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Full Length Research Paper

Genetic diversity within the genus *Cynotilapia* and its phylogenetic position among Lake Malawi's mbuna cichlids

Daud Kassam[1]*, Shingo Seki[1] , Bosco Rusuwa[2], Aggrey J.D. Ambali [2] and Kosaku Yamaoka[1]

[1]Department of Aquaculture, Graduate School of Kuroshio Science, Kochi University, B 200 Monobe, Nankoku-shi, Kochi, 783-8502, Japan.
[2]Biology Department, Chancellor College, University of Malawi, P.O. Box 280, Zomba, Malawi.

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Cynotilapia's **unicuspid teeth, a unique character used to delineate it from all other mbuna genera, leaves evolutionary biologists wondering which is the closest relative to this genus among mbuna cichlids. This genus has only two described species out of the 10-13 species/taxa, whereby the undescribed taxa are either known by their colouration or place where they occur. AFLP genetic marker was used to determine the phylogenetic position of *Cynotilapia* among the mbuna and also the genetic diversity within this genus. Nei's genetic distance, frequency of polymorphic loci and average heterozygosity were used to unravel the genetic diversity. The neighbour-joining (NJ) dendrogram revealed that the genus *Maylandia* is the closest relative to *Cynotilapia*. Genetic distances were higher among all pairs of undescribed taxa than between the two species (*Cynotilapia afra* vs*. C. axelrodi*). Frequency of polymorphic loci and average heterozygosity were also higher within undescribed taxa than in two species. These results, coupled with already known phenotypic differences among these taxa (including colour, a crucial factor in speciation through sexual selection), do provide a strong base to taxonomists who can formally describe these taxa as species. The uncovered genetic differentiation is very important for conservation of this endemic fish fauna**.

Key words: Speciation, AFLP, nuclear genome, polymorphism, heterozygosity, mbuna.

INTRODUCTION

The most speciose fish family, Cichlidae, in east African Great Lakes has attracted biologists from all fields due to its fascinating adaptive radiation and explosive speciation. Speciation in these lakes (Malawi, Victoria and Tanganyika) has been very rapid considering their ages, especially for lakes Malawi and Victoria. Based on geological and palaeolimnological evidence, Lake Victoria basin is said to be ca. 400,000 years old (Johnson et al., 1996, 2000; Talbot and Laerdal, 2000), but within such short timeframe, the lake is currently said to harbour about 535 species (Genner et al., 2004) which are all believed to have descended from one common ancestor. And for Lake Malawi, both molecular and geological evidence indicate that colonization occurred between 500,000 and 2 million years before present (Meyer et al., 1990; Meyer, 1993; Johnson et al., 1996, 2000; Sturmbauer et al., 2001). There seems to be some discrepancies in estimating the cichlid species richness currently found within this lake; for instance, 600, 659, 800 and 850 were estimated by Genner et al. (2004), Turner et al. (2001), Snoeks (2000), and Konings (2001), respectively. Whatever the exact species number is, it is undeniable fact that Lake Malawi has the highest number of species than any other lake in the world. Considering such age evidence, it is undoubtedly that the cichlid species flocks in these two lakes represent the largest

*Corresponding Author. E-mail: kassam@cc.kochi-u.ac.jp, dadeka@yahoo.com, Tel: +81 88 864 5153, Fax: +81 88 864 5148.

known recent adaptive radiations of animals ever known by human kind.

A large group of Lake Malawi's species flock is represented by the most colourful, rock-dwelling species locally known as "mbuna". Almost all mbuna species are restricted to rocky habitats less than 40 m deep (Ribbink et al., 1983). The rapid mbuna and non-mbuna speciation has been attributed to a couple of hypotheses, of which the most dominating in literature are the two classical ones: allopatry (e.g. van Oppen et al., 1997; Arnegard et al., 1999; Markert et al., 1999; Rico and Turner, 2002) and sympatry through sexual selection (e.g. McKaye et al., 1984; Turner and Burrows, 1995; Higashi et. al., 1999; Shaw et al., 2000) or natural selection (Dieckmann and Doebeli 1999; Kondrashov and Kondrashov, 1999). Sexual selection has accelerated the divergence of mate recognition systems, like colour, among mbuna populations and thus may account for rapid speciation.

