Origin and Speciation of Haplochromine Fishes in East African Crater Lakes Investigated by the Analysis of Their mtDNA, Mhc Genes, and SINEs

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The Western Branch of the East African Great Rift Valley is pocketed with craters of extinct or dormant volcanoes. Many of the craters are filled with water, and the lakes are inhabited by fishes. The objective of the present study was to determine the amount and nature of genetic variation in haplochromine fishes inhabiting two of these crater lakes, Lake Lutoto and Lake Nshere, and to use this information to infer the origin and history of the two populations. To this end, sequences of mitochondrial (mt) DNA control region, exon 2 of major histocompatibility complex (Mhc) class II B genes, and short interspersed elements (SINEs) were analyzed. The results indicate that the Lake Nshere and Lake Lutoto fishes originated from different but related large founding populations derived from the Kazinga Channel, which connects Lake Edward and Lake George. Some of the genetic polymorphism that existed in the ancestral populations was lost in the populations of the two lakes. The polymorphism that has been retained has persisted for some 50,000 generations (years). During this time, new mutations arose and became fixed in each of the two populations in the mtDNA, giving rise to sets of diagnostic substitutions. Each population evolved in isolation after the colonization of the lakes less than 50,000 years ago. There appears to be no population structure within the crater lake fishes, and their present effective population sizes are in the order of 10^3 to 10^5 individuals. Comparisons with the endemic haplochromine species of Lake Victoria reveal interesting parallels, as well as differences, which may help to understand the nature of the speciation process.

Introduction

The formation of the East African Great Rift Valley, a complex system of faults and escarpments, was accompanied by extensive volcanic activity that left behind many craters along both the eastern and western branches of the Rift (Schlüter 1997). As the volcanoes became extinct or dormant, many of the craters filled with water, and some of the crater lakes came to be inhabited by fish. The circumstances under which the fish reached the crater lakes have been a subject of speculation for many years. Where the lakes established a temporary or permanent connection with nearby river systems, the origin seems obvious. For some of the crater lakes, however, past or present contacts with rivers could not be documented, and more fanciful explanations, such as transfer of fish fry or fertilized eggs by birds or water mammals, have been invoked.

Genetic characterization of a fish population can provide information about the probable size and composition of the founding population, the time of the founding event, and other related questions. This information can then be used to make inferences not only about the circumstances of the colonization but also about the process of speciation and species divergence. Here we use three genetic systems to probe the origin, population dynamics, and speciation of lacustrine fishes—mitochondrial (mt) DNA, genes of the major histocompatibility complex (Mhc), and short interspersed repetitive elements (SINEs). The mtDNA control region with its high substitution rate and maternal transmission is the tool of choice in efforts to identify the most recent common ancestor (MRCA) of a population (Avise et al. 1987). Our survey of mtDNA control region sequences in lacustrine and fluviatile haplochromine cichlids of the Lake Victoria basin revealed the existence of seven major haplogroups, designated I through VII (Nagl et al. 2000). Each haplogroup is characterized by a set of diagnostic substitutions and by the clustering of its members in a single clade on phylogenetic trees. The haplogroups are distributed differentially over the basin, the most widespread being haplogroup V centered on Lake Victoria, the Lake Edward region (including Lake George and Lake Albert), and the rivers around these lakes. A number of additional haplogroups apparently exist among the fishes of Lake Tanganyika and Lake Malawi, which were involved in the survey only tangentially. Haplogroup V is subdivided further into subgroups VA through VD, which are distinguished by a more restrictive set of diagnostic substitutions. Each of the subgroups, however, may be subdivisible even further on typing additional specimens. In particular, subgroups VB and VC show a heterogeneity indicative of future splitting.

Mhc genes with their high polymorphism and long persistence of allelic lineages are well suited for exploring the population dynamics in the history of a species (Klein et al. 1998). Fish Mhc genes, like those of other jawed vertebrates, fall into two classes, I and II, and each class into two subclasses, A and B, encoding the α and β chains of the αβ heterodimeric protein (Klein et al. 1997; Shand and Dixon 2001). Since most of the work on cichlid Mhc genes was done on the class II B genes (Klein et al. 1993; Ono et al. 1993a, 1993b; Sato et al. 1997; Málaga-Trillo et al. 1998; Figueroa et al. 2000), we focused on the most variable part of these genes, exon 2 encoding the β1 domain of the class II β chain.

Fish SINEs are retroposons that are several hundred base pairs long and contain a region homologous to

Key words: cichlids, haplochromine fishes, Mhc, mtDNA control region, SINE, crater lakes.

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The nucleotide sequences reported in this publication have been submitted to the GenBank database and assigned the following accession numbers: AY164681 to AY164696 (mitochondrial control region), AY211028 to AY211042 (Mhc class II B), AY211024 to AY211027 (SINEs).

DOI: 10.1093/molbev/msg151
Molecular Biology and Evolution, Vol. 20, No. 9,
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a transfer RNA gene in their 5' part and frequently also contain a region corresponding to a segment of a long interspersed repetitive element (LINE) in their 3' part (Okada 1991; Schmid and Maraia 1992; Okada et al. 1997). In a recent study, Terai and colleagues (2003) have shown that haplochromines of the Lake Victoria basin contain two types of SINEs, fixed and polymorphic. The former are found in all individuals of a given species or species assemblage, whereas the latter are present in only some individuals of a population. As there is no indication for selection influencing the evolution of SINEs, these loci provide a nuclear counterpart to the neutrally evolving control region segment of the mtDNA on the one hand and the selection driven evolution of the Mhc genes on the other hand. Furthermore, the presumed random distribution of SINEs among the chromosomes enables sampling of different segments of the nuclear genome.

The two main aims of the present study were (1) to determine the origin, structure, and effective size of the haplochrome populations in Lake Lutoto and Lake Nsphere and to ascertain the genetic relationship between them and (2) to provide a basis for comparing the genetic variability existing in these two isolated populations with that present in the endemic Lake Victoria species and thus pave a way toward a better understanding of the speciation process in these fishes.

Materials and Methods

Localities

Lake Nsphere and Lake Lutoto are located in the Bunyaruguru county in southwest Uganda. Judging from their location and shape, and from the geology of the region (Boven et al. 1998), both lakes originated by flooding of craters of extinct volcanoes. They are at a distance of approximately 20 km from each other (fig. 1). They are small (surface areas of \(1 \text{ km}^2\)) and are surrounded by even smaller lakes (fig. 1), the waters of at least some of which have a high salt concentration. Lake Nsphere (or Chibwera) is located at an altitude of 970 m in the middle of a steppe-like flatland, the Chambura Game Reserve administered by the Queen Elizabeth National Parks. It is connected via a small river, which dries out periodically, with the Kazinga Channel and so with Lake George and Lake Edward (fig. 2). The distance from Lake Nsphere to the Kazinga Channel is approximately 15 km.

