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PHYLOGENETIC ANALYSIS OF HAPLOCHROMINE CICHLID TAXA UTILIZING HETERODUPLEX TECHNIQUES

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INTRODUCTION

The cichlid species flocks of the Great Lakes of Africa represent some of the most remarkable events of explosive speciation ever documented in vertebrates (Mayr 1984, Stiassny 1991). The endemic haplochromine species flock of Lake Victoria, shows extraordinary morphological and ecological diversity (Greenwood, 1984a, 1984b and refs therein) despite a monophyletic origin postulated as being not older than 225,000 years BPE (Meyer et. al. 1990, by mtDNA clock), and possibly less than 14,500 years old, if the lake in fact dried out completely as is currently suspected (Stager et al., 1986). The detailed nature of the evolutionary relationships among the hundreds of recently divergent species, as well as the evolutionary position of the group with respect to other species of Cichlidae, is an area of active investigation. The short evolutionary time period for the group makes molecular analysis difficult, since only the most rapidly evolving sequences will provide phylogenetic information. The determination of a robust phylogeny for the endemic taxa of Lake Victoria is the last major obstacle to its use as a powerful instrument to study the mechanics of vertebrate evolution.

For Lake Victoria Region (LVR) cichlids, morphological, behavioral, and paleontological data have been used to estimate phylogenetic relationships (Van Couvering, 1982, Dominey, 1984, Greenwood, 1991, Lippitsch, 1993). More recently, protein and nucleic acid sequences have been combined with morphological data to investigate cichlid relationships in the African Great Lakes (Sage et al., 1984; Meyer et al., 1990; Sturmbauer and Meyer, 1992, 1993; Ono et al., 1993). These studies have shown the difficulty of identifying sequences of use for discrimination among the recently divergent taxa of Lake Victoria.

The choice of a genomic region for molecular analysis depends strongly on the suspected time since divergence of the groups being studied. In the case of the Lake Victoria fish fauna critical time windows are likely to include: 5000 years or less for recent speciation events (peripheral satellite lake differentiation), around 12000 years for events following reflooding of the lake basin following the most recent desiccation event, and up to several hundred thousand years for initiation of the regional fauna and origination of the major component clades (Meyer et al. 1990, Kaufman ms.). To be useful, a genetic region must accumulate changes to yield measurable genetic differentiation with sensitivity geared toward such critical time windows, which can only be guessed in advance. Mitochondrial DNA sequences have been used extensively for studies of fish at both intraspecific and interspecific levels because of the rapid evolution of the mitochondrial genome (Brown 1985).

However, within Lake Victoria, the analysis of mtDNA sequences, though informative as to the very early divergence of this fauna from the ancestral stock, failed to provide insight into the phylogenetic patterns among taxa that subsequently evolved within the species flock (Meyer et al., 1990).

Several rapidly evolving nuclear gene markers exist, but each has potential drawbacks for phylogenetic studies. Hypervariable VNTR loci and microsatellite STR (short tandem repeat) loci can acquire variation primarily by recombination or replication slippage, rather than by point mutations (Jeffreys, et al., 1985). They could actually evolve too rapidly to be useful for phylogenetic studies, even among species as closely related as the ones considered here. For classical point mutation processes, identification of traditional nuclear restriction fragment length polymorphisms (RFLP) is laborious, and polymorphisms are often too rare or species-specific to be useful (Avise, 1994).

Comparison of the rates of nucleotide substitution for various gene segments (Li, et al., 1985, Gillespie, 1986) suggests that sequences such as introns, with reduced coding constraints, may accumulate changes rapidly enough to warrant study. These sequences are among the most rapidly evolving regions of nuclear genomic DNA, thus having the potential to accumulate changes in sequence very soon after populations diverge. Included by analogy in the class of noncoding sequences are the internal transcribed spacer (ITS) sequences located between the ribosomal RNA genes. The target of this study is the first internal transcribed spacer (ITS 1) of the ribosomal gene cluster. The ribosomal gene cluster consists of repeat units, each containing one 18S, 5.8S, and 28S rRNA gene. Between these genes lie internal transcribed spacers: ITS 1 between the 18S and 5.8S genes, and ITS 2 between the 5.8S and 28S genes. The entire block of genes and spacers is transcribed as one unit, after which the spacers are removed in RNA processing. Adjacent cluster repeats are separated by non-transcribed (NTS) spacer regions. The ITS1 sequence occurs in a conserved location within adjacent genes, flanked by relatively conservative rRNA gene sequences, thus it is possible to develop primers for PCR. This provides a technique to rapidly identify, amplify and analyze the spacer from a large number of individuals from different genera and species. This approach has already proven useful in a variety of organisms (Torres, et al., 1990, Baldwin, 1992, Cordese, et al., 1993; Soltis and Kuzoff, 1993; Volger and DeSalle, 1994). The ITS1 has recently been used in the study of close species relationships in salmonid taxa (Pleyte, et al., 1992).

