Hybridization versus Randomly-Sorting Ancestral Alleles: Genetic Variation in Lake Malawi Cichlids

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CONTENTS:

1. ABSTRACT

Hybridization is believed to be an important mechanism for genetic diversity and speciation in many groups of organisms. One of the most important evolutionary signatures of hybridization is low phylogenetic resolution in rapidly evolving and closely related species. However, low phylogenetic resolution may also be caused by randomly-sorting ancestral alleles. The cichlid fishes of Lake Malawi, Africa, are a commonly studied model of rapid speciation and have mosaic genomes characteristic of young and diverse groups of species. Because of reproductively viable hybrid offspring used in laboratory studies as well as observed populations of hybrid origin in the lake, hybridization is believed to be the major source of the mosaic genomes of Lake Malawi Cichlids. To test this hypothesis, DNA was isolated from individuals of mbuna (rock-dwelling) and non-mbuna cichlid species from Lake Malawi, including individuals from various populations of two mbuna species *Metriaclima zebra* and *Labeotropheus fuelleborni*. The mitochondrial gene ND2 and the nuclear gene DLX2 were amplified using PCR technique and were sequenced. Sequences were aligned and then used to build phylogenetic neighbor-joining distance trees. This study is the first to examine genetic variation among a wide range of species as well as populations of two potentially hybridizing species from the lake in order to examine the relative roles of hybridization and ancestral polymorphisms in the mosaic genomes of Lake Malawi cichlids.

2. INTRODUCTION

2.a The mbuna of Lake Malawi

Lake Malawi is located in southeast Africa and is one of the east African rift lakes (Figure 1). It is world-renowned for its cichlid fishes. The Lake Malawi cichlids are very diverse and have experienced rapid speciation; with between 700 and 1000 species evolving in

lake's short history of less than one million years, the Lake Malawi cichlids are one of the most diverse group of vertebrates on earth (Owen et al. 1990, Turner et al. 2001). At least 300 of these species belong to the rock dwelling fish of the lake called the mbuna – one of the most frequently studied group of fishes in the world (Konings 2001). The rocky habitats in which the mbuna are predominantly found are often separated by stretches of sandy bottom or open water creating isolated populations throughout the lake (Genner and Turner 2005).

2.b Speciation of the mbuna

Speciation of organisms can occur in one of two ways: allopatrically or sympatrically. The mechanisms of speciation and diversity among mbuna are not fully understood. Some degree of allopatric speciation among the mbuna has almost certainly occurred. Lake level fluctuations are an integral part of the natural history of Lake Malawi, and some parts of the lake including the southern basin were dry less than 200 years ago (Owen et al. 1990, Sturmbauer et al. 2001). Such fluctuations are believed to have aided in some degree of allopatric speciation among cichlids within the lake via isolating populations with little to no gene flow (Kornfield 1978, Albertson et al. 2003a, Schon and Martens 2004). But many species have arisen without any obvious habitat barriers and may be attributed to either adaptive processes via competition or nonadaptive processes via sexual selection (Albertson et al. 1999).

Sympatric speciation occurs without obvious geographic barriers and is also believed to be an important source of genetic variation of Lake Malawi cichlids (Kornfield and Smith 2000, Shaw et al. 2000, Danley and Kocher 2001). Sympatric speciation via sexual selection of color morphology is believed to play an important role in mbuna speciation (Panhuis et al. 2001). Mate choice by color morphology in the mbuna creates a pre-zygotic reproductive barrier and

can lead to speciation. Mbuna species are sexually dimorphic with territorial males usually having brighter colors than females, and it has been suggested that the bright colors typical of males have evolved as a result of sexual selection (Seehausen et al. 1999, Genner and Turner 2005).

2.c The mosaic genomes of Lake Malawi cichlids

The cichlid fishes of Lake Malawi have mosaic genomes, which are characteristic of young and diverse groups of species. Mosaic genomes are characterized by populations with high phenotypic diversity and low genotypic diversity. This is the case in Lake Malawi cichlids. Species throughout the lake range tremendously in size, color morphology, jaw structure, and even tooth shape (Liem 1973, Streelman et al. 2004). However, phylogenetic analyses of Lake Malawi cichlids show very low resolution of relationships between species; often different genes tell different stories of the relationships of these fish (Figure 2) (Hulsey et al. 2007). Mosaic genomes are often attributed either to hybridization or retention of ancestral polymorphisms.

2.d Hybridization and genetic diversity in Lake Malawi cichlids

i. Hybridization

It is unclear whether the mosaic genomes of Lake Malawi cichlids are the result of hybridization or ancestral polymorphisms. Recent studies suggest hybridization may have played a major role in the genetic diversity of Lake Malawi cichlids and could be the source of the poor phylogenetic signal found in these fish (Smith et al. 2003, Seehausen 2004, Streelman et al. 2004, Albertson and Kocher 2005). In the event of sympatric speciation, gene flow is not completely restricted – especially when the mechanism of reproductive isolation is pre-zygotic –

and hybridization between species may occur. Until the last decade, observed instances of natural hybridization were extremely rare and were not thought to occur frequently (Albertson et al. 1999). However, recent studies show that introgression, or increased genetic diversity via hybridization, may be an important part of the speciation and maintenance of diversity of Malawi cichlids (Kornfield et al. 2003, Smith et al. 2003, Seehausen 2004, Streelman et al. 2004).

If viable hybrid offspring from parents of two different species fit some resource niche independent of either parent species, the potential for speciation via hybridization is present (Seehausen 2004). In habitats such as Lake Malawi which are characterized by distinct niche partitioning (Ribbink et al. 1983) and rapid speciation (Kornfield 1978), introgression has gained support as an important and perhaps prominent mechanism of diversity in Lake Malawi. Though some studies have now shown that hybridization and introgression occur in Lake Malawi, the rate of occurrence and the importance of hybridization in the genetic variation of Malawi cichlids are not well understood.

ii. Two examples of hybridization in Lake Malawi

Hypotheses of the hybrid origin of some mbuna populations were originally based on evidence from morphological traits coupled with geographic locations relative to parent populations (Stauffer et al. 1996); hybridization now has substantial genetic and observational evidence as well. Smith *et al.*'s 2003 study examines a population of *Metriaclima sp.* at Makanjila Point hypothesized to be hybrid with parent groups *Metriaclima zebra* at Choifu Bay, geographically north of Makanjila Point, and *Metriaclima thapsinogen* at Eccles Reef, geographically southeast of Makanjila Point. The Choifu population has blue dorsal fins, and the Eccles Reef population has red dorsal fins; the dorsal fins of the Makanjila population are an

intermediate blue and red coloration. Smith *et al.* tested genotype probability of both potential parent populations, individuals from the Makanjila population, and simulated hybrids. The data support the theory that the Makanjila population is hybrid in origin and provided some evidence of alleles unique to the hybrid population. Such hybridization events may help explain the maintenance of high levels of genetic variation within populations as well as the origin of new species in the lake (Smith et al. 2003).