Cynotilapia, one of the 13 mbuna genera, is no exception of such factors enhancing its speciation. This genus is fascinating among the mbuna due to its unique unicuspid, conical shaped tooth form (Lewis et al., 1986; Konings, 1990, 2001). The unicuspid tooth form is the major character that taxonomists used to elevate this group into a distinct genus, and due to such tooth shape uniqueness, it is not clear as to which of the other mbuna genera is closely related to this genus. And secondly, *Cynotilapia* consists of about 10 to 13 species/taxa, only two of which are taxonomically described species (*Cynotilapia afra* and *C. axelrodi*). The rest are just recognized by either their colouration (e.g. *C.* sp. "black dorsal") or the place where they endemically occur (e.g. *C*. sp. "chinyankwazi" or *C*. sp. "maleri", named after Chinyankwazi and Maleri Islands, respectively). Therefore, due to lack of molecular data evidence, it is not clear how differentiated are these undescribed taxa and if at all they are differentiated so much to be considered separate species or just allopatric populations of the two described species. These two factors make this genus an ideal material to elucidate its phylogenetic relationships with other mbuna and also determine intrageneric genetic diversity.

Amplified fragment length polymorphism (AFLP, Vos et al., 1995), was used to determine the phylogenetic position and genetic diversity. AFLP is one of the most reliable and promising DNA fingerprinting techniques producing hundreds of informative polymerase chain reaction (PCR)-based genetic markers that provide wide multi-locus genome screening (Bonin et al., 2005). AFLP nuclear markers have proven to be valuable tools in taxonomic and phylogenetic inferences (e.g. Albertson et al., 1999; Giannasi et al., 2001; Buntjer et al., 2002; Ogden and Thorpe, 2002; Seehausen et al., 2003), genetic diversity assessment (Travis et al., 1996; Seki et al., 1999; Ajmone-Marsan et al., 2001, 2002), investigations of population structuring and estimation of gene flow (Jorde et al., 1999; Dearborn et al., 2003). Unlike other markers, reproducibility of AFLPs is usually higher than 95% (Ajmone-Marsan et al., 2001; Bagley et al., 2001). The PCR-based AFLP markers are amenable to automation for high-throughput genotyping at relatively low cost and, being anonymous, do not require prior sequence information. These are some of the factors that make AFLPs most favoured DNA fingerprinting method among molecular biologists.

Therefore, the objectives of this study were to utilize AFLPs in order to 1) determine the phylogenetic position of *Cynotilapia* genus among the mbuna cichlids, and 2) evaluate the genetic diversity among *Cynotilapia* species/taxa.

Table 1. Samples used, collection sites and specimen number (n). For *Cynotilapia* specie/taxa, number in parentheses represents specimens used for genetic diversity experiment. Locations are shown in Figure 1.

Taxa	Locality	n
Labeotropheus fuelleborni, Lfue	West Thumbi Island	2
L. treawavasae, Ltre	"	2
Petrotilapia nigra, Pnig	"	2
P. genalutea, Pgen	"	1
P. sp. "mumbo blue", *P* "mbl"	"	1
Tropheops sp. "red cheek", *T* "rch"	"	2
T. sp. "orange chest", *T* "orc"	"	2
Maylandia zebra, Mzeb	"	2
Dimidiochromis kiwinge	Malembo	1
Tilapia rendalli	"	1
Cynotilapia afra, Cafr	West Thumbi Island	2 (12)
C. axelrodi, Caxe	Chadagha point	3 (10)
C. sp. "maleri", *C* "mal"	Maleri Island	3 (10)
C. sp. "black dorsal", *C* "bdo"	Mbenji Island	2 (3)
C. sp. "black eastern", *C* "bea"	Gome point	2 (6)
C. sp. "chinyamwezi", *C* "cmz"	Chinyamwezi Island	2 (6)
C. sp. "chinyankwazi", *C* "cnz"	Chinyankwazi Island	2 (5)