In earlier studies we amplified and sequenced fifteen species of East African cichlid fishes at the ITS 1 locus (Booton, 1995). While informative, the levels of variation were low in the ITS 1 in LVR taxa, with some species containing identical ITS 1 sequences. To rapidly screen a larger sample of species (up to 600+ species are hypothesized to be located in the LVR, LK, in prep) we are now utilizing heteroduplex screening prior to full sequence analysis of this region (Sorrentino et al., 1991). Here we present initial results of the examination of the ITS1 sequences by heteroduplex screening of this region in other LVR taxa. Results using this method are presented. This methodology allows us to rapidly screen greater numbers of taxa for possible variation at the ITS 1 locus prior to sequence analysis.

METHODS AND MATERIALS

Taxa: The species included in the first round of ITS 1 sequence analysis included two tilapiines: *Oreochromis niloticus* (a widespread species) and *Oreochromis esculentus* (an endemic to the Lake Victoria basin). Also included were the widely distributed *Pseudocrenalabrus multicolor* (we examined the LVR subspecies, *P. m. victoriae*); *Astatoreochromis alluaudi* (as the LVR representative of a genus that extends south to the Lake Tanganyika Basin); *Astatotilapia burtoni* (a representative riverine haplochromine from the Lake Tanganyika Basin); and *Astatotilapia nubila,* a widespread, generalized LVR taxon. Finally, we examined a series of LVR lacustrine endemics: *Lipochromis taurinus* a paedophage from Lake Edward; *(Harpagochromis)* "kachira deep", *Yssichromis fusiformis* and *Y. laparogramma* , zooplanktivores from Lake Victoria;

Neochromis nigricans and the undescribed (*Neochromis*) "kruising", epilithic algal scrapers from Lake Victoria; *Ptyochromis xenognathus* , an oral sheller from Lake Victoria; *Xystichromis phytophagous* , a macrophyte eater from Lake Victoria (our specimens drawn from the refugium in Lake Kanyaboli, Kenya); and the undescribed (*Paralabidochromis*) "rock kribensis", an invertebrate eater from Lake Victoria. Additional taxa which were used the initial heteroduplex analysis included: *(Psammochromis)* "constellation", *Haplochromis* "barred pygmy", *Garuochromis angustifrons*, and *Pyxichromis orthostoma*. Genera for the undescribed forms are listed parenthetically because their placement is a proposal pending further investigation (Kaufman, in prep.); they will be referred to in this paper using their proposed generic classification, with temporary species names. All material was collected through the efforts of the Lake Victoria Research Team, involving the authors and researchers of several other institutions. Identifications are by LK through comparison with other voucher specimens All wild caught specimens of East African cichlids from which DNA was obtained were deposited as voucher samples in the fishes collection of the Muzeum of Comparative Zoology, Harvard University.

DNA Preparation, PCR Amplification and DNA Sequencing.

Field collected material was preserved in 95% EtOH, changed after one hour, until DNA could be extracted in the laboratory. DNA was extracted from muscle tissue which was excised from the right epaxial musculature of each specimen. To prepare for DNA extraction, tissue was sheared by razor blade in the presence of ABI lysis buffer (Applied Biosystems Inc., O.1 M Tris, 4M Urea, 0.2M NaCl, O.01 M CDTA, and 0.5% n-laurelsarcosine). Chopped tissue was placed in a 1.5 ml centrifuge tube to total volume of 1ml, and 10µl of a 20mg/ml solution of proteinase K was added. Samples were incubated overnight at 50° C. Following digestion, DNA was extracted by two phenol/chloroform, and one chloroform extraction. DNA was precipitated by addition of 2 volumes of 95% EtOH, followed by a 70% EtOH wash. DNA was quantified by spectroscopy and quantifications were confirmed by electrophoresis on a 1% agarose gel.