Lake Lutoto (or Rutoto, also known as Lake Nkugute) is located at an altitude of approximately 1,250 m in a mountainous area (the height of nearby Mt. Kasunju is 1,950 m [fig. 1]). It is connected by a small, rapidly flowing brook to Lake Mugogo at a distance of approximately 3.5 km, the difference in altitude between the two lakes being approximately 180 m. Lake Mugogo drains into the Chambura River system and so into the
Kazinga Channel at a distance of approximately 40 km. Although tilapias (Oreochromis spp.) were apparently introduced into Lake Lutoto some time ago, the studied species seem to be the only haplochromines in the lake.

Fishes

As far as we could determine, the species of haplochromines living in Lake Nshere and Lake Lutoto have not been described. For reasons that will become apparent later, we regard them as two different species, distinct from those inhabiting other East African lakes and rivers. We refer to them tentatively as Haplochromis ‘‘Nshere’’ and Haplochromis ‘‘Lutoto.’’ The adults of both species have an average standard body length of 55 mm and hence belong to the category of small haplochromines. Males of H. ‘‘Nshere’’ are dark black with prominent orange-yellow egg dummies. The profile of their heads is straight. Females are a pale greenish color. H. ‘‘Lutoto’’ males have dark vertical bars on a dark greenish background. Their head profile forms a smooth concave line (fig. 3). The fishes were caught by angling, approximately 50 individuals in each lake, in less than 30 min using three fishing rods. Apparently, the fishes are abundant in both lakes.

Polymerase Chain Reaction (PCR), Cloning, and Sequencing

Amplifications were performed in the PTC-200 Programmable Thermal Controller (MJ Research, Biozym, Hessisch Oldendorf, Germany) or in the GeneAmp PCR System 9700 (Applied Biosystems, Weiterstadt, Germany). For the amplification of Mhc class II B exon 2, two Hot Start PCR systems were applied. In one system, 100 ng of genomic DNA were added to a reaction mixture of Mg2+-free 1 × PCR buffer, pH 8.5 (Invitrogen, Karlsruhe, Germany), 0.2 mM of each of the four deoxynucleoside triphosphates (Amersham Biosciences, Freiburg, Germany), 1 μM of each of the sense and antisense primers, 2.5 units of Taq DNA polymerase (Amersham Biosciences), and 0.4 units of Pfu DNA polymerase (Stratagene, Amsterdam, The Netherlands). The reaction mixture was overlaid with 2.5 mM Mg2+ HotWax Beads (Invitrogen) to initiate the reaction at high temperature. The amplification consisted of DNA denaturation for 1 min at 94°C, annealing for 15 sec at the appropriate annealing temperature, and 7 min extension at 72°C, followed by 34 cycles of 15 sec denaturation at 94°C, 15 sec annealing at the required temperature, and 2 min extension at 72°C. The reaction was completed by a final primer extension for 7 min at 72°C. Alternatively, 100 ng of genomic DNA were added to a reaction mixture of 1 × PCR buffer (Qiagen, Hilden, Germany), 2.5 mM MgCl2, 0.2 mM of each of the four deoxynucleoside triphosphates (Amersham Biosciences), 1 μM of each of the sense and antisense primers, 2.5 units of HotStar Taq DNA polymerase (Qiagen), and 0.4 units of Pfu DNA polymerase (Stratagene). To activate the enzyme, the amplification was initiated by a DNA denaturation step of 15 min at 95°C. The PCR was carried out with HotStar Taq DNA polymerase under the conditions given above. PCR products were purified by agarose gel electrophoresis and extraction using the QIA EX II Gel Extraction Kit (Qiagen) and cloned into the pUC18 vector with the help of the Sure Clone Ligation Kit (Amersham Biosciences). The primers used in the various PCRs are listed in table 1. The DNA was sequenced with the help of the Dyenamic Direct Cycle Sequencing Kit with 7-deaza dGTP (Amersham Biosciences). Sequencing reactions were processed by the LI-COR Long ReadIR 4200 DNA sequencer (MWG Biotech, Ebersberg, Germany).

<table>
<thead>
<tr>
<th>Locus</th>
<th>Orientation</th>
<th>Sequence 5′→3′</th>
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</thead>
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<td>CR F</td>
<td>TAAATCCTCTCTACTTCTCA</td>
</tr>
<tr>
<td></td>
<td>CR R</td>
<td>TCAAAAATAATGATAAACAAAC</td>
</tr>
<tr>
<td>Mhc class II B</td>
<td>F</td>
<td>TCTGAGTTATRGTGGRTGGCTGTC</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>GCAGTACSTCCTCTTCTSAGCTTCT</td>
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<td>1357 F</td>
<td>CAGCTGGATGAGTAGTGGAARSTTCTG</td>
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<td>1357 R</td>
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<tr>
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<td>1303 R</td>
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<td>1802 F</td>
<td>TGTAGTGTTIGTGTTAAGTCCCTTACTG</td>
</tr>
<tr>
<td></td>
<td>1802 R</td>
<td>CAAAAGCTGATATTTGTTAAGTCTGATAGA</td>
</tr>
</tbody>
</table>

Note.—CR indicates control region; F indicates forward; R indicates reverse.
Southern DNA Blotting and Hybridization

Five micrograms of genomic DNA were digested with Eco RI restriction endonuclease for 18 h under the conditions recommended by the supplier (Roche Diagnostics, Mannheim, Germany), and fragments were separated by agarose gel electrophoresis and blotted onto Hybond-N nylon filters (Amersham Biosciences). Prehybridization, hybridization, and probe labeling were carried out using the AlkPhos Direct Hybridization and Detection Kit (Amersham Biosciences). One hundred nanograms of DNA were used to label the probe. After the overnight hybridization, the filters were washed according to the AlkPhos Direct protocol. After the application of the chemiluminiscent detection reagent CDP-Star of the kit, Hyperfilm ECL (Amersham Biosciences) was exposed to the blot for 6 h and developed.

Phylogenetic Analysis

The multiple alignments of sequence data were made by using ClustalW 1.82 (Thompson, Higgins, and Gibson 1994) and checked by eye. The phylogenetic trees were drawn by the neighbor-joining (NJ) method (Saitou and Nei 1987) and evaluated by 1,000 bootstrap replications.

In the analyses of mtDNA control region sequences, the sites 518 to 535 of the alignment in Nagl et al. (2000) were eliminated because they contain many single nucleotide repeats and the sequences are not reliable. Sites with indels were also deleted. NJ trees were drawn using p-distances. Unless the substitution rate varies extensively in different lineages, p-distance is better in obtaining a correct tree topology than distances based on a complex substitution model, even when the substitution pattern of the nucleotide changes follows a complex model, particularly for closely related sequences (Nei and Kumar 2000). The alignment of group V sequences (fig. 4 [only variable sites are shown]) includes sequences from Nagl et al. (2000). The NJ tree for the group V sequences (fig. 5) was drawn based on the alignment in figure 4 with 810 shared sites.