MATERIALS AND METHODS

Sample collection

Species/taxa collected, locality and number of specimens, for both the phylogenetic and genetic diversity experiments are shown in Table I, and the exact localities in Lake Malawi are shown in Figure 1. In July/August 2004, fish were captured by leading them into microfilament nets with the aid of SCUBA, except for *Tilapia rendalli* and *Dimidiochromis kiwinge* which were obtained from local fishermen. *Tilapia rendalli* was used as an outgroup while *D. kiwinge* represented non-mbuna haplochromine cichlid. Since most of our collection sites are within Lake Malawi National Park, only a portion of the unpaired fins (ca. 1 cm^2) was removed from each individual and preserved in 100% ethanol, then the fish was immediately released at the collection site. Preserved finclips were transported to Kochi University, Japan for genetic analyses. DNA

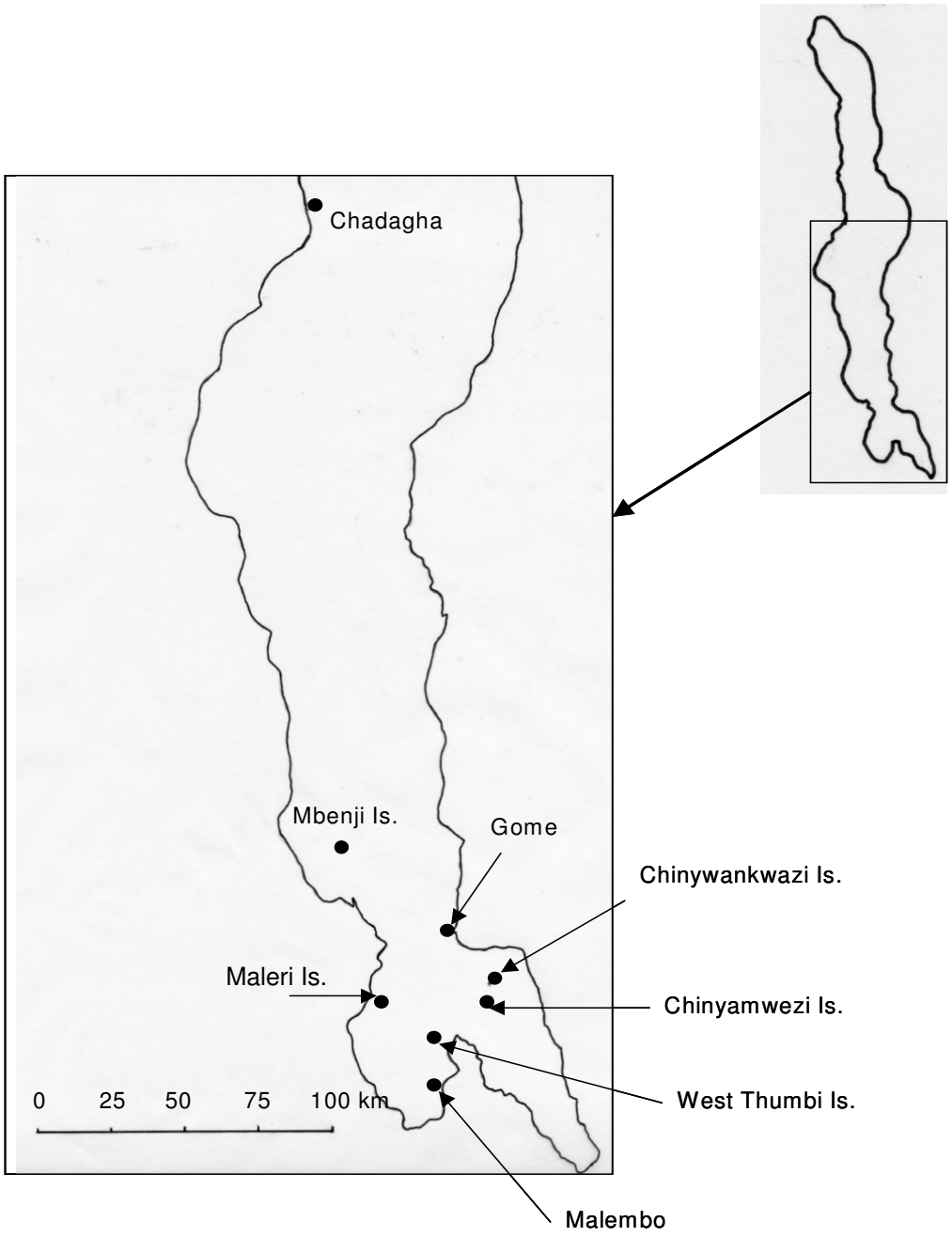

Figure 1. Map of Lake Malawi showing sampling localities.

was extracted from the fin tissues by the standard proteinase-K digestion, phenol-chloroform extraction protocol.

Phylogenetic relationships experiment

The AFLP procedure was performed following the AFLP Plant Mapping Kit of PE Applied Biosystems (Foster city, CA) with minor modifications. The genomic DNA was digested with two endonucleases, EcoRI (5 units) as the rare cutter and MseI (1 unit) as frequent cutter (New England Biolaborataries). Double stranded synthetic adapters were ligated to sticky ends of the resultant fragments to serve as templates for the PCR primers. The restriction-ligation was carried out as single step by incubating the reaction mixture at 23℃ for 10 h using the TaKaRa PCR Thermal Cycler (TaKaRa). Two rounds of PCR (Preselective and Selective, performed by GeneAmp PCR System 9700) using primers complimentary to the synthetic