Robust genetic evidence to support hypothesized hybridization between the introduced species *Cynotilapia afra* and the native species *M. zebra* at Thumbi West Island (Stauffer et al. 1996) was provided by Streelman *et al*. in 2004. The introduction of *C. afra* at Thumbi West Island is a well-documented species introduction that took place in the 1960's (Munthali and Ribbink 1998). Throughout the early 1980's *C. afra* individuals were confined to the point of introduction on the southeast point of the island (Ribbink et al. 1983). In 1991, the dorsal fins of *C. afra* were observed with blue barring, suggesting hybridization with *M. zebra* (Stauffer et al. 1996). Genetic analysis of individuals from locations around Thumbi West Island showed that many individuals which appeared to be hybrids exhibited a mixture of *C. afra* and *M. zebra* genomes, providing phenotypic and genetic evidence for hybridization. The *C. afra* genome has much greater contributions to individual genomes from the northern part of the island, whereas individuals from the southern part of the island exhibited a mixture of *C. afra* and *M. zebra* (Streelman et al. 2004).

The populations at Thumbi West Island allow for the direct observation of habitat colonization, contact between previously isolated populations, hybridization, selection with gene flow, and divergence of genes and phenotypes – all important parts of cichlid evolution (Kornfield and Smith 2000). The Thumbi West Island study not only provides further evidence

for the occurrence of hybridization in Lake Malawi but also sheds light on the potential effects of human interaction with native populations. While studies of currently evolving populations offer a unique and important way to understand how the evolution of such a diverse group of organisms has occurred and roles of hybridization, questions regarding the frequency and importance of hybridization remain unanswered.

2.e Hybridization versus ancestral polymorphism retention in Lake Malawi cichlids

While studies now definitively show that hybridization occurs in Lake Malawi, the extent of hybridization and the importance of its contribution to the low resolution of phylogenies of the lake's species are not known. A popular argument to date is that low resolution in phylogenetic trees among phenotypically diverse populations is a result of extensive hybridization (Seehausen 2004). But the low-resolution phylogenies consistently produced by genetic analysis of Malawi cichlids may also be the result of ancestral polymorphisms that have not yet sorted. Because Malawi cichlids have evolved within the last one million years, there may not have been sufficient time for ancestral alleles to sort among populations.

The purpose of this study will be to examine genetic variation in two genes, DLX2 and ND2, within two common mbuna species, *Metriaclima zebra* and *Labeotropheus fuelleborni*. *M. zebra* and *L. fuelleborni* individuals have been crossed in laboratory experiments and produce viable hybrid offspring (Albertson et al. 2003b). The resemblance of laboratory hybrids of these two species to some Lake Malawi *Tropheops* species suggests that hybridization of these two species may occur in the wild (Albertson and Kocher 2005). The extent to which variation in DLX2 and ND2 in these two species may be attributed to hybrid versus ancestral origin will be examined through observed patterns in existing variation.

This study addresses the following questions:

1) How much variation exists in both ND2 and DLX2 within mbuna and non-mbuna species in Lake Malawi, and what is the level of variation of ND2 and DLX2 within *M. zebra* and *L. fuelleborni* individuals?

2) How much of total variation in ND2 and DLX2 is also found in *M. zebra* and *L. fuelleborni*?

3) Are any polymorphisms found in ND2 and DLX2 in *M. zebra* and *L. fuelleborni* unique, i.e. seen in no other species in the lake?

This study will answer some of the questions surrounding whether the mosaic genomes of Lake Malawi cichlids are a result of novel mutations via hybridization or ancestral polymorphisms. A survey of individuals from ten locations throughout the lake will also be useful in its depth and breadth; it may help us better understand the rates at which polymorphic sites occur within Malawi cichlids simply by providing us with a larger number of individuals to examine than previous studies allowed. These data may be used in future studies to compare intraspecific as well as interspecific mutation rates for *M. zebra* and *L. fuelleborni* with mutation rates of other African cichlids or even more ancient cichlid lineages to understand mutation rates on a larger scale.

3. MATERIALS AND METHODS

3.a DNA Isolation and Sequencing

To examine whether genetic variation is attributed to novel alleles from hybridization or randomly-assorted ancestral polymorphisms, the ND2 and DLX2 genes of *M. zebra* and *L. fuelleborni* were combined with sequence from several species previously analyzed in other

studies (Hulsey et al. 2007). *M. zebra* and *L. fuelleborni* individuals were collected from 12 locations throughout the southern end of Lake Malawi (Figure 3). All other species were collected from various locations in the lake.

DLX2 is a 0.949kb nuclear developmental gene (Panganiban and Rubenstein 2002). The sequences used in this study contain three exons and two introns. As a nuclear gene, DLX2 is inherited bi-parentally; ND2, a mitochondrial gene, is inherited maternally. ND2 is a 1.049kb mitochondrial coding gene often used in phylogenetic studies of cichlids. Because mitochondrial and nuclear genes have different modes of inheritance, phylogenetic analyses of these genes may show different relationships among the Lake Malawi cichlids.