In the mtDNA analyses, estimations of the mutation rate and population size, as well as Tajima’s test for neutrality, were based on an alignment containing sequences from the neighboring riverine and Lake Malawi fishes and a few Lake Tanganyika species as an outgroup with 804 shared sites (data not shown). The major branching patterns among the group V sequences of the NJ tree (not shown) based on the latter alignment were identical to those shown in figure 5. The GenBank accession numbers of the sequences that are not included in the group V NJ tree are as follows. Lake Tanganyika: Petrochromis orthognathus (U12549), Lobochilotes labiatu (U12550), Trophus duboisi (AF400737); Lake Malawi: Protomelas fenestratus (AF213625), Champrochromis pilorhynchos (U12553), Mylochromis lateristriga (AF213624), Crytocara morii (U12554), Lethrinops auritus (U12551), Stigmochromis woodii (AF213626), Labeotropheus trewavasae (AF213623), Pseudotropheus sp. “msobo” (AF213622), Melanochromis melanopterus (AF213619), Melanochromis parallelus (AF213621), Petrotilapia sp. (U12547), Metriaclima callainos (AF213620), Rhampochromis sp. (U12548); Haplochromines of the rivers and lakes in the Lake Victoria region: AF213559 to AF213609.

Since in the mtDNA control region the transition-transversion ratio is relatively high and the substitution rate varies extensively across sites (e.g., Tamura and Nei 1993), Kimura’s two-parameter distance with gamma correction was used for time estimation. The gamma parameter (a = 0.12) was estimated by the maximum-likelihood method using the tree topology of the NJ tree and the baseml in PAML 3.12 program (Yang 2002). In the maximum-likelihood analysis, the transition-transversion rate ratio was estimated as 7.1. Observed base frequencies were 0.31, 0.31, 0.16, and 0.23 for A, T, G, and C, respectively.

The average distance (h) between the two descendant clusters of interior nodes of the mtDNA control region NJ tree was computed as

\[ h = \sum_{i=A,j=B} d_{ij} / (2nm) \]

where \( d_{ij} \) is the distance between sequences i and j, A and B are the two descendant clusters of the node, and n and m are the numbers of the sequences in the clusters A and B, respectively.

The average distance between the mtDNA control region sequences of Lake Malawi and Lake Victoria fishes was 0.0445 ± 0.0073. Assuming that the separation of these two groups occurred 1 to 2 MYA (see also Sturmbauer et al. 2001), the mutation rate for the mtDNA control region was estimated as 2.2 – 4.5 \( \times 10^{-8} \) per site per year. This rate is similar to the mutation rate (1.8 \( \times 10^{-8} \)) estimated for other fish (snook [Donaldson and Wilson 1999]), but much lower than the rate for human mtDNA control region (0.75 \( \times 10^{-7} \) [Tamura and Nei 1993]).

An NJ tree of the cichlid Mhc class II B exon 2 sequences was drawn using all the available cichlid and closely related sequences in the GenBank database. In the NJ tree shown in figure 5, only representative sequences were included because of space limitations, and medaka fish (Oryzias latipes) sequences were used as an outgroup. In the alignment, there were 160 shared nucleotide sites after sites with missing nucleotides or indels were excluded. Jukes-Cantor distances were used.

Detailed information regarding the fish samples and the sequence data obtained for mtDNA control region, Mhc class II B exon 2, and SINE 1357 are available as Supplementary Material online.

Computer Simulation of Coalescence Process

The coalescence theory (Hudson 1983; Tajima 1983) and the polymorphism found at the SINE loci were used to obtain the lower limit of the effective population size. According to the theory, allelic lineages that exist in a current population have a genealogy that can be traced back to an MRCA. For a neutral nuclear locus of a population with an effective size N, the time (t) during which exactly j allelic lineages exist in the population...
Fig. 4.—Nucleotide alignment of mtDNA control region, haplogroup V sequences borne by East African haplochromines. Two sequences are from haplogroups II and IV and are shown for comparison. The majority consensus sequence is given at the top and identity with the consensus is indicated by dashes (–). Indels introduced to optimize the alignment are indicated by asterisks (*). Only variable sites are shown, and the numbering (to be read vertically downward) at the top is that used by Nagl et al. (2000 [in the electronic attachment for that article]). The Lake Lutoto and Lake Nshere sequences are the result of the present study; all others are from Nagl et al. (2000). A short segment (site from 518 to 535) has been omitted from the alignment and the identification of different haplotypes for the crater lake fishes because repeated stretches of a single nucleotide are difficult to resolve on the sequencing gels. Individual sequences are identified by a species abbreviation or sample number, followed by the GenBank accession code and locality number (in parentheses [see fig. 2]). For the Lake Lutoto and Lake Nshere sequences, the sequence types follow the species abbreviation and the numbers of samples that had the sequence types are shown instead of the GenBank accession code. Species abbreviations are as follows: *Anu,
FIG. 5.—Neighbor-joining tree of mtDNA control region haplogroup V sequences. The tree is based on the alignment in figure 4. The sequence designations are as in figure 4. Subgroups of group V are enclosed in brackets. Encircled numbers indicate important nodes; other numbers at nodes are bootstrap values (only those $>50\%$ are shown).

Astatotilapia nubila; Asve, A. velffer; Enci, Enterochromis cinctus; Gassi, Gaurochromis simpsoni; Halli, Haplochromis lividus; Halu, H. "Lutoto"; Hans, H. "Nshere"; Havo, H. rockkrakensis; Havhb, H. sp. "velvet black"; Limi, Lipochromis melanopterus; Neni, Neochromis nigricans; Pabe, Paralabidochromis beadlei; Pach, P. chilotes; Papl, P. plagiodon; Preq, Prognathochromis venator; Prsi, Psammochromis riponianus; Psra, Ptyochromis sauvagei; Ptxe, P. xenognathus; Ysla, Yssichromis laparagramma. Substitutions diagnostic for the bracketed subgroups are highlighted.

The accession numbers of H. "Nshere" (Hans 1), H. "Lutoto" (Halu 1), and H. "Nshere" (Hans 2) sequences are AY164680/AY164695 (Hans 1), AF213578 (Hans 2), AF213580 (Hans 3), AF213579/AY164695 (Hans 4), AY164687 (Hans 5), AY164688 (Hans 6), AY164689 (Hans 7), AY164691 (Hans 8), AY164692 (Hans 9), AF213577/AY164682/AY164683 (Halu 1), AY164681 (Halu 2), AF213575 (Halu 3), AF213576 (Halu 4), AY164684 (Halu 5), AY164685 (Halu 6), and AY164686 (Halu 7). Some of the accession numbers are from Nagl et al. (2000). The samples sequenced in Nagl et al. (2000) were resequenced and confirmed in this study.
corresponds to a random number generated according to the probability density of the exponential distribution with a mean of $2/[j(j-1)]$ in units of $2N$ generations (Simonsen, Churchill, and Aquadro 1995). In the computer simulation starting with $n$ lineages, the coalescence process was repeated until $n_a - 1$ lineages remained in the population, where $n$ is the number of genes sampled in either the $H$. “Nshere” or $H$. “Lutoto” populations, and $n_a$ is the number of alleles shared between $H$. “Nshere” or $H$. “Lutoto” and other lake populations. The time 

$$t_a = \sum_{j=n}^{n_a} t_j$$

required to reach this stage was recorded, the expectation of $t_a$ being

$$E(t_a) = 2 \frac{1}{n_a} - 1$$

in units of $2N$. By applying various values of $N$, we searched for the case in which the probability that $t_a > 50,000$ years (the geological estimate of the age of Lakes Nshere and Lutoto being < 50,000 years) was smaller than 0.05. For this value of $N$, the hypothesis that $n_a$ allelic lineages persisted for 50,000 years could be rejected at the 5% significance level. This value of $N$ represents the lower limit for the effective population size of a crater lake population.