oligonucleotide adapter sequence amplified and labeled the fragments. First, the preselective amplicatification was done with one selective base on each primer (EcoRI+A and MseI+C) for 24 cycles set at 94℃ denaturation (20 s), 56℃ annealing (30 s), and 72℃ extension (2 min). The initial

hold was at 72°C for 2 min and the final extension was at 60°C for 30 min. The amplified product was diluted 10-fold in TE0.1 buffer and stored at 4°C since selective amplification was performed within a one month time frame. Then selective amplification was performed with an additional two-base extension using the following 10 different primer combinations: E-AAG+M-CAG, E-ACA+M-CAG, E-AAC+M-CAG, E-AAG+M-CTT, E-ACA+M-CTT, E-AAC+M-CTT, E-AAG+M-CAT, E-ACA+M-CAT, E-AAG+M-CAA and E-ACA+M-CAA. Selective amplifications were done according to the ABI protocol, and only EcoRI primers were 5′ end-labeled with blue (EcoRI+ACA, 5-FAM), yellow (EcoRI+AAC, NED) and green (EcoRI+AAG, JOE) fluorescent tag.

One µl of the selective amplified product was mixed with 9.5 µl of deionized formamide and 0.5 µl of GeneScan 500 ROX (Perkin-Elmer Applied Biosystems, Foster city, CA) internal size standard. The samples were electrophoresed on a 16-capillary ABI 3100 Genetic Analyzer (Perkin-Elmer Applied Biosystems, Foster city, CA). Raw fragment data were then collected with GeneScan analysis software version 3.7 (PE ABI), and the resulting GeneScan tabulated files were imported into MS-Excel. The AFLP fragments between 50 to 500 base pairs (bp) were manually scored for presence (1) or absence (0) and fragments were considered homologous if their difference was no more than 0.5 bp. The 0, 1 coded raw data matrix was used to construct a neighbour-joining (NJ)-dendrogram from the mean character difference distance by using the neighbour-joining module in PAUP* 4.0 (Swofford, 2002).