The location of the ITS1 sequence within the rRNA gene region, as well as primer locations used for amplification, heteroduplex, and sequencing are shown in Figure 1. Primers used in the study of ITS 1 are summarized in Table 1. Ribosomal RNA ITS 1 amplifications were performed as follows: 100 ng of genomic template DNA was added to each reaction mixture (final volume 100µl) which contained 10pM of each amplification primer, 2.5mM MgCl2, 1.5mM dNTP's, and 1-2.5U Taq DNA Polymerase (ProMega Corp.). A number of combinations of forward and reverse primers were used for complete ITS 1 amplification, although the greatest success was obtained using primer combination 1712C and ITS2. Primer pair 236C-ITS and ITS2-Cln was used to amplify the 3' half of ITS 1 for the initial heteroduplex analysis. The PCR reactions were carried out as follows: denaturation at 94° C for 1min., annealing at 50-52°C for 1.5 min., and extension at 72 °C for 3 min., 35 cycles. Reactions were performed in a Perkin Elmer Cetus PC1 thermocycler. 10µl of PCR products were electrophoresed on a 1% agarose gel to determine size and approximate quantity before sequencing. Separate PCR reactions from the same individual which produced bands of the expected size were pooled. The PCR product pools were either used directly for sequencing or cloned by T/A cloning using a commercially available T/A cloning kit following manufacturers instructions (Invitrogen, Inc.; Marchuk, Mitchell, and Collins, 1991). Sequencing was performed using the dsDNA Cycle Sequencing System (BRL Inc.). Sequencing gels were scored manually.

Table 1. Internal Transcribed Spacer One Amplification and Sequencing Primers.

Primer	sequence 5'->3'	Forward /Reverse	Melting Point °C
1262C	gtggtgcatggccgttctta	Forward	55.7
1262C-Cln	gcggatccgtggtgcatggccgttctta	Forward	73.7
1712C	agcgccgagaagacgatcaaa	Forward	58.6
1/F	cacaccgcccgtcg	Forward	49.3
SSU2C	gtgaacctgcggaaggatca	Forward	54.8
236C-ITS	ggaccgtggctcgttgg	Forward	54.3
538RE-ITS	ttgccacattcgtagacggg	Reverse	55.8
ITS2	gctgcgttcttcatcgacgc	Reverse	58.1
ITS2-Cln	cgaagcttgtcgacgctgcgttcttcatcgacgc	Reverse	78.1

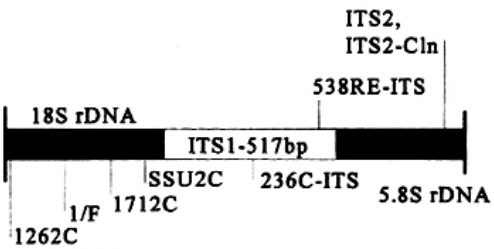

Fig. 1. ITS 1

ITS 1 sequencing and amplification primers. Primers below are forward primers, those above are reverse primers. Cln designation on primer is a cloning primer. Spacer size is given for a represenative taxa, *Astatoreochromis alluaudi*.

Gel preparaton.

Agarose Gels: Agarose gels of 2.5% were used for preliminary agarose heteroduplex analysis. Gels were 15cm x 15cm. Electrophoresis was carried out at 180 Volts for 3.5 hours. Gels were stained in an Ethidium Bromide solution (3mg/L) for 15 minutes prior to photography.

Heteroduplex gels: Gel plates were assembled according to manufacturer's instructions. Sequagel MD (National Diagnostics, Inc.) solution was supplied as a 2X liquid concentrate. A 40cm x 30cm x 1mm gel requires approximately 150 mls of a 1X gel solution. A 19 cm x 16 cm x 1mm gel requires approximately 36 mls. We recommend the use of 19 cm x 16 cm gel if PCR products are approximately 300 bp.