Computer Simulation for Founding Population Size and the Number of Alleles Retained

To examine the relationship between the minimum number of $Mhc$ alleles retained in the population, the founding population size, and the population growth rate after the founding event, another computer simulation was conducted. The simulation was a slight modification of that used by Vincek et al. (1997) who assumed over-dominant selection for $Mhc$ loci with the selection coefficient $s = 0.01$. The alleles for the founding population of $N_b$ individuals were drawn at random from the parental population. After the founding event, population growth started immediately with rate $r$ and continued until the number of individuals in the population reached 10,000. Then, the number of alleles retained in the population was recorded. This process was repeated 1,000 times for each combination of the values of $N_b$ and $r$. The ranges of the $N_b$ and $r$ values varied from 5 to 500 and from 0.01 to 0.5, respectively. The allele frequencies in the ancestral population were assumed to be those reported for the $H{\text{LA-DRB}}1$ locus in Caucasians (Imanishi, Wakisaka, and Gojobori 1991). In a preliminary study, we also assumed that the frequencies of alleles in the ancestral population are equal. The results for these two situations were essentially the same.

Results

We divide the description of the results into two parts. In the first part we characterize the mtDNA control region, SINE, and $Mhc$ class II B loci of $H$. “Nshere” and $H$. “Lutoto.” In the second part we use this information to make inferences regarding these two populations. We begin with mtDNA.

mtDNA Control Region

Control regions of mtDNA were sequenced from 28 fish caught in Lake Nshere and 20 specimens from Lake Lutoto. Ten distinct $H$. “Nshere” and seven $H$. “Lutoto” haplotypes were found (fig. 4) among the 48 sequences obtained. The $H$. “Nshere” sequences showed more variability (one to seven differences) than those of $H$. “Lutoto” (one to three differences). Phylogenetic analysis revealed all 48 sequences to belong to haplogroup V characterized by the presence of T, C, C, A, and A at sites 27, 87, 96, 348, and 825, respectively (see Nagl et al. 2000 and fig. 4). Each of the two sets of sequences forms a separate cluster (a clade on a phylogenetic tree [fig. 5]) and each is identified by distinct diagnostic substitutions. All Lake Nshere sequences have A and T at sites 107 and 830; respectively, whereas all Lake Lutoto sequences share T, T, and G at sites 100, 347, and 830, respectively (fig. 4). We denote the control region subgroup of the VB haplogroup present in $H$. “Lutoto” as VE and that of $H$. “Nshere” as VF. The $H$. “Lutoto” sequences appear to be most closely related to a particular subset of VB sequences with which they share an A at site 635 (fig. 4). Similarly, the $H$. “Nshere” sequences are most closely related to another subset of VB sequences that have a G at site 635. Some of the VF sequences also share an A at site 167 with some members of this VB subset, but this nucleotide is also present in some members of the VC subgroup (figs. 4 and 5). These observations lead us to three conclusions. First, the haplochrome fishes inhabiting Lake Lutoto and Lake Nshere originated in an area in which the haplogroup VB is common. Second, the fishes of these two lakes originated from two distinct but related populations, probably occupying different parts of the area of wide VB-haplogroup distribution (see Discussion). Third, the fish populations of the two lakes have been isolated from other populations long enough to attain fixation of diagnostic substitutions. A network presentation of mtDNA haplotypes of the VB group and the crater lake fishes is available as Supplementary Material online.

Using the mutation rate of $2.2 - 4.5 \times 10^{-8}$ per site per year (see Material and Methods), we estimate the age of the nodes that separate the crater lake fishes from fishes of other localities (nodes 3 and 4 in fig. 5) as $0.18 \pm 0.06$ to $0.09 \pm 0.03$ MYA for $H$. “Nshere” and $0.12 \pm 0.05$ to $0.06 \pm 0.03$ MYA for $H$. “Lutoto” (table 2). These estimates give the upper limit for the time when the isolation of the crater lake fish occurred. The common ancestral nodes of the Lake Nshere and the Lake Lutoto fishes (nodes 5 and 6 in fig. 5) are estimated to date to $0.13 \pm 0.04$ to $0.06 \pm 0.02$ and $0.080 \pm 0.04$ to $0.04 \pm 0.02$ MYA, respectively. In contrast, the common ancestral node (node 7 in fig. 5) of the Lake Victoria fishes is dated to $0.23 \pm 0.08$ to $0.12 \pm 0.04$ MYA (table 2). These estimates suggest that the isolation of Lake Victoria fishes from other fishes occurred somewhat earlier than that of the crater lake fishes.
Table 2
Estimated Divergence Times of Groups of East African Haplochromine Fishes Indicated by Name of Locality or mtDNA Control Region Haplogroup

<table>
<thead>
<tr>
<th>Node</th>
<th>h (× 10⁻³)</th>
<th>Time Estimate (MYA)</th>
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</thead>
<tbody>
<tr>
<td>Lake Malawi versus Lake Victoria + rivers</td>
<td>44.5 ± 7.3</td>
<td>2</td>
</tr>
<tr>
<td>Lake Victoria (group V) versus rivers</td>
<td>30.5 ± 5.3</td>
<td>1</td>
</tr>
<tr>
<td>1.0 VR versus (VA + VB + VC)</td>
<td>7.2 ± 2.0</td>
<td>0.32 ± 0.09</td>
</tr>
<tr>
<td>2.0 VB versus VC</td>
<td>4.4 ± 1.2</td>
<td>0.20 ± 0.05</td>
</tr>
<tr>
<td>3.0 Lake Nshere versus VB</td>
<td>4.1 ± 1.2</td>
<td>0.18 ± 0.06</td>
</tr>
<tr>
<td>4.0 Lake Lutoto versus VB</td>
<td>2.6 ± 1.2</td>
<td>0.12 ± 0.05</td>
</tr>
<tr>
<td>5.0 MRC (Lake Nshere)</td>
<td>2.9 ± 1.0</td>
<td>0.13 ± 0.04</td>
</tr>
<tr>
<td>6.0 MRC (Lake Lutoto)</td>
<td>1.8 ± 1.0</td>
<td>0.08 ± 0.04</td>
</tr>
<tr>
<td>7.0 MRC (VC)</td>
<td>2.6 ± 1.0</td>
<td>0.23 ± 0.08</td>
</tr>
</tbody>
</table>

Note.—MRCA indicates most recent common ancestor. The separation of Lake Malawi sequences from Lake Victoria sequences and those of the neighboring river system and lakes were assumed to have occurred 1 or 2 MYA. The node numbers in the first column correspond to the numbers in figure 5. h is half the average distance between the two descendant clusters of the node.