Genetic diversity experiment

As indicated in Table 1, only *Cynotilapia* species/taxa were used in this experiment. Although Konings (2001) regarded populations from the Chinyamwezi and Chinyankwazi islands as one species (*C.* sp. “chinyankwazi”), we included both populations in this study in order to assess their genetic differentiation. Restriction-ligation of genomic DNA, preselective and selective amplifications were done as explained above with the only exception of primer combinations for selective amplification. The following five primer pairs were used for the selective amplification: E-AAG+M-CAG, E-ACA+M-CAG, E-AAC+M-CAG, E-AAG+M-CAT and E-ACA+M-CAT.

Using the 0, 1 coded binary matrix, genetic diversity among species/taxa was estimated by using the unbiased Nei’s genetic distance as recommended by Nei (1978) for a small number of individuals. In order to assess genetic variation within each taxa, we estimated average heterozygosity (H) and also frequency of polymorphic loci (f). Frequency of polymorphic loci was calculated as f = Lp/L, where Lp = number of polymorphic loci and L = total number of loci. All these 3 parameters were calculated in POPGENE software version 1.32 (Yeh et al, 1997).

RESULTS

Phylogenetic relationships

From the combined data of 10 primer combinations for all species/taxa, a total of 2068 DNA fragments were scored, out of which 1978 were polymorphic, thus representing 95.6%. Although these primer pairs were chosen arbitrarily, some combinations showed the potential of revealing many characters than others, for instance, the best combination was E-ACA+M-CAG (minimum number of fragments scored = 60, maximum = 135, mean ± SE = 98.9 ± 9.1, thus mean number of fragments per individual) and the least was E-AAC+M-CTT minimum number of fragments scored = 47, maximum = 74, mean ± SE = 59.5 ± 5.4).

The NJ dendrogram generated (Figure 2), which is robust as indicated by the high bootstrap values, revealed that, among the mbuna cichlids, the genus *Maylandia* is the closest relative of *Cynotilapia* (supported by a higher bootstrap value of 99%). The tree topology also indicated that *Cynotilapia* sp. “black eastern” is the basal taxon for all *Cynotilapia* species/basal taxon. Another interesting result worthy noting is the clustering of the *Petrotilapia* and *Tropheops* as sister genera (supported by a 59% bootstrap value).

Genetic diversity

The whole data set, with all five primer combinations, consisted of 993 characters of which 942 were polymorphic (94.9%). Genetic diversity among species/taxa as indicated by the unbiased Nei’s genetic distance (Table 2) shows that the smallest distance was between *C. afra* and *C. axelrodi* (D = 0.03) while the largest distance was between *C. axelrodi* and *C.* sp. “chinyamwezi” (D = 0.16). Overall, all pairwise genetic distances among undescribed taxa were larger than the distance between 2 taxonomically described species (Table 2).

As for the genetic variation within each species/taxa, *C.* sp. “maleri” showed highest polymorphic loci frequency since 570 of the 681 scored were polymorphic (f) = 0.84, while 321 of the 608 fragments scored for *C. axelrodi* showed lowest polymorphic loci frequency (f) = 0.53 (Table 3). Average heterozygosity was lowest in *C. axelrodi* (H) = 0.147 and highest in *C.* sp. “black eastern” (H) = 0.295. Both parameters, i.e. frequency of polymorphic loci and average heterozygosity, were mostly higher in undescribed taxa than in 2 described species (f ≥ 0.61 and H ≥ 0.228).

DISCUSSION

Utilization of AFLP nuclear markers in the present study has revealed the following: *Cynotilapia* is closely related to *Maylandia* while *Tropheops* is closely related to *Petrotilapia* and also that *Cynotilapia sp. “black eastern”* is the basal taxon for *Cynotilapia* species/taxa. And also AFLPs have revealed genetic differentiation among *Cynotilapia* species/taxa, thus supporting the present status of regarding the undescribed taxa as distinct species (see Konings, 1990, 2001).