Total genomic DNA was isolated for sequencing from axial muscle at the University of New Hampshire. A DNA template for the polymerase chain reaction (PCR) was provided using a 1 µl aliquot of this solution. The entire ND2 gene was PCR amplified using primers from Kocher et al. (1995). The DLX2 gene was amplified using the same technique. Amplifications were carried out in a Perkin-Elmer DNA thermocycler. The PCR reaction volume was 15µl [16µl of H_2O, 2.5µl 10X $MgCl_2$ PCR buffer, 1.25 µl $MgCl_2$, 2.0µl dNTPs (10mM), 1.25µl of each primer (10µM), 0.25 µl of TAQ, and 1 µl DNA (~15-20ng)]. Thermal cycling conditions consisted of an initial denaturation step of 94 ºC (45 min), 52 C (45 sec), and 72 ºC (2 min 30 sec). A final incubation of 72 ºC for 4 min was added to ensure complete extension of amplified products. The 1.047 kb ND2 and 0.949 kb DLX2 PCR products were separated from unincorporated primers and dNTPs using electrophoresis in low melting point agarose gel run in Tris-acetate buffer (pH 7.8). Ethidium bromide (1.5mg/µl) was added to the gels for visualization. Positively amplified DNA was then purified using an enzymatic combination of 1

μl of Exonuclease I (10.0 U/μl) and 1 μl shrimp alkaline phosphatase (2.0 U/μl) per 10μl of PCR.

Treated PCR products were used as templates for sequencing reactions (Applied Biosystems terminator cycle sequencing reactions). Sequences were read at the Automated DNA Sequencing Facility at the University of Washington. Sequencher version 4.1 (Gene Codes, Ann Arbor, MI) was then used to assemble the complete gene sequences. Sequences were aligned for analysis using Clustal X (Thompson et al. 1999), and codon positions were defined using MacClade 4.0 (Madison and Madison 2000).

3.b Phylogenetic Analysis

Phylogenetic neighbor-joining distance trees were built for DLX2 and ND2 using PAUP* 4.0b10 (Swofford 2002). *Rhamphochromis esox* was used as an outgroup for both trees. DLX2 sequences were analyzed as contigs rather than individual alleles, and for this reason some sequences were not used due to gaps of unreadable sequence created by multiple indels. A total of 285 individuals were included in the DLX2 tree, and 212 individuals were included in the ND2 tree.

4. RESULTS

4.a Polymorphism frequency in M. zebra and L. fuelleborni

Total percent variation in *L. fuelleborni* and *M. zebra* haplotypes in ND2 and DLX2 are shown in Table 1. Total variation in DLX2 was 0.11% among *M. zebra* individuals and 0.15% among *L. fuelleborni* individuals. The total variation in ND2 was on average lower than DLX2 with 0.12% for *M. zebra* individuals and 0.04% for *L. fuelleborni*. The percent variation of *M.*

zebra and *L. fuelleborni* haplotypes most divergent from the common mbuna haplotype are also shown in Table 1 (the most divergent haplotypes are marked in Figures 5 and 6). The most divergent *M. zebra* and *L. fuelleborni* haplotypes in ND2 differed from the most common mbuna haplotype by 0.29%. In DLX2, the most divergent *M. zebra* haplotype differed from the common haplotype by 0.84%, and the most divergent *L. fuelleborni* haplotype differed from the common haplotype by 0.42%.

4.b DLX2 phylogenetic tree

The DLX2 phylogenetic neighbor-joining tree (Figure 5) indicated a common haplotype shared by 81.1% of all individuals. Fourteen haplotypes were found among the 18.9% of individuals differing from the common haplotype. No novel haplotypes shared mbuna and non-mbuna species; 7 haplotypes were specific to non-mbuna species, and 7 were specific to mbuna species. One of the haplotypes specific to mbuna species was *M. zebra*-specific, and 3 were *L. fuelleborni*-specific. One haplotype was specific to *M. zebra* and included individuals from 3 separate locations (2 Eccles Reef, 2 Mazinzi Reef, and 1 West Reef). Three haplotypes were specific to *L. fuelleborni*; 2 contained only individuals from Domwe Island, and the other included individuals from 3 separate locations (2 Domwe Island, 1 Makanjila Point, 4 West Reef). One haplotype was shared by 4 *L. fuelleborni* individuals from 3 separate locations (1 Choifu Bay, 1 Otter Point, 2 Makanjila Point) as well as 9 other mbuna individuals (3 *Gengochromis mento*, 1 *Tropheops* red cheek, 1 *Tropheops* intermediate, 2 *Labeotropheus trewavasae*, 1 *Metriaclima patricki*, 1 *Metriaclima aurora*.)

4.c ND2 phylogenetic tree

The ND2 phylogenetic neighbor-joining distance tree shows distinct differences between mbuna and non-mbuna haplotypes (Figure 6). With the exception of 2 *Placidochromis milomo* individuals (Figure 8), no non-mbuna clustered with mbuna species. Similarly, no mbuna clustered with specific non-mbuna haplotypes. Four haplotypes were specific to *L. fuelleborni* species. Two *L. fuelleborni*-specific haplotypes contained only individuals from Chinyamwezi. One *L. fuelleborni*-specific haplotype was restricted to Eccles Reef individuals and included 80% of the individual sequenced from that location. The fourth *L. fuelleborni-specific* haplotype contained 4 individuals from Zimbawe Rock, 1 individual from Domwe Island, and 1 *L. trewavase* individual from Otter Point. All of the individuals from 6 out of the 10 populations of *L. fuelleborni* were found with the common mbuna haplotype.

M. zebra individuals were predominantly split between 2 major haplotypes (Figure 8). One haplotype was the most common mbuna haplotype (also shared with a majority of *L. fuelleborni* individuals*)*, and the other haplotype was also shared with other mbuna species as well as 2 individuals from the non-mbuna species *Placidochromis milomo*. Three novel *M. zebra*-specific haplotypes sorted by location and contained 2 individuals each (2 Zimbawe, 2 Masinge, and 2 Domwe Island). Nine of the 12 populations' individuals were found exclusively in 1 of the 2 major haplotypes.

5. DISCUSSION

5.a Polymorphism frequency in M. zebra and L. fuelleborni

Table 1 focuses on variation in both ND2 and DLX2 for *M. zebra* and *L. fuelleborni*. Total percent variation in each gene for both species is shown; the percent variation of the most

divergent haplotype from the common mbuna haplotype for *M. zebra* and *L. fuelleborni* is also shown (the most divergent haplotypes are marked in Figures 5 and 6). *M. zebra* total variation of ND2 was slightly greater than DLX2; *L. fuelleborni* individuals had much greater total variation in DLX2 than in ND2. The percent variation of the most divergent haplotype of each species from the common mbuna haplotype was greater for DLX2 than ND2.