Heteroduplex and homoduplex preparation

For heteroduplex production, two different PCR products were mixed by combining 4 ml of each PCR product into a 0.75 ml microcentrifuge tube with 7µl of deionized water, and overlayed with a drop of mineral oil. DNA was then denatured and renatured as follows: 96 ^{o} C for 5 min, 55^{o} C for 10 min, 45^{o} C for 10 min, 37^{o} C for 10 min, 26^{o} C for 15 minutes or more. Next, 3 µl of loading buffer was added. As controls, two homoduplexes were used in these experiments: 1) a mixture of

two PCR products from the same individual that was processed through the melting and re-annealing cycles and 2) a reaction that contained the same mix but was not processed through the melting and reannealing cycles.

Electrophoresis
1 µl of gel loading buffer was added for each 5 µl of reaction mixture. Finally, for a 40 cm x 30 cm gel, 6 µl of the hetero/homo duplex mixture was loaded into the wells. When using a 19 cm x 16 cm gel, 3 µl of the mixture was loaded. One lane was loaded with 5 µl (for 40 cm x 30 cm gel), or 1.5 µl (for 19 cm x 16 cm gel) of the positive control (AT Biochem product), and one lane with a 1 kb DNA marker (Life Technologies Inc.).

For a 40 cm x 30 cm gel: Pre-running was for 30 min at 800 V. Samples were then electrophoresed for 16 hours. For a 19 cm x16 cm gel: Pre-running was for 30 min at 360 V, followed by loading and running for 4 hours. The gel was stained in 0.6 x TBE, 3mg/L ethidium bromide for 10 minutes, and the gel was then exposed to UV light to visualize the DNA banding pattern. The gel was photographed using a video imaging system.

Phylogenetic Data Analysis: The primary sequences from the taxa in this study were aligned using the sequence alignment program Eyeball Sequence Editor (ESEE) for the PC (Cabot and Beckenbach, 1989). This alignment was used to produce data sets which were suitable for analysis is PAUP and MEGA (Swofford, 1990, Sudhir, et al., 1993). Phylogenetic analyses which used distance methods were done using the computer program MEGA. Within MEGA the corrected proportion of nucleotide substitutions were estimated using the Kimura two parameter model. Distances derived by this method were then used to produce a phylogenetic tree by the neighbor-joining algorithm. Maximum parsimony analysis was also performed on the data using the cladistic analysis program PAUP, as well as using the maximum parsimony option in MEGA. Bootstrapping of the data was performed in MEGA and PAUP to examine the consistency of the data.

RESULTS
Full sequences of ITS 1 were obtained for two tilapiine and thirteen haplochromine cichlid species. The aligned ITS region spans 553 bases, although most taxa have an ITS 1 between 509-512 nucleotides in length. Variation in overall size was due to multiple insertion/deletion (indels) events. In initial ITS 1 sequencing no differences were observed between the two species of *Oreochromis*, or between *Xystichromis* and *Ptyochromis*. This alignment was used to calculate the corrected proportion of nucleotide substitutions using the Kimura two parameter model. Distances were also calculated considering only the transition and transversion changes in data. Trees produced from these different data sets had the same gross topology as that shown but were less well resolved due to data truncation.

The calculated distances were used to produce a phylogenetic tree using the neighbor-joining (NJ) algorithm in MEGA, with 1000 bootstraps (Saitou and Nei, 1987). As representatives of the subfamily Tilapiinae within the African Group of Stiassny's cichlid phylogeny, (Stiassny 1991), the two *Oreochromis* species were defined as the outgroup for an analysis of the other taxa. *P. multicolor* showed the most divergence from the main group of Lake Victoria haplochromine species, due to the accumulation of nine unique nucleotide substitutions compared to all other taxa. Although the level of resolution was low using distance methods, the numerous indel events can be treated as additional informative sites using maximum parsimony (MP) analysis. Phylogenetic trees using maximum parsimony methods were produced in MEGA and PAUP. The initial consensus ITS 1 trees obtained from maximum parsimony and neighbor-joining methods is shown in Figure 2.

The trees indicate that the haplochromine taxa of Lake Victoria represent a monophyletic group. Resolution of absolute branching order is resolved for some, but not all LVR lacustrine taxa. The riverine *Astatotilapia, A. burtoni* , represents a sister group to the Lake Victoria endemics, which include the marginal lacustrine *A.nubilis*. Clustering of *Yssichromis* and *Lipochromis* conforms to a group predicted by the analysis of Lipittsch (1993). The *Xystichromis* and *Ptyochromis* exhibited identical ITS sequences, again confirming a group consistent with Lippitsch (1993).. The only difference between the two trees is in the clustering of *Astatoreochromis* with the riverine *Astatotilapia* (*burtoni*) in the NJ and MP tree of MEGA while *Astatoreochromis* groups with *Pseudocrenalabrus* in the MP anlaysis of PAUP.