Phylogenetic relationships

The revelation that the genus *Cynotilapia* is closely related the genus *Maylandia* is consistent with what

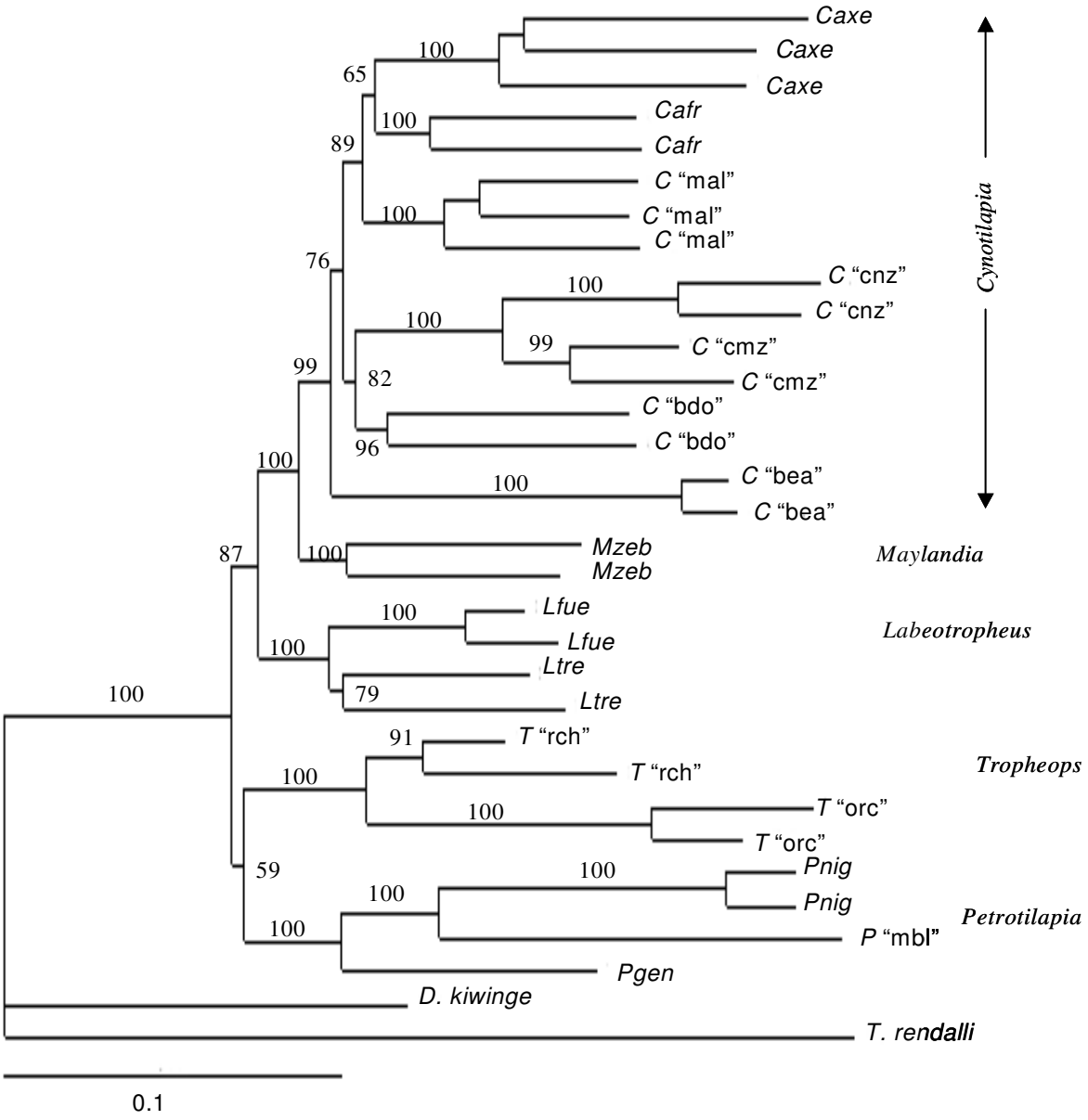

Figure 2. The dendrogram indicating the phylogenetic position of the genus *Cynotilapia* among Lake Malawi's mbuna cichlids as estimated by the NJ algorithm (PAUP* 4.0). Bootstrap values are shown above branches from 1000 replicates. The scale bar indicates 10% character difference. Species/taxa abbreviations are defined in Table 1.