5.b DLX2 phylogenetic tree

In the DLX2 tree (Figure 5), 231 out of 285 individuals of mbuna and non-mbuna species share a common haplotype. However, 54 individuals sorted into 14 novel haplotypes (Figure 5, inset). Non-mbuna and mbuna species shared none of the 14 novel alleles. *L. fuelleborni* and *M. zebra* individuals shared no novel haplotypes; of the three novel *L. fuelleborni* haplotypes, two were exclusive to individuals from Domwe Island. The third *L. fuelleborni*-specific and the *M. zebra*-specific haplotypes contained individuals from various populations, many of which were not geographic neighbors in the lake.

The DLX2 tree did not show any common novel haplotypes shared by *L. fuelleborni* and *M. zebra*. Thus, there were no common novel alleles of *L. fuelleborni* and *M. zebra* from the same or even geographically close populations. The relationships in the DLX2 tree provided no support for the hypothesis of common hybridization between *L. fuelleborni* and *M. zebra* individuals in Lake Malawi. The DLX2 tree instead provided support for the retention of ancestral polymorphisms as a source of the mosaic genomes of Lake Malawi cichlids. Sorting of haplotypes by non-mbuna and mbuna individuals would be expected, followed by sorting of haplotypes by species and then by populations given enough time. However, such sorting is not observed in the DLX2 tree. For example, *L. fuelleborni* individuals from Domwe Island sort

with other mbuna and even non-mbuna species for the common haplotype. Domwe Island individuals also sort with other mbuna species for a haplotype divergent from the common haplotype. Even further, *L. fuelleborni* individuals from Domwe Island also sort in three novel haplotypes specific to *L. fuelleborni* – two of which contain only individuals from Domwe Island. The same is true for *L. fuelleborni* individuals from Makanjila Point; individuals share the common mbuna/non-mbuna haplotype, an mbuna-specific haplotype with other mbuna species, and a *L. fuelleborni* haplotype with individuals from other locations. The divergence of haplotypes between mbuna and non-mbuna species, between specific mbuna species, and between populations of species support retention of ancestral polymorphisms as a cause of the mosaic genome of Lake Malawi cichlids.

5.c ND2 phylogenetic tree

In the ND2 tree, non-mbuna specific haplotypes showed high confidence (bootstrap values of 100) (Figure 6). The only two non-mbuna individuals to sort with the otherwise mbuna-specific haplotypes were the same species, *Placidochromis milomo*. Three *L. fuelleborni*-specific haplotypes showed geographic influence with novel haplotypes sorting strongly by location. The haplotype specific to Eccles Reef included 80% of individuals sequenced for that location, suggesting very close correlation with geography. Two of the novel haplotypes were specific to Chinyamwezi. The only *L. fuelleborni*-specific haplotype containing individuals from multiple locations included four individuals from Zimbawe Rock, one from Domwe Island, and one *L. trewavase* individual from Otter Point (a species that is likely a close relative of *L. fuelleborni*) (Figure 7). Domwe Island and Zimbawe Rock are geographically close (Figure 4.a), suggesting that geography may play a role in this haplotype even though it

included individuals from two locations. The sorting of *M. zebra* populations between the two major haplotypes also strongly suggests some geographic influence (Figure 8).

Despite the strong evidence for geographic sorting of alleles in the ND2 tree, the tree provided no evidence of hybridization between the two species, because no evidence for sharing of novel alleles was present. The second common *M. zebra* haplotype included all of the *M. zebra* individuals from Otter Point and West Reef, and a majority of the individuals form Domwe Island (80.0%), Makanjila Point (88.9%), and Thumbi West Island (60%). If hybridization between *M. zebra* and *L. fuelleborni* individuals was often occurring, we would expect to see some *L. fuelleborni* individuals sharing the second common *M. zebra* haplotype. Similarly, the novel *L. fuelleborni* haplotype at Eccles Reef is shared by 80% of *L. fuelleborni* individuals from that location but no *M. zebra* individuals.

6. CONCLUSIONS

The results from both the DLX2 and the ND2 tree do not support common hybridization between *L. fuelleborni* and *M. zebra* individuals in populations throughout southern Lake Malawi. The sorting of both *L. fuelleborni* and *M. zebra* populations suggests some geographic influence but it provides no evidence of hybridization between the two species as there was no evidence for sharing of novel alleles – nor allele sharing in space. Furthermore, the divergence of haplotypes among mbuna and non-mbuna groups, among mbuna species, and among specific populations of species provides support for retention of ancestral alleles as the source for the mosaic genomes of Lake Malawi cichlids.

Hybridization is known to occur in Lake Malawi (Smith et al. 2003, Streelman et al. 2004), and our data cannot rule out isolated hybridization events between *L. fuelleborni* and *M.*

zebra individuals. However, our data provide robust evidence that hybridization between these two species is not a common occurrence. Because both species in both trees show that a majority of individuals share a common haplotype with many other mbuna species, we do see evidence for ancestral polymorphisms. The lack of geographic sorting of individuals with novel haplotypes in the DLX2 tree provides even further support for retention of ancestral polymorphisms rather than hybridization as a source of genetic variation. Our data exclude recent hybridization as an explanation for shared polymorphisms and point instead toward the retention of ancestral polymorphisms.

