993) is shown in figure 3. The branch that separates the outgroups from the Malawi cichlids was supported with 100% bootstraps. The branch that separates *Rhamphochromis* and the outgroups from the remaining Malawi cichlids had weak bootstrap support (54%). No other branches had more than 20% bootstrap support. If it is assumed that the divergence between *Eretmodus* and the Malawi cichlids began 7.5 MYA, the substitution rate is 1.1×10^{-9} changes per base per year. A linearized tree places the base of the Malawi clade at 0.70 MYA, which is about one-third of that estimated for ND2.

The same calculation for the base of the tree finds an estimated divergence time for *Oreochromis* of 17.7 MYA, which is nearly double that found for ND2. The ND2 sequences came from *O. niloticus*, whereas the nuclear gene sequences are from the commercial red tilapia which is a hybrid of *O. niloticus* and *O. mossambicus*. Given that the genus *Oreochromis* appears to be monophyletic, on the basis of mtDNA sequences from 13 species (Klett and Meyer 2002), the fact that a species hybrid was used for the nuclear gene sequence probably does not explain the different degrees of divergence observed between mtDNA and nuclear genes.

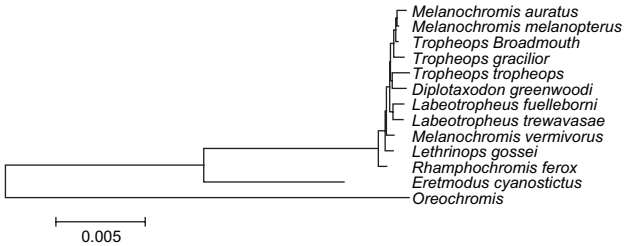

FIG. 3.—Phylogenetic tree estimates for concatenated nuclear loci constructed using the minimum evolution criterion (Rzhetsky and Nei 1993).

Relative Ratio Tests

The relative branch lengths in figures 1 and 3 give the appearance that the Malawi radiation is older from the perspective of the mitochondrial ND2 gene than it is from the perspective of the nuclear loci (i.e., assuming that the time of separation of the Malawi cichlids from the outgroup *Eretmodus* is the same for both groups of genes). To test for a disparity in relative branch lengths, we used the relative ratio test of Muse and Gaut (1997) with simple three-taxon trees that included a representative mbuna (*M. auratus*) and a representative of the possibly basal genus *Rhamphochromis*, as well as the outgroup *Eretmodus*. This comparison is highlighted in the top panel of figure 4, which also shows the estimated dates for the nuclear loci and the mtDNA assuming a 7.5 MYA date for ancestry with *Eretmodus*. For this test, the nuclear loci were concatenated and treated as a single locus to compare with the ND2 data. The HKY substitution model (Hasegawa, Kishino, and Yano 1985) was applied globally for each data set (i.e., a single transition/transversion ratio was determined for each data set).

The LRT of the hypothesis that the relative branch lengths from the outgroup *Eretmodus* to the ingroup (the two Malawi taxa) are the same in the two data sets yielded a test statistic (two times the likelihood ratio or 2LR) of 4.061 which is statistically significant for the number of degrees of freedom ($P = 0.04389$, 1 df). In effect the apparent disparity in relative branch lengths receives modest support from this test. If the separation of *Eretmodus* was really at the same time for both nuclear and mitochondrial genes, then the base of the mitochondrial tree for the