Table 2. The unbiased Nei's genetic distances among taxa (Nei, 1978) showing genetic distances among *Cynotilapia* species/taxa. Abbreviations are defined in Table 1.

Taxa	*Cafr*	*Caxe*	*C* "mal"	*C* "bdo"	*C* "cnz"	*C* "bea"
Caxe	0.03					
C "mal"	0.06	0.14				
C "bdo"	0.06	0.07	0.05			
C "cnz"	0.05	0.06	0.04	0.05		
C "bea"	0.11	0.14	0.07	0.10	0.10	
C "cmz"	0.14	0.16	0.08	0.10	0.11	0.08

Table 3. Genetic diversity within *Cynotilapia* species/taxa as revealed by frequencies of polymorphic loci (f) and average heterozygosity (H).

Taxa	No. of variable bands	No. of fixed bands	f	H
C. afra	437	217	0.67	0.188
C. axelrodi	321	287	0.53	0.147
C. sp. "maleri"	570	111	0.84	0.273
C. sp. "black dorsal"	314	205	0.61	0.228
*C.*sp."chinyankhwazi"	447	183	0.71	0.248
C. sp. "black eastern"	467	107	0.81	0.295
C. sp. "chinyamwezi"	427	124	0.78	0.289

previous researchers (e.g. Fryer and Iles, 1972; Konings, 1990, 2001) had hypothesized, basing their conclusion on the phenotypic (colour to be specific) resemblance of these two genera. Such concordance, to some extent, suggests that colour is more reliable in determining closely related genera/species than tooth shape. However, such conclusion has limitations since both characters, colour and tooth shape, have shown to evolve independently multiple times, even in so distantly related species/genera. For instance, similar colouration between Lake Malawi's *Melanochromis* genus and Lake Tanganyika's *Julidochromis* does not imply that these two genera are closely related but rather same colour pattern evolved in parallel (Fryer and Iles, 1972; Kocher et al., 1993). And also similar tooth shapes have been shown to evolve in distantly related lineages, and vice versa, of Lake Tanganyika's Eretmodini cichlids (see Ruber et al., 1999). With such parallel/convergent evolution in these two characters, which are usually taxonomically utilized, it implies that in order to determine species/genera phylogenetic relationships, molecular data evidence is also required.

Again, the finding that *Petrotilapia* is sister genera to *Tropheops* is surprisingly interesting because we expected *Tropheops* to be closer to *Maylandia* (both once lumped in the *Pseudotropheus* genus). From the genus *Pseudotropheus*, Meyer and Foerster (1984) separated the then *Pseudotropheus* zebra complex and described it as a distinct genus now known as *Maylandia*, and at the same time, Trewavas (1984) proposed *Tropheops* to be a subgenus of *Pseudotropheus* considering its distinguished steep sloping snout and a small ventrally placed mouth. So the genus *Pseudotropheus* was split into three groups, viz; genus *Maylandia*, genus *Pseudotropheus* and its subgenus *Tropheops*. However, recently other researchers (see Konings, 2001) have advocated the elevation of the subgenus *Tropheops* into a distinct genus, and since then, Tropheops has been treated as a distinct genus (see Allender et al., 2003; Won et al., 2005). So our results, indicating the occupation of distinct position (not even closer to *Maylandia*) in our phylogenetic tree, corroborate the idea advocated by Konings (2001) that *Tropheops* be regarded a genus on its own.