Mhc Genes

The specimens used were the same as those tested in the mtDNA part of the study: 28 from Lake Nshere and 20 from Lake Lutoto. Altogether, 128 and 36 sequences were obtained from fishes of these two lakes, respectively. After the elimination of identical sequences and exclusion of single nucleotide differences, these numbers reduced to 14 and five different H. “Nshere” and H. “Lutoto” sequences, respectively (fig. 6). The number of different sequences per individual ranged from one to five (see Supplementary Material online for detailed information), indicating that at least some of the sequences were derived from different loci. To identify these loci, we subjected the sequences to phylogenetic analysis (fig. 7) and regarded sequences containing distinct, well-separated clades on the phylogenetic tree as representing separate loci (LC1 through LC7). Although these locus assignments must be regarded as tentative, our confidence in them is based on finding orthologous loci in related cichlid species from other localities (fig. 7). Southern blot analysis of genomic DNA isolated from these fishes and hybridized with exon 3 class II B-specific probes revealed the presence of multiple bands corresponding to different loci (fig. 8). (DNA of H. “Nshere” and H. “Lutoto” was not available in the quantity and quality necessary for Southern blot analysis of these fishes.)

The set of cichlid genes obtained so far is split into three major, well-separated lineages, which we refer to as I, II, and III (fig. 7). Lineage I, as presently defined, encompasses most known haplochromine class II B sequences, including those of haplochromines from Lake Victoria and Lake Malawi, as well as class II B sequences of Alcolapia (Oreochromis alcalicus), the tilapiine cichlids from Lake Natron and Lake Magadi, and Oreochromis niloticus. The lineage is divided further into the sublineages A and B described previously by Figueroa et al. (2000). Sublineage A comprises the loci LC4, LC5, and LC6 and sublineage B the loci LC1, LC2, and LC3. Both sublineages contain genes of both haplochromine and tilapiine cichlids. Lineage II, by contrast, contains no known genes of haplochromines from the Lake Victoria or Lake Edward regions (Figueroa et al. 2000), but does contain sequences derived from Lake Malawi and Tanganyika haplochromines and from the tilapiine O. niloticus (but not Alcolapia). Lineage III contains Mhc class II B sequences from Lake Malawi haplochromines and from the tilapiine cichlid O. niloticus, but no sequences from Lake Victoria or Lake Edward region haplochromines are present in this lineage. To what extent

Fig. 6.—Nucleotide alignment of Mhc class II B exon 2 sequences borne by H. “Nshere” (Hans) and H. “Lutoto” (Hali). Symbols are as in figure 4. Only variable sites are shown of 167 bp obtained and the numbering (to be read vertically downward) begins with codon 1 of exon 2. Sequences are designated by the locus (LC1 through LC7) followed by the GenBank accession number. The alleles are identified by ignoring single nucleotide differences (a nucleotide difference that appears in a single sequence data). The frequencies of the sequences in samples from Lake Lutoto, Lake Nshere, and the Lake Victoria region (LV) are shown on the right. The accession numbers of sequences of the LV region samples are AF232204 and AF232210 for LC3-AY211032, AF232207 for LC3-AY211033, AF232098 for LC4-AY211037, and AF232072 for LC6-AY211041.
FIG. 7.—Neighbor-joining tree of *Mhc* class II B exon 2 sequences of East African cichlid fishes. *H. 'Nshere' and *H. 'Lutoto' sequences produced in the present study are identified as *Hans* or *Hala*, respectively, followed by the number of the individuals and the GenBank accession number, and are shaded. Other sequences are from Figueroa et al. (2000), Mañá-Trillo et al. (1998), and Ono et al. (1993a, 1993b). I, II, and III indicate lineages, A and B indicate sublineages, and LC1 through LC7 indicate different loci. Individual sequences are identified by species.
the separation of sequences into these clades is influenced by
the choice of primers used for PCR amplification is
unclear at present.

One unique sequence of \( H \) ‘‘Nshere’’ is included in
lineage II; all other sequences of fishes from the two crater
lakes are included in lineage I and are approximately
equally distributed between the sublineages IA and IB
(fig. 7). Thus, a minimum of three class II \( B \) loci must exist
in fishes from crater lakes (IA, IB, and II). Additional loci
are defined by distinct clades within lineage I. Altogether,
seven possible class II \( B \) loci are defined in the
haplochromines of the two crater lakes (fig. 7). The
predicted number of loci is also supported by the results of
the Southern blot analysis (fig. 8).

Distribution of SINEs

Terai and his coworkers (2003) identified a set of
SINEs that have been inserted into the genomes of
endemic Lake Victoria and Lake Edward region haplo-
chromines after the divergence of the Lake Victoria from
the Lake Malawi flock and persist in most of the species
and populations as presence or absence polymorphisms.
To test for the presence or absence of these SINEs in the
individual \( H \) ‘‘Nshere’’ and \( H \) ‘‘Lutoto’’ specimens, we
used primers specific for the flanking regions of SINEs
1350, 1840, 1424, 1801, 1807del, 1909, and 1919 in PCR.
Of the seven SINEs tested, two were found to be poly-
morphic in \( H \) ‘‘Nshere’’ (1918 and 1901), whereas only
one (1918) was polymorphic in \( H \) ‘‘Lutoto’’; all other
SINEs were fixed in the two populations (table 3 [see also
Terai et al. 2003]). Comparison of observed and expected
frequencies of the genotypes at the polymorphic SINEs
revealed both populations to be in Hardy-Weinberg
equilibrium (table 3). This result suggests that neither of
the two populations is structured.

One of the fixed SINEs (1357) described by Terai
et al. (2003) was shown by these authors to display sequence
polymorphism, revealing the existence of at least 14 alleles
in the haplochromines of the Lake Victoria basin. To
obtain additional information about the \( H \) ‘‘Nshere’’ and
\( H \) ‘‘Lutoto’’ populations, we sequenced the 1357 locus
of individual fishes from these populations (fig. 9). The
sequences obtained in the present study were shorter than
those from Terai et al. (2003) in that they lacked 36 bp of the
5′ region and 38 bp of the 3′ region. For this reason,
alleles \( D1 \) and \( D3 \), which differ at site 31 in the missing
region, could not be distinguished in the sequences
obtained in this study. Of the 14 alleles, at least four \((D1
or D3, D4, D5, and E1) were found in \( H \) ‘‘Nshere,’’ but
only one \((D1 or D3) was detected in \( H \) ‘‘Lutoto.’’ No new
SINE 1357 alleles were found in either of the two
populations.