After primary determination of an ITS 1 gene tree, a larger sample of LVR haplochromines was initiated using a heteroduplex analysis. This procedure takes advantage of the altered mobility of DNA hybrids which differ by as few as one nucleotide substitution. Briefly, PCR products from different taxa are denatured and allowed to reanneal together with a control (driver) PCR products whose sequence is known. If multiple bands are observed on the heteroduplex gel matrix, there is an assumed difference between the two tested PCR products. If a single band is observed on the heteroduplex gel, the PCR products are believed to be identical (Sorrentino et al., 1991, White et al., 1992,). This method has been used to detect single nucleotide substitutions for disease screening (Higsmith, 1993). This method works optimally on DNA strands of less than 400 base pairs. This, combined with the fact that most of the observed variation in the ITS 1 was in the 3' end of the spacer, led us to initially use the primer pair 236C-ITS/ ITS 2-Cln for heteroduplex analysis. Heteroduplex screening was done using a multiple step process in our experiments. The first step in this procedure was to screen taxa in an agarose heteroduplex method which is methodologically identical to standard heteroduplex analysis except that the heteroduplex products are electrophoresed on an 2.5% agarose gel. Initially, we used PCR products from those taxa that we had previously determined the ITS 1 sequences. By this method, it was empirically determined that ITS 1 sequences which differ by a few indel events, or as few as five nucleotide substitutions, could be visualized as multiple banding patterns by the agarose method. In this initial, less expensive, agarose screening those taxa which produced multiple bands were slated for later sequence analysis. Those taxa which appeared identical in the agarose heteroduplex screening were then analyzed by the more traditional heteroduplex analysis using a proprietary polyacrylamide matrix (Sequagel MD). Those taxa which produced mulitple bands in this heteroduplex analysis were also slated for sequence analysis. Those taxa which did not produce multiple bands were assumed to be identical to the driver DNA ITS 1 product.

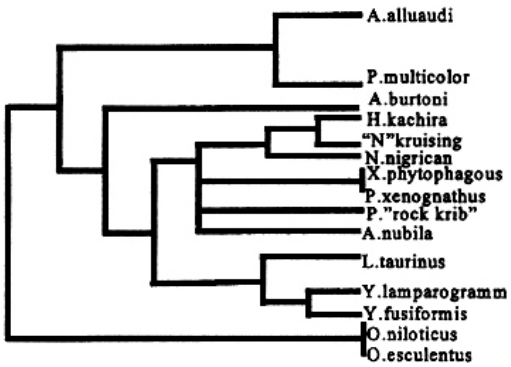

Figure 2. Initial consensus ITS 1 gene tree from 15 taxa.

A heteroduplex result is shown in Figure 3. In these experiments, we used *Yssichromis fusiformis* as the driver DNA. For heteroduplex analysis , PCR product from *Y. fusiformis* was mixed with *Astatoreochromis alluaudi* (lane A), *Haplochromis barred pygmy* (lane B), *Pyxichromis orthostoma* (lane C), and *Gaurochromis augustifrona* (lane D and E; two individuals).