And finally, our study does not support the suggestion by Konings (2001) that *Cynotilapia* sp. "black eastern" is closely related to C. sp. "black dorsal", rather *C.* sp. "black dorsal" is sister taxa to *C.* sp. "chinyankwazi" and *C.* sp. "chinyamwezi" (see Figure 2). Konings suggested the close relationship of these two taxa based on their similarity in black colouration, but as stated above, similar colour patterns are prone to parallel evolution and do not always reflect close relationship.

Genetic diversity and conservation implications

The larger Nei's genetic distances revealed in this study among undescribed taxa (together with higher frequency of polymorphic genes and average heterozygosity) than between two taxonomically recognized species, just support that these taxa maybe distinct species as Konings (1990, 2001) had treated them. Due to a bias towards the use of microsatellite markers (e.g. van Oppen et. al., 1997; Arnegard et al., 1999, Markert et. al., 1999; Shaw et al., 2000; Rico and Turner, 2002) than AFLPs to assess genetic diversity in these cichlids, leaves us with no other studies to compare our results with. However, when genetic distance was calculated between other well known mbuna species, (e.g. *Labeotropheus fuelleborni* vs. *L. trewavasae*, D was 0.079 whereas D was 0.124 for *Petrotilapia nigra* vs. *P. genalutea*), the magnitude of such distances is similar to what we have found among *Cynotilapia* species/taxa. Such comparable distances just emphasize that these taxa have differentiated so much to be recognized as distinct species.

Another important finding is the genetic diversity unraveled between *Cynotilapia* sp. "chinyamwezi" and C. sp. "chinyankwazi", in which the former is regarded as just a population of the later. The genetic distance between these two (D = 0.11) is even one of the largest among *Cynotilapia* species/taxa. With such differentiation, it can be suggested that there is little or no gene flow

between these two and they can as well be regarded distinct species. The genetic differentiation revealed between these two taxa (which occupy islands ≈ 5 km apart, separated by sandy beach), is not surprising since mbuna cichlids are known to be habitat specialists and lack dispersal capabilities over sand beaches or deep waters (see Fryer and Iles, 1972; van Oppen et. al., 1997; Arnegard et al., 1999, Markert et. al., 1999; Rico and Turner, 2002). Rico and Turner (2002) even uncovered a significant genetic differentiation between taxa that were separated by a 35 m of habitat discontinuity. Konings (2001) reported that these two taxa are morphologically indistinguishable, but we suggest that a better quantitative approach should be utilized to explore their morphological similarity/variation. Geometric morphometrics can be used to determine morphological divergence due to its robustness in revealing morphological differences not discernible through qualitative approaches (e.g. Kassam et al., 2003 a,b, 2004 a,b).

Assessing genetic diversity, through average heterozygosity and frequency of polymoprhic genes, seems a reasonable approach since these two parameters are the raw materials for natural selection and may allow species to persist or evolve (Sterns, 1992). The genetic diversity revealed in this study within the undescribed taxa implies that these taxa can withstand future environmental extremes and thus avoid instant extinction. So in conclusion, we propose that allopatric speciation is the plausible mechanism that has led to *Cynotilapia's* speciation, especially among the taxa included in this study since they are all spatially separated with barriers (e.g. sandy beaches or deep waters) in between them, limiting gene flow and thus enhance rapid speciation among the taxa.

Cynotilapia's preference for the open waters when feeding on zooplanton makes them more vulnerable to overfishing which is major problem in Lake Malawi. Although *Cynotilapia* is not caught as target genus, it is not uncommon to see large numbers of these species caught together with target genera such as *Copadichromis* (locally known as "utaka", D. Kassam, personal observation). From a conservation perspective, the genetic diversity parameters uncovered through this study can serve as a basis to monitor these endemic *Cynotilapia* taxa in order to detect if there is any future decrease in genetic diversity. Overfishing may lead to reduction in population size which subsequently results into reduced heterozygosity due to inbreeding depression. Therefore, close monitoring of these genetic diversity parameters is essential for the conservation and management of these precious endemic fish fauna.

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