Present Population Sizes of \( H \) ‘‘Nshere’’ and \( H \) ‘‘Lutoto’’

Information about the effective population sizes of \( H \)
‘‘Nshere’’ and \( H \) ‘‘Lutoto’’ is provided by the mtDNA
ccontrol region and SINE data. In the case of the mtDNA
control region sequences, we computed the nucleotide
diversity (average number of nucleotide differences per
site, \( \pi \) [see Nei 1987]) as being 3.41 \( \times 10^{-3} \) and
7.46 \( \times 10^{-4} \) for the \( H \) ‘‘Nshere’’ and \( H \) ‘‘Lutoto’’
populations, respectively. In a mutation-drift equilibrium,
\( E(\pi) = 2Nf/\pi \), where \( Nf \) is the effective population size of females and \( \pi \) is the
mutation rate per generation. By taking \( \pi = 2.2 \times 10^{-8}
\) to 4.5 \( \times 10^{-9} \) per site per year (see Materials and Methods)
and assuming that 1 generation = 1 year in cichlid fishes,
we obtain \( Nf = 3.4 \times 10^5 \) to 7.7 \( \times 10^6 \) for \( H \) ‘‘Nshere’’
and \( Nf = 0.8 \times 10^4 \) to 1.7 \( \times 10^5 \) for \( H \) ‘‘Lutoto.’’

The Lower Limit for the Effective Population Size of
Crater Lake Fishes

Since polymorphism at some of the SINE loci is
shared by the crater lake fishes and fishes of the
neighboring region, we used the number of shared alleles
and the coalescence theory to obtain the lower limit of the
effective population size for \( H \) ‘‘Nshere’’ and \( H \) ‘‘Lutoto.’’ As described in Materials and Methods,
we computed the probability of \( n_a \) allelic lineages persisting
for 50,000 years by the coalescence algorithm (Hudson

\( \text{abbreviations, GenBank accession codes (see Ono et al. 1993a, 1993b; Málaga-Trillo et al. 1998; Figueroa et al. 2000), and locality (LM: Lake Malawi,
LT: Lake Tanganyika), or mtDNA haplotype. Species abbreviations are as follows: Aska, Astatotilapia katavi; Asna, A. rubida; Asve, A. velifer; Auhu,
Aulonocara hansbaenschii; Cyfr, Cyphotilapia frontosa; Enci, Enterochromis cinctus; Gasi, Gaurochromis simpsonii; Habb, Haplochromis sp. ‘‘big
blue’’; Habi, H. sp. ‘‘black’’; Hahn, H. sp. ‘‘high neck’’; Hali, H. lividus; Haro, H. rockhribensis; Hast, H. sp. ‘‘stone’’; Habv, H. sp. ‘‘velvet black’’;
Meau, Melanochromis auratus; Meze, Metriaclima zebra; Neni, Neochromis nigricans; Or1, Oreochromis alcalicus; Or1a, Oryzias latipes; Orni,
Oreochromis niloticus; Pabe, Paralabidochromis beadlei; Ptsa, Pyrrichromis sauvagei; Ptxe, P. xenognathus; Puny, Pandanopia nyererei; Yspi, Ysichromis pyrrhocephalus. Numbers at nodes are bootstrap values (only those >50% are shown).

\[ \text{Origin and Speciation of East African Crater Lake Fishes} \]
Table 3
Observed and Expected Genotype Frequencies (%) at SINE Loci in Crater Lake Cichlids

<table>
<thead>
<tr>
<th>Locus</th>
<th>H. “Nshere” (n = 28)</th>
<th>H. “Lutoto” (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>++/+</td>
<td>+/-</td>
</tr>
<tr>
<td>1918</td>
<td>obs 27</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>exp 29</td>
<td>50</td>
</tr>
<tr>
<td>1801</td>
<td>obs 22</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>exp 27</td>
<td>50</td>
</tr>
<tr>
<td>1424</td>
<td>obs 100</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>exp 100</td>
<td>0</td>
</tr>
<tr>
<td>1919</td>
<td>obs 0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>exp 0</td>
<td>0</td>
</tr>
<tr>
<td>1350</td>
<td>obs 100</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>exp 100</td>
<td>0</td>
</tr>
<tr>
<td>1840</td>
<td>obs 100</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>exp 100</td>
<td>0</td>
</tr>
<tr>
<td>1807</td>
<td>obs 100</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>exp 100</td>
<td>0</td>
</tr>
</tbody>
</table>

Note.—n is the number of individuals tested; na is the number of individuals for which data were not obtained; obs indicates observed genotype frequencies; exp is the expected genotype frequencies; +/+, +/-, and -/- indicate homozygote for the presence of SINE insertion, heterozygote, and homozygote for the absence of SINE insertion, respectively; +d indicates SINE insertion variant with 216-bp deletion. None of the $\chi^2$ values indicated significant deviation from Hardy-Weinberg equilibrium.

1983; Tajima 1983) for various population sizes (N) and searched for the value of N for which $n_a$ lineages persist for more than 50,000 years in 5% of the iterations of the computer simulation.

We can assume $n_a = 2$ in the case of the presence/absence polymorphism of SINES 1918 and 1801 in H. “Nshere” and 1918 in H. “Lutoto”; and $n_a = 4$ for the alleles determined by sequencing SINE 1357 and found to be shared between H. “Nshere” and other lake populations. Using the $n_a = 2$ value, we can reject the hypothesis that N was smaller than 6,000 individuals at the 5% significance level for the past 50,000 years of existence of the H. “Nshere” and H. “Lutoto” populations. The 6,000 individuals thus represent the lower limit for the effective sizes of these two populations. Using the $n_a = 4$ value, the results of the computer simulation indicate that $N = 24,000$ is the lower limit of the effective H. “Nshere” population size that assures the persistence of at least four allelic lineages for the last 50,000 years.

Population History

Tajima (1989) designed a test that examines

$$d = k - S \sqrt{\left( \sum_{i=1}^{n-1} 1/i \right)} = 0$$

by using the statistic

$$D = d \sqrt{\hat{V}(d)}$$

where $S$ is the number of segregating sites in nucleotide sequences, $n$ is the number of sequences obtained, and $k$ is the average number of nucleotide differences per sequence. This test is based on relationships existing under the mutation-drift equilibrium

$$E(S) = M \left( \sum_{i=1}^{n-1} 1/i \right)$$

and $E(k) = M$, where $M = 2N_v \nu$ for the mtDNA sequences and $\nu$ is the mutation rate per sequence in this case. It was developed for testing the neutrality of a locus under the assumption that a population is in the mutation-drift equilibrium. However, if we assume that neutrality holds at the locus, the test can also be used for examining a population size change in the past. A negative value of $D$ indicates an excess of $S$ relative to $k$, which suggests a recent rapid accumulation of mutations by population expansion. The results of the Tajima test applied to the H. “Nshere” and H. “Lutoto” populations are shown in Table 4. Tajima’s $D$ statistic is negative for both H.
The Nucleotide Differences in mtDNA Control Region and Tajima's Test of Neutrality

<table>
<thead>
<tr>
<th></th>
<th>H. “Nshere”</th>
<th>H. “Lutoto”</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>28</td>
<td>20</td>
</tr>
<tr>
<td>π</td>
<td>$3.41 \times 10^{-3}$</td>
<td>$7.46 \times 10^{-4}$</td>
</tr>
<tr>
<td>k</td>
<td>3.12</td>
<td>0.60</td>
</tr>
<tr>
<td>S</td>
<td>14.00</td>
<td>6.00</td>
</tr>
<tr>
<td>D</td>
<td>-0.45</td>
<td>-2.06*</td>
</tr>
</tbody>
</table>

Notes.—n is the number of samples from which sequences are obtained; π is the average nucleotide difference per site; k is the average nucleotide difference per sequence; S is the number of segregating sites; D is Tajima’s D statistic.