M +| +| |A| B| C| D |E| homo

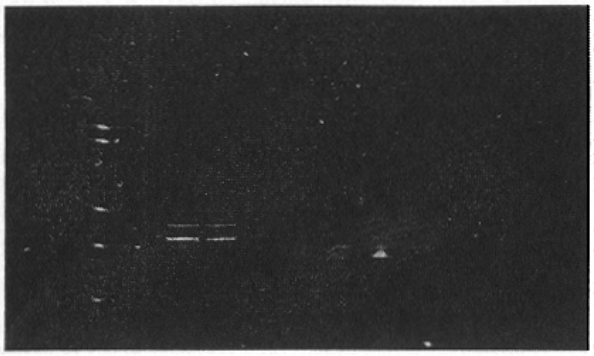

Fig 3. Sequagel MD Heteroduplex Gel

Lane M was 1kb marker (Life Technologies product) and lane H was a homoduplex of *Y. fusiformis* PCR products. Multiple banding patterns were observed (indicating heteroduplexes) in A, B, C, D, and E, which is partially expected from our earlier ITS 1 sequencing work. The level of variation (number of nucleotide substitutions) indicated by heteroduplex screening was then determined by DNA sequencing of *Gaurochromis angustifrona* and *(Haplochromis)* "barred pygmy".One taxa, *(Haplochromis)* "barred pygmy" differed by two nucleotide substitutions from the driver PCR product. *Gaurochromis angustifrona* differed by one nucleotide substitution from the driver. One apparent homoduplex, *(Psammochromis)* "constellation" (heteroduplex gel not shown) was also sequenced to determine if assumed homoduplexes were in fact identical. No differences were found between this *(Psammochromis)* "constellation" sequence and the driver PCR sequence.

DISCUSSION

Sequence divergence among the first fifteen taxa involved in this study was low at ITS1, but nonetheless informative. The low level of variation is not unexpected, given that the entire species flock is monophyletic (Lippitsch 1993), with an age geologically constrained by the formation of the Lake Victoria basin (ca. 750,000 BPE), and estimated at ca. 225,000 years by mitochondrial clock (Meyer et al. 1990). Despite the low degrees of variation, structure was discerned and it was informative. A large number of insertion and deletion events were found in ITS 1. These were most prevalent in comparisons between haplochromines and tilapiines, and to a lesser extent in the comparisons involving *Pseudocrenilabrus* relative to other taxa. This is similar to other studies which have observed numerous indels in species ranging from salmon to beetles (Pleyte, et al., 1992; Vogler and DeSalle, 1994).

Analysis of phylogenetic gene trees produced nearly identical branching patterns using both distance and parsimony methods (as well as other methods not shown), although bootstrap values were low for many branches. This is due to the small number of phylogenetically informative sites. Although this indicates that the data should be interpreted cautiously, it does yield some interesting findings. The ITS 1 sequences, though very closely related, confirmed independently derived basal phylogenies, and also provided useful information from within the Victoria region superflock. The ITS1-based phylogeny is generally consistent, where comparison is possible, with cladograms derived independently from morphological characters, and from mtDNA (Stiassny 1991, Lippitsch 1993, Meyer et al. 1990). *Pseudocrenilabrus* arose early, close to the divergence event that separated the tilapiine and haplochromine tribes of the "African Group" of the family Cichlidae, and thus appears to be the most primitive extant haplochromine clade. *Astatoreochromis alluaudi* was the sister group to the riverine *Astatotilapia burtoni,* which was in turn the sister group to a monophyletic assemblage including both Lake Victoria and Lake Edward taxa. The ITS1 phylogeny placed *L. taurinus* as a sister group to two species of *Yssichromis* , an association that would not

seem likely on the grounds of superficial morphology, but which is well supported by lepidological characters (Lippitsch 1993). Also in agreement with Lippitsch is the grouping of the lacustrine *Astatotiapia nubila* with other LVR haplochomines. In contrast, placement of *Neochromis* in close proximity to *Harpagochromis sp.* is sharply at variance with the weight of morphological evidence, which would place *Neochromis* with *Xystichromis* plus *Haplochromis* (Greenwood 1981, Lippitsch 1993, pers. com.).

It is particularly noteworthy that taxa drawn from Lake Edward/George rather than Victoria nonetheless fell amongst the Victorian taxa. This supports Greenwood's contention that the Lake Victoria radiation is one part of a larger Lake Victoria-Edward/George- Kyoga-Kivu regional fauna and further supports the notion that Meyer et al.'s evidence for monophyly of the Lake Victoria haplochromines applies to the regional haplochromine fauna as a whole.