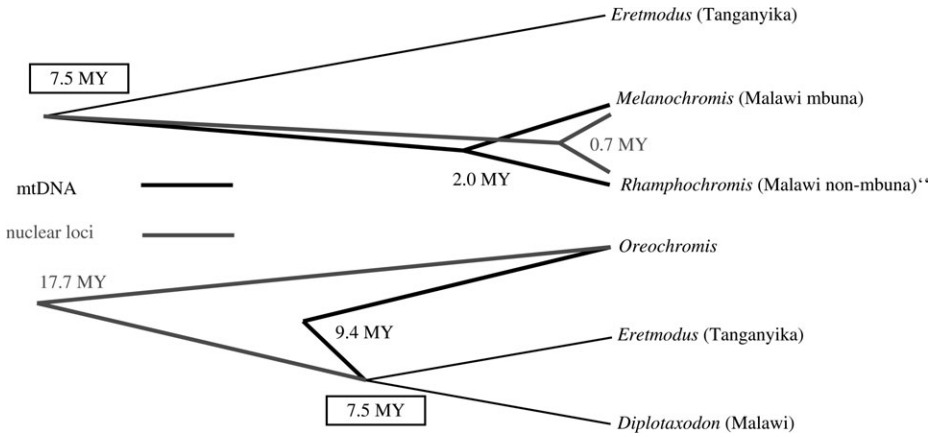

FIG. 4.—Relative branch lengths for nuclear genes (gray) and mtDNA (black), based on the assumption that *Eretmodus* from Lake Tanganyika and Malawi cichlids last shared a common ancestor 7.5 MYA. The top panel shows the estimated time depth for divergence within Lake Malawi between representative mbuna and nonmbuna, based on linearized branch length estimates of the minimum evolution trees. The lower panel shows the differences in the estimated time depth for common ancestry with *Oreochromis*.

Malawi radiation (represented by *M. auratus* and *Rhamphochromis*) is deeper than it is in the case of the nuclear tree.

Another apparent disparity in relative branch lengths concerns the position of *Oreochromis*. The ND2 analysis suggests a divergence date a little less than 10 MYA, which is consistent with other reports and summaries based on mitochondrial data (Kocher et al. 1995; Salzburger and Meyer 2004). However, the nuclear data show a greater relative divergence of *Oreochromis* (table 1 and figs. 3 and 4) consistent with a divergence date of about 18 MYA. Evidence from fossils is not particularly helpful. A morphological analysis using cichlid fossils suggested that the divergence of tilapiine (which includes *Oreochromis*) and haplochromine cichlids was older than the 45 MYA date of the fossils described in the study (Murray 2001), a history that is not suggested by any molecular data (Albertson et al. 1999; Klett and Meyer 2002; Sugie et al. 2004; Salzburger et al. 2005). It also does not appear to be the case that the relative divergence between *Oreochromis* and Lake Malawi cichlids varies greatly among the nuclear loci, relative to the divergence between *Eretmodus* and the Malawi cichlids. From table 1, which shows the mean divergence per base pair for these comparisons, the *Oreochromis* divergence varies from 1.8 times (in the case of *mitfb*) to 4.6 times (in the case of UNH001) the value for *Eretmodus* (values obtained by dividing OD2 by OD1 in table 1). The ratio of the weighted average across loci is 2.3. In contrast, the relative divergence for *Oreochromis* in the case of ND2 is only 1.3 times that found for *Eretmodus* (fig. 1).

A relative ratio test between the ND2 data and concatenated nuclear loci was conducted using a Malawi cichlid, *D. greenwoodii*, in addition to *Eretmodus* and *Oreochromis*. This comparison is highlighted in the lower panel of figure 4, which also highlights the estimated dates for *Oreochromis* coancestry, assuming a 7.5 MYA date for *Eretmodus*. Assuming the HKY model for both loci and a global transition/transversion ratio for each of the data sets, the test was highly significant (2LR = 16.425, $P = 5.0613 \times 10^{-5}$, 1 df). To test for variation among the nuclear loci in the relative branch length from *Oreochromis* to the pairing of *Eretmodus* and *Diplotaxodon*, a LRT was done in which a model that constrained the *Oreochromis* branch to be a constant multiple of the branch lengths of *Eretmodus* and *Diplotaxodon* was compared to one without that constraint. The test for the seven loci for which sequence data could be obtained for all three taxa was not significant (2LR = 8.08843, $P = 0.2317$, 6 df). This is consistent with a relatively constant position of *Oreochromis* to *Eretmodus* and the Malawi cichlids, across nuclear loci.

It is possible that the mitochondrial ND2 tree, which is based on protein-coding sequence, is different from the nuclear loci because of natural selection. This was tested by estimating the branch lengths for a simple tree (again using *M. auratus*, *Rhamphochromis*, and *Eretmodus*) and estimating the synonymous and nonsynonymous branch lengths under the codon substitution model of Muse and Gaut (1994). The likelihood of this unconstrained tree was then compared to that for a model in which the nonsynonymous branch lengths were all a constant multiple of the corresponding synonymous branch lengths using the HyPhy program. The test statistic did not approach statistical significance (2LR = 0.819123, $P = 0.66394$, 2 df).