* D is significant at 5% level.

“Nshere” and H. “Lutoto,” but the result is significant only for the latter, suggesting that the population of H. “Lutoto” expanded recently. The population size estimates shown in the earlier section were obtained by $\pi$, which is equivalent to $k/m$ where $m$ is the number of nucleotide sites examined. If we use the relationship

$$E(S) = \left( \sum_{i=1}^{n-1} 1/i \right) (2N_v \nu)$$

$N_v$ is estimated as $2.4 \times 10^4$ to $4.7 \times 10^4$ for H. “Lutoto.” Although a large error is associated with the estimate using $S$, it is likely that the female population size of H. “Lutoto” is currently larger than $0.8 \times 10^4$ to $1.7 \times 10^4$.

Founding Population Size

Among the exon 2 Mhc class II B gene sequences we obtained, we found 14 different ones in H. “Nshere” and five different ones in H. “Lutoto.” All these sequences, with one exception, belong to lineage I; the exceptional sequence found in H. “Nshere” belongs to the lineage II sequences of *Metriaclima zebra* (fig. 5). The lineage I sequences can be divided into six loci (see fig. 5), or they can be treated as one locus. By considering these two extreme possibilities, the numbers of alleles that were passed onto the founding populations of the crater lakes from the ancestral population are one to four for H. “Nshere” and one for H. “Lutoto” in the former case and 12 for H. “Nshere” and five for H. “Lutoto” in the latter case.

In a computer simulation similar to that described by Vincek et al. (1997), we examined the number of alleles retained for various founding population sizes ($N_b = 5$ to 500) and growth rates ($r = 0.01$ to 0.5) (see Materials and Methods). Only representative values ($r = 0.01, 0.05, 0.5$) are shown in figure 10. In figure 10A, the average numbers of alleles ($n_{ave}$) retained when the number of individuals reached 10,000 in 1,000 replications of computer simulation are shown in relation to the founding population size ($N_b$). In figure 10B, instead of the average number of alleles ($n_{ave}$), the 95% upper limit of the number of alleles ($n_u$) retained in the population is shown. That is, in at least 95% of the replications, the number of alleles retained in the population was smaller than $n_u$. For each $N_b$ value, we can reject that the number of alleles retained in the population is smaller than $n_u$ at the 5% significance level. Thus, $N_b$ values in figure 10B can give a lower limit of the founding population size for the observed number of alleles $n_u$. The result of the simulation suggests that four or five alleles can be retained even in the case of a small founding population (5 to 10 individuals) if the growth rate of the population after the founding event is not small (< 0.1 to 0.2). The founding population must contain at least 20 breeding individuals to retain 12 alleles, even for a growth rate as high as 0.5. For a relatively low growth rate (0.01), the founding population must be at least 50 to 70 to retain four to five alleles and at least 300 individuals to retain 12 alleles.

Discussion

Mitochondrial DNA haplotyping assigns the H. “Nshere” and H. “Lutoto” populations to the VB group, which is widely distributed throughout the Lake Edward region. On the phylogenetic tree (fig. 5), the set of VB sequences closest to those of H. “Nshere” stemmed from localities 39 (Kazinga Channel), 40 (Lake Edward), 44 (Lake George), and 45 and 46 (Lake Albert [see fig. 2]). Similarly, the set of VB sequences closest to those of H. “Lutoto” is derived from the same area (localities 39 and 40), although here related sequences were also found in Lake Albert. We suggest, therefore, that the founders of the two crater lake populations originated in the Kazinga Channel but that haplotypes closely related to those they carried were distributed across much of the entire region. Since the nearest neighbors of H. “Nshere” and H. “Lutoto” on the tree are different subsets of the VB subgroup, we suggest further that the founders came from different parts of the Kazinga Channel or from different populations and that the colonizations of the two lakes occurred independently. There is certainly no evidence that H. “Nshere” originated from H. “Lutoto,” or vice versa.

Geologists date the earliest volcanic activity in the Lake Lutoto and Lake Nshere region to approximately 50,000 years ago (Boven et al. 1998). This date places an upper limit on the origin of the two lakes and hence also on the time of their colonization by fishes. The date is younger than the estimated time required for the mtDNA control region haplotypes now present in H. “Nshere” and H. “Lutoto” to coalesce to the MRCA. The difficulty with coalescence time estimates, however, is that the calibration of the mtDNA molecular clock is unreliable. For the East African cichlid fishes, the clock is commonly calibrated by the geological age of Lake Malawi (Meyer et al. 1990; Nagl et al. 2000; Sturmbauer et al. 2001), which, however, is uncertain (Schlüter 1997). The estimates range from 0.5 to 4 Myr, depending on the part of the lake examined and the dating method. In an earlier publication (Nagl et al. 2000), we calibrated the clock on both the 2 Myr and 4 Myr age of Lake Malawi. Here, we also take the younger age estimate (1 Myr) into account. Thus calibrated, the molecular clock dates the MRCA of the mtDNA control region of H. “Nshere” to 60,000 or 130,000 years ago and that of H. “Lutoto” to 40,000 or 80,000 years ago (the two alternatives represent datings based on the 1 or 2 Myr age of Lake Malawi). Both estimates for H. “Nshere” exceed
the upper limit set by the geological dates, but the H. "Lutoto" estimates come reasonably close to it. Of course, large errors are associated with these time estimates (table 2), and the geological dating of the crater lakes is subject to uncertainty (Boven et al. 1998). It is therefore difficult to tell whether or not the age of the MRCAs of the crater lake fishes really exceeds the geological date of the lakes. In principle, there can be two reasons for the discrepancy between molecularly and geologically estimated dates. First, the coalescence time to the MRCA need not coincide with the time of lake colonization and emergence of a species because polymorphism could have passed through the speciation phase. In the case of H. "Nshere" and H. "Lutoto," however, there is no indication that any of the mtDNA control region haplotypes now present in the population existed before the emergence of these species. Second, Lake Malawi could in fact be younger than 1 Myr or the divergence of the Lake Victoria basin cichlids from the Lake Malawi fishes might have significantly postdated the emergence of the lake (Stemberger et al. 2001). Be this as it may, a large standard error of the molecular clock estimate leaves the door open for a younger age of the MRCA. In relative terms, the ages of H. "Nshere" and H. "Lutoto" may be comparable to those of the endemic Lake Victoria species.