The limited degree of variation that we observed had the ancillary benefit of permitting alignment with little ambiguity along the entire ITS1 among the taxa studied. The calculated distances in most comparisons are small, with no differences found between the two *Oreochromis* species that we examined (*O. esculentus* and *O. niloticus*), or within certain smaller groups of Lake Victoria haplochromines. The largest distances are found between *Pseudocrenilabrus* and other taxa, ranging from 4.97% to 7.37%. The genetic distances between *Astatoreochromis alluaudi* and the other haplochromine genera range from 4.90% for *Pseudocrenilabrus* , to 0.99% for *Astatotilapia burtoni*, its sister genus. The average distances between *A. alluaudi* and the remaining haplochromines is approximately 1.4%. Expansion of the ITS 1 analysis using heteroduplex analysis appears to be useful for these closely related species. We have shown that differences as little as one nucleotide substitution can be determined by heteroduplex analysis, and identical sequences can be excluded from further analysis. Using this method we will be able to rapidly screen large numbers of taxa at this locus prior to sequencing, and to incorporate those results into future phylogenetic analyses.

In summary, while the ITS1 contains low levels of variation, it is able to discriminate between a hypothesized sister taxon of the Lake Victoria flock and genera within the flock. It also provides evidence of structure within the flock; some of this structure is consistent with data from other sources, but in one instance (*Neochromis*) it is not. The ITS 1 sequence is very likely, however, to help in testing the validity of haplochromine genera erected by Greenwood, as well as monophlyly of these genera across lakes: i.e., the structure and history of the radiation. Since Lake Victoria may have been dry between 14,500 and 12,400 BPE, the modern assemblage must have derived from reinvasion by the products of earlier cladogenesis events. Thus, although the regional superflock is monophyletic, the haplochromines of Lake Victoria itself did not evolve *in situ* from a single ancestor. Rapid addition of a much larger sample of Lake Victoria cichlid taxa using heteroduplex screening analysis to the ITS 1 database is now possible. This information, as well as examination of other regions of the genome will be needed to more robustly establish the relationships among Lake Victoria Region cichlids.

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REFERENCES

Avise, J. 1994. Molecular Markers, Natural History, and Evolution. Chapman and Hall, N.Y.

Baldwin, B. G., 1992. Phylogenetic Utility of the Internal Transcribed Spacers of Nuclear Ribosomal DNA in Plants: An Example from the Compositae. *Molecular Phylogenetics and Evolution* 1(1): 3-16.

Booton, G., 1995. Molecular Genetic Analysis of the Phylogenetic Relationships of Lake Victoria Cichlid Fish. Ph.D. thesis. The Ohio State University.

Brown, W. M., 1985. The mitochondrial genome of animals, pp. 95-130. *In* R. MacIntyre (ed.), *Molecular Evolutionary Genetics*. Plenum, New York.

Cabot, E. L. and A. T. Beckenbach. 1989. Simultaneous editing of multiple nucleic acid and protein sequences with ESEE. *Computational and Applied Bioscience*, 5: 233-234

Cordese, F., R. Cooke, D. Tremousaygue, F. Grellet, and M. Delseny 1993. Fine Structure and Evolution of the rDNA Intergenic Spacer in Rice and Other Cereals. *J. Mol. Evol.*, 36: 369-379.

Dominey, W. J. 1984. Effects of Sexual Selection and Life History on Speciation: Species Flocks in African Cichlids and Hawaiian *Drosophila*, 231-250. *In* A. A. Echelle and I. Kornfield (eds.), *Evolution of Fish Species Flocks,* University of Maine Press, Orono.

Gillespie, J.H., 1986. Rates of Molecular Evolution. Annual Reviews of Ecological Systematics. Annual Reviews Inc., 17: 637-665.

Greenwood, P. H. 1984a. African Cichlids and Evolutionary Theories, 141-154. *In* Anthony A. Echelle and I. Kornfield (eds.), *Evolution of Fish Species Flocks*, University of Maine Press, Orono.

Greenwood, P. H. 1984b. What *Is* a Species Flock?, 13-19. *In* Anthony A. Echelle and I. Kornfield (eds.), *Evolution of Fish Species Flocks,* University of Maine Press, Orono.

Greenwood, P. H. 1991. Speciation. Keenlyside, Miles H.ed. Cichlid Fishes: Behaviour, ecology, and evolution. First London Chapman and Hall 86-102 Fish and Fisheries Series 2.

Higsmith, Jr., W.E. 1993. Carrier screening for cystic fibrosis. *Clinical Chemistry* 39:706-707.

Jeffreys, A. J., V. Wilson, & S. L. Thein 1985. Individual-specific 'fingerprints' of human DNA. *Nature*, 316, 76-79.