Discussion

In contrast with studies based on the mitochondrial genome, we found essentially zero phylogenetic resolution among 12 nuclear regions. This is not simply because the data set is small. Not only do we not find phylogenetic signal among the mbuna, which are a particularly closely related group on the basis of mtDNA and AFLP studies and which have long been known to share genetic variation (Moran and Kornfield 1993), but also we find no consistent pattern between mbuna and nonmbuna species or among nonmbuna species. If a phylogenetic tree interpretation is demanded of the data, then it appears as if the 11 species and six genera of Malawi cichlids form a star phylogeny. When a distance approach is used, weak support is found for *Rhamphochromis*, forming a basal connection to the rest of the Malawi cichlids (fig. 3).

Previous studies of Lake Malawi cichlids using the mitochondrial genome had suggested that members of the informal mbuna taxon formed a closely related group that, with some exceptions, was close to being monophyletic. This pattern was also found in the analysis of published ND2 gene sequences (fig. 1). These sequences also yield strong support for a basal position of the nonmbuna genus *Rhamphochromis*, among the *Malawi* cichlids, which is consistent with some previous work (Meyer 1993; Moran, Kornfield, and Reinthal 1994; Meyer, Montero, and Spreinat 1996) but is partly in conflict with some reports that had placed *Rhamphochromis* together with the genus *Diplotaxodon* as the basal group in the Malawi radiation (Shaw et al. 2000; Salzburger et al. 2005). These two genera are the only ones among those that have multiple species represented in the study that appeared to be monophyletic or nearly so.

The pattern of variation found among Malawi cichlids at nuclear loci is similar in some respects to that observed at nuclear loci among haplochromine cichlids from Lake Victoria. Nagl et al. (1998) studied four randomly selected nuclear loci and discovered that variation was widely shared among 12 endemic Lake Victoria species as well as among some closely related riverine species. Something like this is observed among the mbuna of Lake Malawi, which share mitochondrial (Moran and Kornfield 1993) and microsatellite variation (Van Oppen et al. 2000; Won et al. 2005) as well as nuclear locus variation (table 1). Nagl et al. (1998) explained their observation of shared variation in Lake Victoria by recent colonization of the lake by a large number of riverine founders. In the case of the Malawi mbuna, the relative roles of recent shared ancestry and gene flow, as causes of shared variation, still remain to be resolved, though speciation was clearly quite recent in some cases (Won et al. 2005).

Like Lake Malawi, Lake Victoria has had a history of desiccation, possibly as recently as 15,000 years ago (Johnson et al. 1996; Sturmbauer et al. 2001). However, a study of the mitochondrial control region in a large number of species suggests that the major radiation dates to 100,000 years ago (Verheyen et al. 2003). This same analysis also suggested that present day mitochondrial variation descended from two mitochondrial lineages that were present among the founders of the Lake Victoria flock. This is consistent with the report from nuclear genes that showed widespread shared variation between species from Lake Victoria and related riverine species (Nagl et al. 1998).

The estimated time depth of the tree for the mitochondrial ND2 gene among Malawi cichlids (near 2 MYA—based on a linearized tree, see *Results*) also suggests that multiple mtDNA lineages persist since the founding of the Malawi flock, which may have occurred within the past 1 Myr since the last desiccation of the lake (Delvaux 1996). However, the nuclear gene variation among Malawi cichlids does not suggest the presence of multiple lineages that predate the founding of the lake. If this had occurred, then we would expect that some variation would persist and appear as phylogenetically informative sites among the nonmbuna and mbuna genera. Rather, the paucity of informative sites is suggestive of a small founding population for the Malawi species flock (i.e., a bottleneck) that radiated rapidly into different groups.

It is important to emphasize that the presence of just a small number of phylogenetically informative sites cannot simply be explained by gene exchange or by persistence of ancestral variation. If ancestral variation had persisted in multiple lineages of the Malawi radiation, then this would be expected to appear as phylogenetically informative sites. This is true even if there was widespread gene exchange among the lineages early in the radiations, as has been suggested. Gene exchange could well have occurred and contributed to the radiation (Seehausen 2004). Hybridization would also be expected to lead to a lack of phylogenetic resolution, but it would not necessarily remove parsimony-informative variation at individual base positions. In order for hybridization and gene flow across species to contribute to the loss of old informative variation, those processes would have had to occur at a rate sufficient that genetic drift within and among diverging species lineages could lead to the loss of polymorphisms from the entire species flock.