Both the SINE and the mtDNA data indicate that the populations of H. "Nshere" and H. "Lutoto" are quite large. The lower limit of N (effective size of a whole population) from the SINE data is 6,000 for H. "Lutoto" and 24,000 for H. "Nshere," and the Nf (female effective population size) estimate from the mtDNA control region data is 38,000 to 77,000 for H. "Nshere" and 8,000 to 17,000 for H. "Lutoto." For lakes that have each a surface area of less than 1 km², these are indeed large effective population sizes. In a large population, selection—where applicable—gains the upper hand over random genetic drift. It can lead to the rapid fixation of mutations introducing adaptive modifications. This potential does not seem to have been exploited by fishes of Lake Nshere and Lake Lutoto, but has apparently been capitalized on by fishes in some other crater lakes and by haplochromines of Lake Victoria. In West Africa, the volcanic crater lakes Barombi Mbo and Bermin in Cameroon harbor 11 and nine tilapiine cichlid species, respectively (Trewavas, Green, and Corbet 1972; Stiassny, Schliewen, and Dominey 1992), which apparently arose in situ from a single ancestor species by sympatric speciation (Schliewen, Tautz, and Pääbo 1994). Similarly, Lake Tana in Ethiopia provides a home for 14 morphotypes of large barbels (Barbus intermedius complex), all adapted to different ecological niches, exploiting different food resources, and derived from a common ancestral species within the lake (Nagelkerke, Sibbing, and Osse 1995; Dixon et al. 1996). The tremendous morphological diversity of the endemic Lake Victoria haplochromines is well documented (Greenwood 1981; Seehausen 1996). The difference between Lake Victoria on the one hand and Lake Nshere and Lake Lutoto on the other is, of course, that Lake Victoria, being the third largest lake in the world, offers many different ecological niches for adaptive radiation that the crater lakes do not. By contrast, Lake Barombi Mbo and Lake Bermin on the one hand and Lake Nshere and Lake Lutoto on the other are comparable in size. Whether they are also comparable in the number of ecological niches they potentially provide for fishes is not known to us. The reasons why cichlids adaptively radiated in the West African but not in the two East African crater lakes are unclear.

The lower limits of N (6,000 for H. "Lutoto" and 24,000 for H. "Nshere") obtained in this study were based on the assumption that the size remained constant over 50,000 years. If this was the case, the founding populations of these two species must have been quite large. Although a bottleneck phase is not excluded by these considerations, if it did occur it must have been of short duration and followed by very rapid expansion of the population to its present size. The lower limit of the bottleneck is indicated by the Mhc data. It is 10 to approximately 300 breeding individuals, depending on how the data are interpreted. These numbers make the hypothesis involving the "seeding" of the lakes by fish eggs or fry transmitted by animals an unlikely proposition. Even if birds were able to deliver up to 300 founders into each of the two lakes, one would not expect the eggs or fry to be of the same origin in terms of species and locality, whereas all the available evidence suggests a monophyletic origin of both H. "Nshere" and H. "Lutoto" in a single founding event. Most likely, the founders of H. "Nshere" and H. "Lutoto" reached the lakes by way of rivers, and perhaps the way to Lake Lutoto was longer or more difficult than the way to

FIG. 10.—The results of computer simulations showing the relationship between the founding population size and the number of alleles retained in the population. Nf is the number of individuals in the founding size; r is the growth rate. (A) 6nave is the average number of alleles retained in the population. (B) 6nave is the 95% upper limit of the number of alleles. Nf gives the lower limit of the founding population size for retaining 6nave alleles. The number of alleles in the population exceeds Nf in less than 5% of iterations.
Lake Nshere, so fewer fish reached the former than the latter body of water. In both cases, however, the genetic diversity of the founding populations might have been greater than was ultimately retained. This conclusion is indicated by a comparison of the genetic variability currently found in the crater lake populations and that present among the Kazinga Channel fishes. Some loss of diversity apparently occurred by random genetic drift during the expansion phase after the founding event. The isolation of the crater lake population might have been another factor contributing to the loss of variability.

All the genetic systems we tested consistently indicate that compared with H. "Nshere," H. "Lutoto" is genetically a more homogeneous population. Whereas in H. "Nshere," we detected 10 different mtDNA control region haplotypes, found four of the six Mhc class II B loci to be polymorphic with two to four alleles per locus, could establish the presence or absence of polymorphism at two of the five SINE loci, and found four alleles at the SINE 1357 locus, in H. "Lutoto" we found seven mtDNA alleles, all the Mhc class II B loci to be monomorphic, one of the SINE loci to be polymorphic, and only one SINE 1357 allele. The reason for this difference between H. "Nshere" and H. "Lutoto" populations is not immediately apparent. The founding population of H. "Lutoto" might have been smaller than that of H. "Nshere," as mentioned earlier, or, as the Tajima test indicates, the H. "Lutoto" population might have experienced a recent bottleneck and currently be in a recovering phase as a consequence of some event such as a change in water level.

In contrast to molecular diversification, morphological diversification reveals just the opposite trend when the populations of the crater lakes and Lake Victoria are compared. The two crater lake species are rather similar in appearance, the differences between them in body shape and coloration being inextensive. Similarly, the morphological variation within each crater lake population is relatively minor (H. Tichy and E. Schraml, unpublished data). By contrast, the endemic Lake Victoria species have diverged much more extensively from each other morphologically and each shows considerable intraspecies variation (Greenwood 1981; Seehausen 1996). These disparate trends can be attributed to the differences in opportunities for adaptation to distinct environmental niches on the one hand and to opportunities to mix diverging populations in the large and small lakes on the other hand.

At the molecular level, the most striking difference between speciation of haplochromines in the crater lakes and in Lake Victoria is in the fixation of substitutions. Although, as stated earlier, the endemic haplochromines in the crater lakes and in Lake Victoria had approximately the same length of time to evolve, the crater lake species each accumulated two or three diagnostic substitutions in the control region of their mtDNAs since the time of their origin from the founding populations, but the endemic Lake Victoria species tested thus far have not accrued any such fixed changes (Nagl et al. 1998, 2000). This difference is most likely attributable to the different degrees of isolation of the populations. Whereas the crater lake populations apparently evolve in complete physical isolation with only one species per lake, in Lake Victoria the reproductive barriers between the emerging species may be leaky. The barriers may be sufficiently strong to segregate characters under selection, differentiating the populations morphologically and behaviorally for longer periods of time, but not strong enough to assure fixation of neutral mutations by random genetic drift. Occasional breaches of the barrier may keep these mutations in a polymorphic state. An important implication of these deductions is the tentativeness of the early phases of speciation in the endemic Lake Victoria haplochromines. We suggest that as long as the environmental conditions in the lake remain stable, the different species may evolve in isolation from one another. Dramatic changes in the conditions may, however, usher in a phase of a limited gene flow between some of the species and so prevent fixation of neutral mutations. The ease with which the Lake Victoria haplochromines produce interspecies hybrids in aquaria attests to the weakness of the reproductive barriers between the species.

Acknowledgments

We thank Erwin Schraml for help in sample collecting during the expeditions, as well as Sabine Rosner for technical and Jane Kraushaar for editorial assistance.

Literature Cited


Accepted April 20, 2003