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9. FIGURES

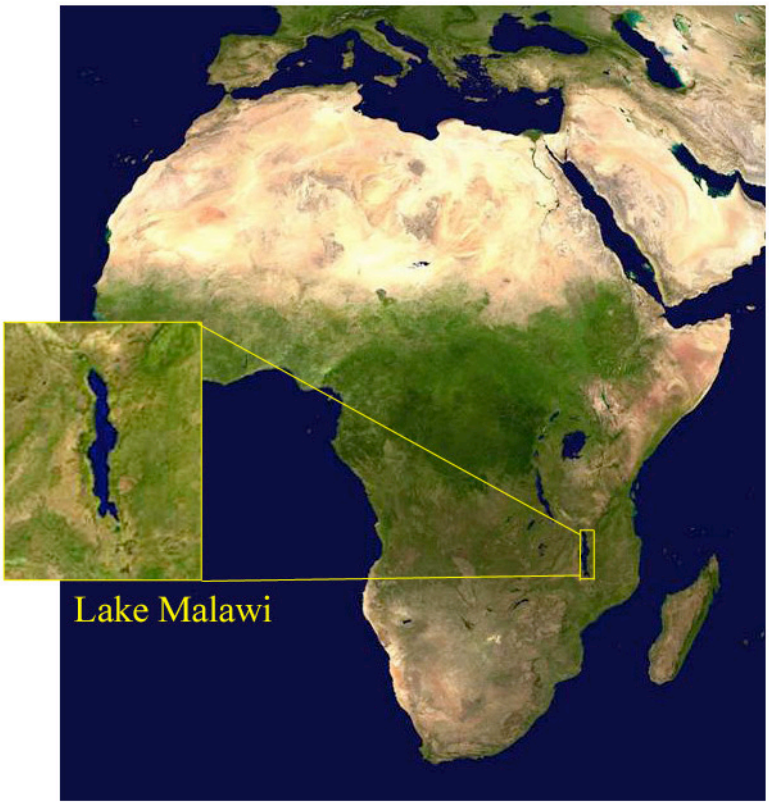
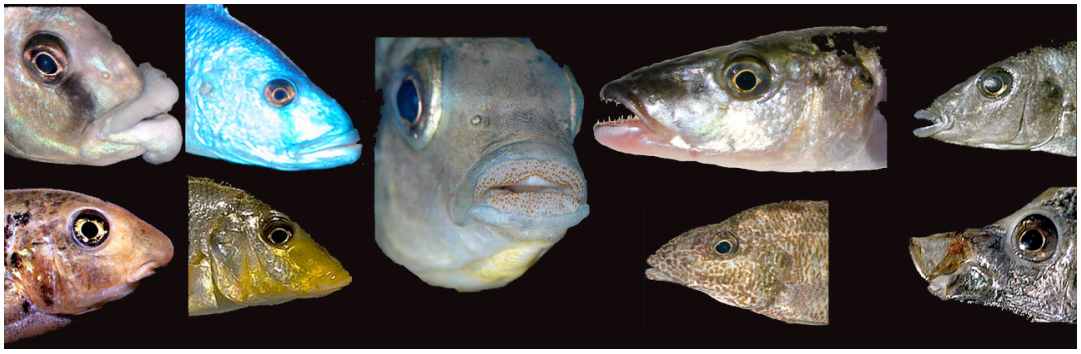


Figure 1. Lake Malawi, Lake Tanganyika, and Lake Victoria make up the East African Rift lakes. These lakes are the result of tectonic activity in southeast Africa. Lake Malawi is roughly 360 mile long, 50 miles wide, and over 1000 meters deep. It is home to the most diverse and rapidly evolving group of vertebrates in the world, the cichlid fish of Lake Malawi.

A)



B)

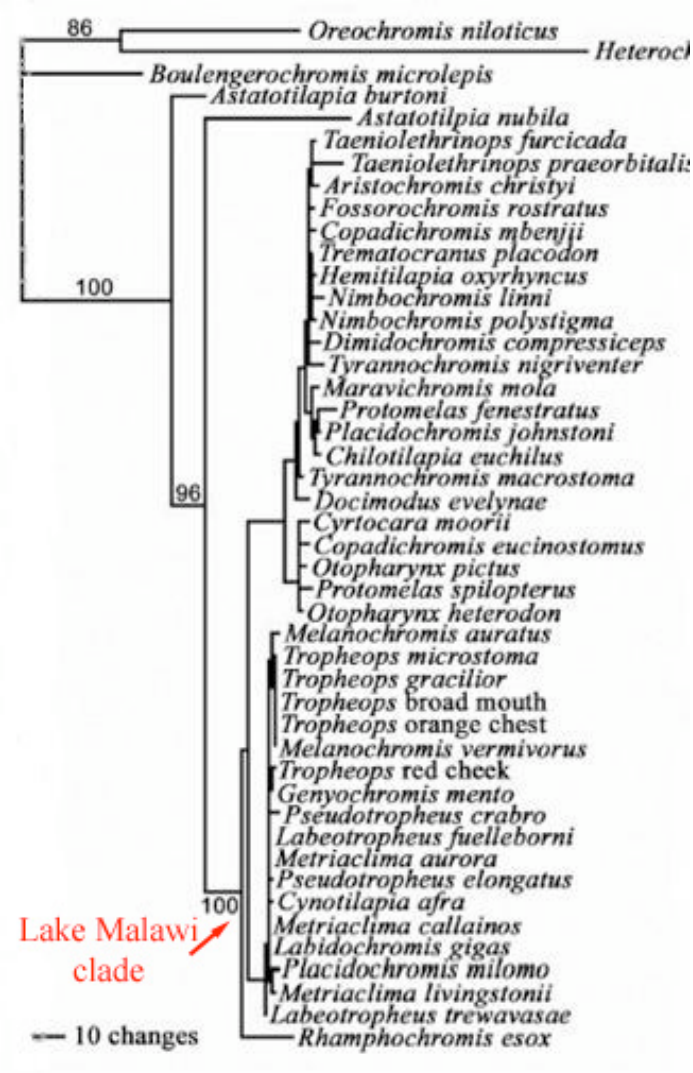
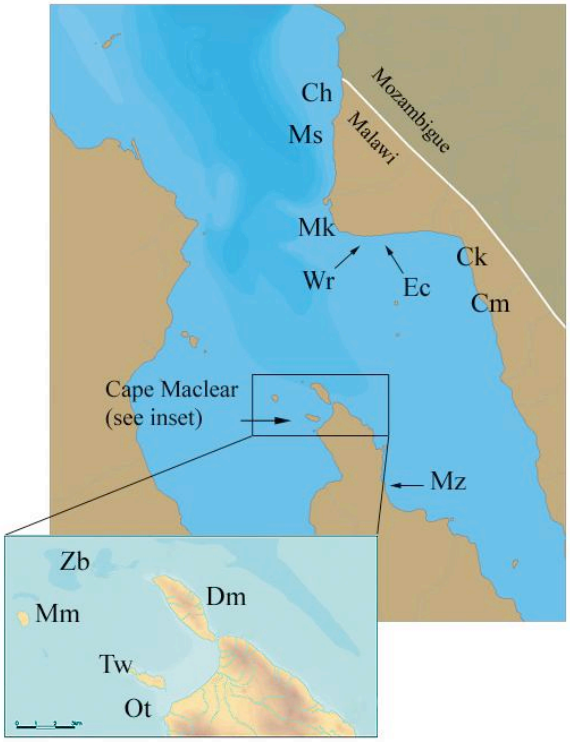


Figure 2. A) Lake Malawi cichlids are incredibly morphologically and trophically diverse, with variable color patterns, jaw structures, and tooth shapes. B) An ND2 phylogenetic tree showing little resolution of relationships of the species from the Lake Malawi clade (Hulsey et al. 2007). All individuals shown in A) are included in the ND2 phylogeny.