Li, W-H., C. C. Luo, and C. I. Wu 1985. Evolution of DNA Sequences. pp. 1-94. In R. J. MacIntyre (ed.), Molecular Evolutionary Genetics. Plenum, N.Y.

Lippitsch, E., 1993. A phyletic study on lacustrine haplochromine fishes (Perciformes, Cichlidae) of East Africa, based on scale and squamation characters. *J. Fish Biol.*, 42: 903-946.

Marchuk, D., A.S. Mitchell, and F.S. Collins, 1991. Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. *Nucleic Acid Research*, 19:1154.

Mayr, E. 1984. Evolution of Fish Species Flocks: A Commentary, 3-11. *In* Anthony A. Echelle and I. Kornfield (eds.), *Evolution of Fish Species Flocks*, University of Maine Press, Orono.

Meyer, A., T. D. Kocher, P. Basasibwaki and A. C. Wilson. 1990. Monophyletic origin of Lake Victoria cichlid fishes suggested by mitochondrial DNA sequences. *Nature*, 347, 550-553.

Ono, H., C. O'hUigin, V. Vincek, and J. Klein, 1993. Exon-Intron Organization of fish major histocompatability complex II B genes. *Immunogenetics* 38(3): 223-234.

Pleyte, K. A., S. Duncan, and R. Phillips, 1992. Evolutionary Relationships of the Salmonid Fish Genus *Salvelinus* Inferred from DNA Sequences of the First Internal Transcribed Spacer (ITS 1) of Ribosomal DNA. *Molec. Phy. Evol.* 1(3): 223-230.

Sage, R. D., P. V. Loiselle, P. Basasibwaki and A. C. Wilson 1984. Molecular Versus Morphological Change Among Cichlid Fishes of Lake Victoria, 185-202. *In* A. A. Echelle and I. Kornfield (eds.), *Evolution of Fish Species Flocks*, University of Maine Press, Orono.

Saitou, N., and M. Nei, 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4(4): 406-425

Soltis, P., and R. Kuzoff, 1993. ITS Sequence Variation within and among Populations of Lomatium grayi and L. laevigatum (Umbelliferae). *Mol. Phy. Evol.* 2(2):166-170.

Sorrentino, R., C. Iannicola, S. Costanzi, A. Chersi, and R. Tosi. 1991. Detection of complex alleles dy direct analysis of DNA heteroduplexes. *Immunogenetics*. 33:118:123.

Stager, J. C., P. N. Reinthal, and D. A. Livingstone, 1986. A 25,000-year history for Lake Victoria, East Africa, and some comments on its significance for the evolution of cichlid fishes. Fresh. Biol. 16: 15-19.

Stiassny, M. L. J. 1991. Phylogenetic intrarelationships of the family Cichlidae: an overview, 1-35. *In* Miles H. A. Keenlyside (ed.), *Cichlid Fishes: Behaviour, ecology, and evolution*, Chapman and Hall, London.

Sturmbauer, C. and A. Meyer 1992. Genetic divergence, speciation and morphological stasis in a lineage of African cichlid fish. *Nature*, 358: 578-581.

Sturmbauer, C, and A. Meyer, 1993. Mitochondrial phylogeny of the endemic mouthbrooding lineages of cichlid fishes from Lake Tanganyika in eastern Africa. *Mol. Biol. Evol.* 10(9): 751-768.

Swofford, D. L. 1990. PAUP- Phylogenetic Analysis Using Parsimony (Version 3.0) Computer program distributed by the Illinois Natural History Survey, Champaign, Illinois.

Torres, R. A., M. Ganal, and V. Hemleben, 1990. GC balance in the internal transcribed spacers ITS 1 and ITS 2 of nuclear ribosomal RNA genes. *J. Mol. Evol.* 30:170-181.

Van Couvering, 1982. Fossil cichlid fishes of Africa. Special papers in Paleontology. Paleontological Ass. Lond. 29: 1-103.2

Vogler, A. P., and R. DeSalle, 1994. Evolution and Phylogenetic Information Content of the ITS-1 Region in the Tiger Beetle Cicindela dorsalis. *Mol. Biol. Evol.* 11(3): 393-405.

White, M. B., M. Carvalho, D. Derse, S.J. O'Brien, and M. Dean. 1992. Detecting single base substitutions as heteroduplex polymorphisms. *Genomics* 12: 301-306.