Dating Discrepancies

We have focused on the time point of coancestry for *E. cyanostictus* because this species is a member of the oldest extant lineage of cichlids that are endemic to Lake Tanganyika and is therefore likely to have an age that approaches the age of this lake. If this date is assumed to be shared by both nuclear and mitochondrial loci, then relative ratio tests indicate, with a test statistic that is on the margin of statistical significance, that the Malawi radiation as viewed from the mitochondrial ND2 gene is deeper than the same radiation as viewed from the nuclear loci. The greater depth of the mitochondrial tree is not in the direction that would be expected on the basis of population genetic considerations. Genes of sex-limited inheritance are expected to have a coalescent time roughly one-quarter of diploid autosomal genes (assuming an even sex ratio and equal variance in reproductive success by the two sexes), so the expected time depth of the mitochondrial tree, at each node that joins separate taxa, is less than for autosomal loci by a factor that is a function of the effective population size of the ancestral species at that node. Consideration of the coalescent time depth of different genes also raises the point that the normal stochastic variance among genes can be quite large (Hudson and Coyne 2002; Hudson and Turelli 2003). The only variance in the relative ratio test is that which arises under the mutational model that is assumed. If coalescent variance were also included in the test, then it is probable that the difference between nuclear and mitochondrial tree depths would not be statistically significant.

The nuclear and mitochondrial trees differ even more for the apparent depth of the node that joins *Oreochromis* to the other taxa in the study. Nuclear genes suggest a time depth of approximately 18 MYA, which is nearly double the estimate for the mitochondria. Also the nuclear loci were consistent with each other in showing a greater divergence for *Oreochromis* than does the mitochondria. The fact that the nuclear sequences came from a hybrid of two *Oreochromis* species probably does not account for this difference as mitochondrial-based phylogenetic studies have shown *Oreochromis* to be a monophyletic genus

(Klett and Meyer 2002). The time point discrepancy important for the general understanding of the radiation of tilapiine cichlids in Africa.

Given the apparent relative time discrepancies, we must consider the possibility that the time point of divergence of *Eretmodus* and the lineage that led to the Malawi radiation may not be shared by the nuclear and mitochondrial genes. For example, it is possible that this node is actually much older for the mitochondrial tree than for the nuclear tree. For instance, the ND2 tree includes considerable depth that has persisted beyond the node and that was already present in the founding population of the Malawi clade. This is a valid interpretation of the apparent discrepancies around the dating of the *Oreochromis* divergence. However, if this were the case, it would also have strong implications for the timing of the base of the Malawi radiation. If the time point of *Eretmodus* on the ND2 tree is actually much older, then so would the apparent age of the Malawian radiation on the ND2 tree be. If the time point of the *Eretmodus* on the nuclear gene tree is actually much younger, then so would be the Malawian radiation for nuclear genes. Given that this interpretation generates even larger discrepancies and that the nuclear gene tree time depth for the Malawi radiation (0.7 MYA) roughly corresponds to the estimated time since the last desiccation of the lake (Delvaux 1996), it seems simpler to consider the divergence time point between *Eretmodus* and the Malawian lineage as being shared by the two types of loci. It is also important to keep in mind the current uncertainty over dates when the lake might not have been habitable for fish. For example, it is possible that Lake Malawi did not in fact dry out within the past 1 Myr. Preliminary results of a scientific drilling suggest that the lake has not dried out in the past 1.5 Myr (Scholz et al. 2005).

The finding that the relative divergence between *Eretmodus*, *Oreochromis*, and the Malawi cichlids is roughly consistent across the nuclear loci suggests that the cause of the discrepancy may lie with the mtDNA and that for some reason the mtDNA history has not accurately tracked the phylogenetic history of these species. However, this suggestion is tentative at this stage, and a better understanding of the discrepancy must await additional data from more nuclear loci, and particularly for more genera of Malawi cichlids.

Supplementary Material

Supplementary Tables 2 and 3 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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