Figure 3. *Metriaclima zebra* (top) and *Labeotropheus fuelleborni* (bottom) are two mbuna species found throughout Lake Malawi. These two species have been crossed in laboratory studies and produce reproductively viable hybrids; they are also believed to hybridize in Lake Malawi. Individuals from populations of both of these two species were used in this study to examine the extent to which hybridization occurs between these species in Lake Malawi.

A)



B)

	Dlx2		ND2	
Location	**Mz**	**Lf**	**Mz**	**Lf**
Chinyamkwazi (Ck)	-	6	-	10
Chinyamwezi (Cm)	-	6	-	9
Choifu Bay (Ch)	16	6	6	10
Domwe Island (Dm)	10	8	10	9
Eccles Reef (Ec)	15	7	6	10
Makanjila Point (Mk)	6	6	9	10
Masinge (Ms)	8	-	9	-
Mazinzi Reef (Mz)	16	-	7	-
M. benetos (Mz)	7	-	7	-
Mumbo (Mm)	10	7	8	9
Otter Point (Ot)	8	2	8	5
Thumbi West Isl. (Tw)	11	-	5	-
West Reef (Wr)	10	9	6	8
Zimbawe Rock (Zb)	10	6	9	10

Figure 4. (A) 12 locations in southern Lake Malawi from which *Metriaclima zebra* and *Labeotropheus fuelleborni* individuals were collected. (B) The number of *M. zebra (Mz)* and *L. fuelleborni (Lf)* individuals for which sequences of DLX2 and ND2 were successfully obtained. A location where a species is not found is indicated with a dash (-).

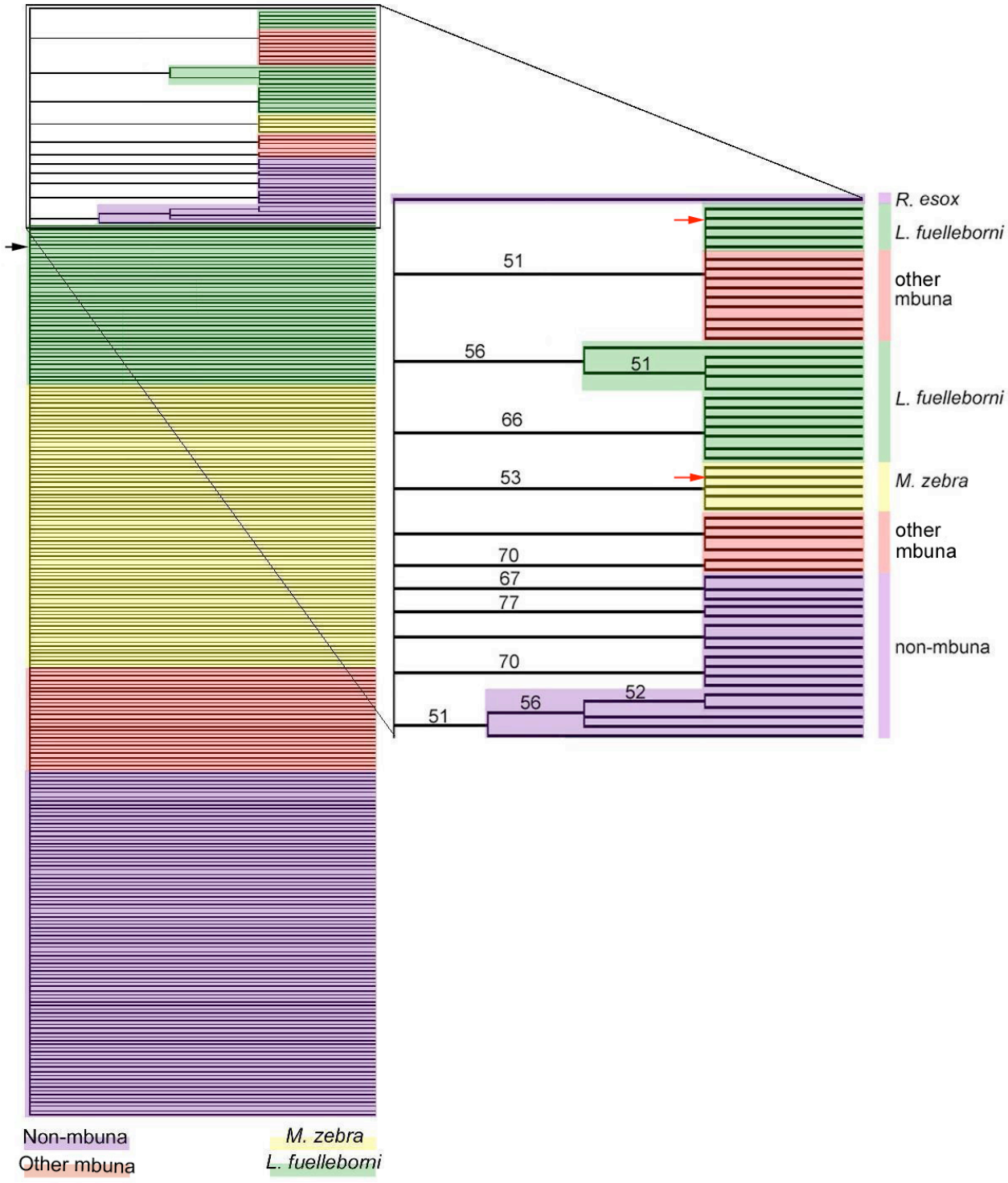


Figure 5. Dlx2 phylogenetic distance tree constructed using PAUP 4.0. *L. fuelleborni* sequences are shown in green, *M. zebra* in yellow, other mbuna in red, and non-mbuna in purple. Inset shows close-up of section of tree with individuals of novel haplotypes. Confidence shown with bootstrap values. Common haplotype marked with black arrow; most divergent haplotype of *M. zebra* and *L. fuelleborni* marked with red arrows.

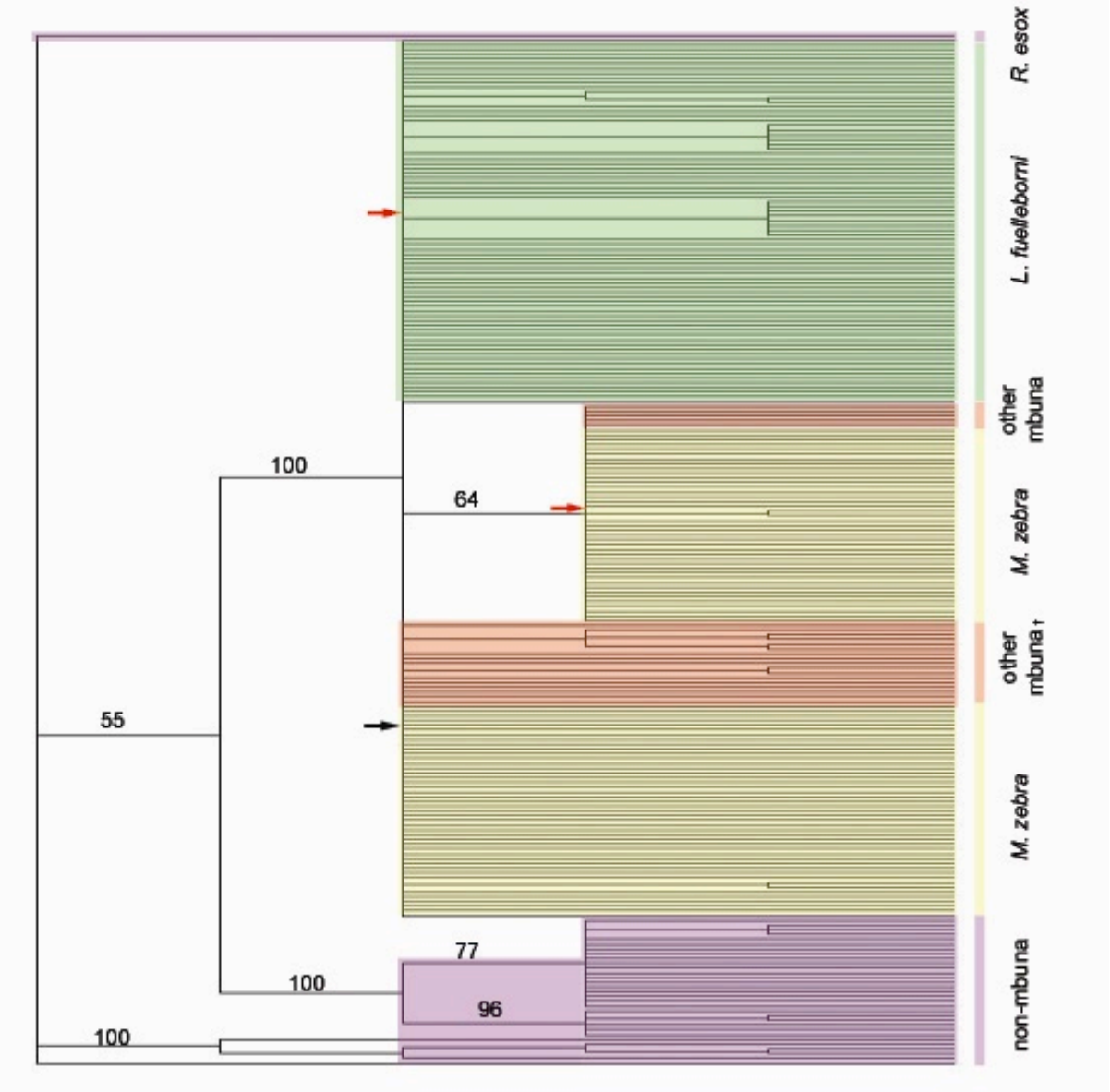


Figure 6. ND2 phylogenetic distance tree constructed using PAUP 4.0. *L. fuelleborni* sequences are shown in green, *M. zebra* in yellow, other mbuna in red, and non-mbuna in purple. Confidence shown with bootstrap values. †This group also contained 3 *M. zebra* individuals and 2 non-mbuna individuals. Common haplotype marked with black arrow; most divergent haplotype of *M. zebra* and *L. fuelleborni* marked with red arrows.

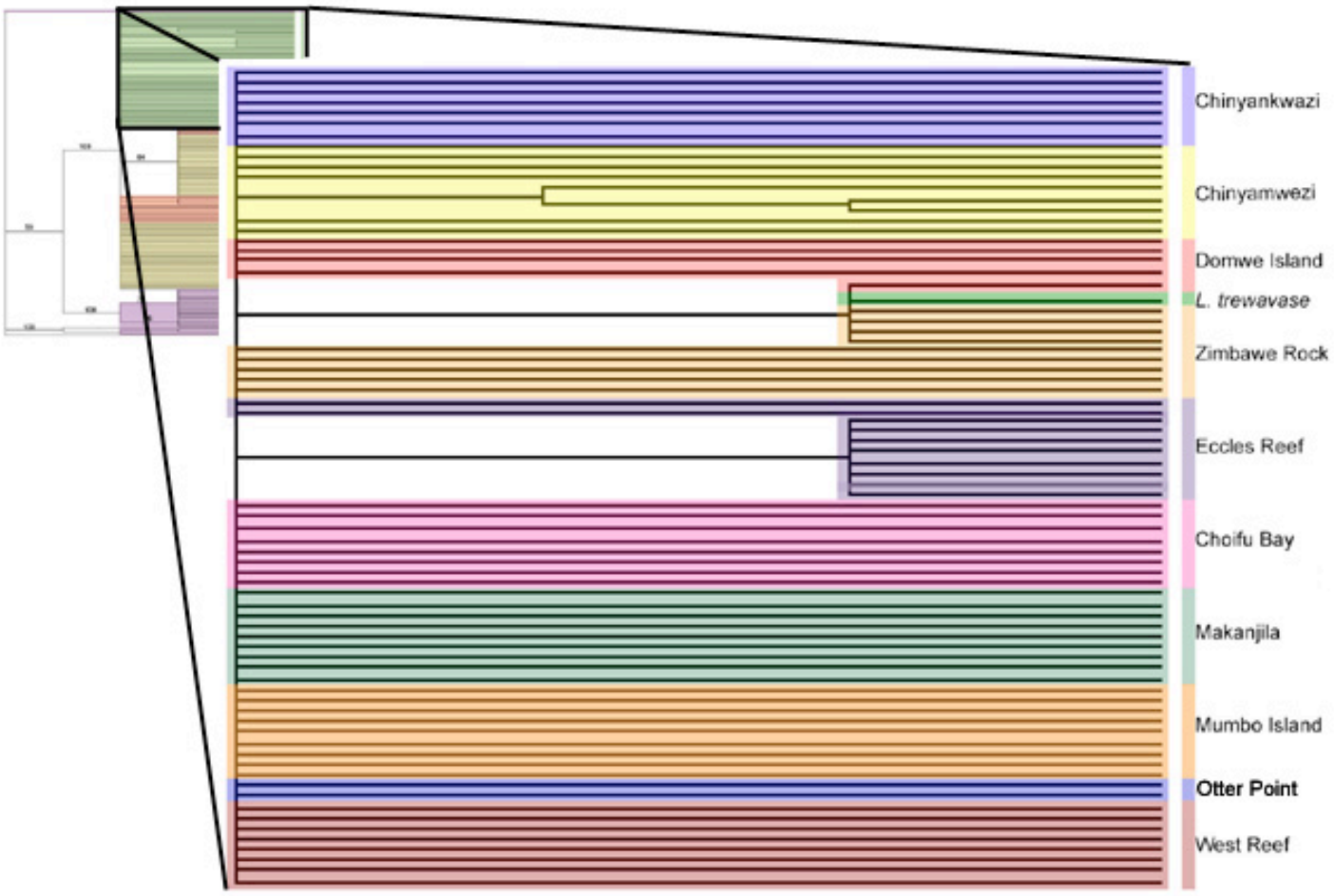


Figure 7. The inset figure shows the *L. fuelleborni* individuals from the ND2 phylogenetic distance tree. Populations are specified by color and labeled. *L. trewavase* individual collected from Otter Point.

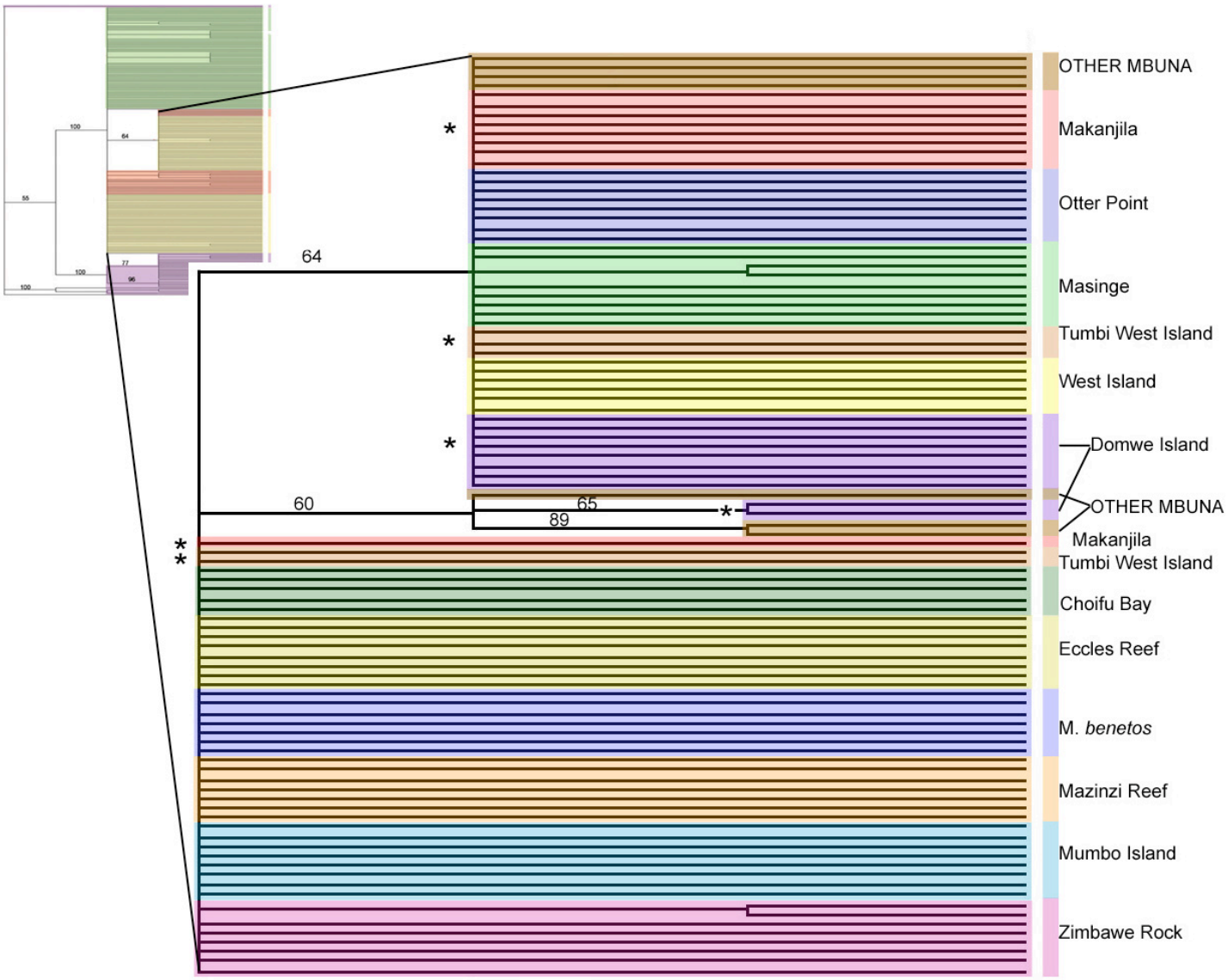


Figure 8. The inset figure shows the *M. zebra* individuals from the ND2 phylogenetic distance tree. Populations are specified by color and labeled. Stars (*) indicate populations that have individuals in both the major haplotypes. Values represent bootstrap confidence.

	Total variation in gene (%)		Diver. from common hap. (%)	
	M. zebra	***L. fuelleborni***	***M. zebra***	***L. fuelleborni***
ND2	0.12	0.04	0.29	0.29
DLX2	0.11	0.15	0.84	0.42

Table 1. Percent total variation in ND2 and DLX2 for both *M. zebra* and *L. fuelleborni*, and percent divergence from most common mbuna haplotype in ND2 and DLX2 for *M. zebra* and *L. fuelleborni*.