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Phylogeography and Evolution of the Tanganyikan Cichlid Genus *Tropheus* Based upon Mitochondrial DNA Sequences

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Abstract. Lake Tanganyika harbors the oldest and most diverse species flock of cichlid fish. Many species are subdivided into numerous genetically and phenotypically distinct populations. Their present distribution and genetic structure were shaped by a series of lake level fluctuations which caused cycles of isolation and admixis and promoted dispersal events. One of the best examples of this phenomenon is the genus *Tropheus*. We present a comprehensive mtDNA phylogeny based upon 365 individuals of 55 populations from all over Lake Tanganyika, which suggests an almost-contemporaneous origin of eight major mitochondrial lineages about 700 Ka ago. While the distribution of seven lineages is restricted to particular sections of the lake shore, one lineage was found to have a much more widespread distribution. This particular lineage is subdivided into four sublineages which seem to have originated from a single dispersal event about 400 Ka. By using a molecular clock estimate in combination with geological data we derived a hypothetical scenario for the colonization history of *Tropheus*. Thereby we show a high degree of concordance between major changes of the lake level in the recent history of Lake Tanganyika and three distinct diversification events in this genus.

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Introduction

For more than 100 years the Great East African lakes have captured the attention of evolutionary biologists. These lakes have produced enormously diverse cichlid fish faunas which represent outstanding examples for the study of explosive speciation and adaptive radiation. Lake Tanganyika, with an age of 9 to 12 million years (Ma), is by far the oldest of the East African lakes (Cohen et al. 1993) and harbors the morphologically, behaviorally, and genetically most diverse flock of cichlid fish (Fryer and Iles 1972; Greenwood 1984; Mayr 1984; Meyer 1993). Many of the about 200 described species are subdivided into geographically and genetically distinct populations that vary mainly in their coloration. One of the best examples of this phenomenon is the endemic genus *Tropheus*, of which six nominal species and more than 70 distinctly colored local variants are currently described (Poll 1986; Schupke 1994). Except for *T. duboisi*, the overall morphology remained highly similar in this genus. *Tropheus* is abundant in the upper littoral zone in all types of rocky habitats, where it feeds on epilithic algae and takes shelter from predators, whereas sandy or muddy shores and river estuaries are strictly avoided. There is strong evidence from ecological and genetic studies that *Tropheus* is not capable of crossing greater distances

over unsuitable habitats or open water (Brichard 1978; Sturmbauer and Meyer 1992; Sturmbauer et al. 1997), as a consequence of its pronounced habitat specificity, site fidelity, and territoriality.

So far, *Tropheus* is one of the most intensively studied genera from Lake Tanganyika. Ethological studies on *T. moorii* demonstrated complex behavioral patterns and a highly developed social organization (Wickler 1969; Nelissen 1976; Yanagisawa and Nishida 1991; Sturmbauer and Dallinger 1995). There is no pronounced sexual dimorphism. Both sexes keep territories, and unlike many other maternal mouthbrooders *Tropheus* forms temporary breeding pairs (Sturmbauer and Dallinger 1995). Mouthbrooding is very complex and performed exclusively by females (Nelissen 1976; Kawanabe 1981; Yanagisawa and Sato 1990; Yanagisawa and Nishida 1991; Sturmbauer and Dallinger 1995). Previous phylogeographic studies on *Tropheus* demonstrated surprisingly large genetic differences among populations (Sturmbauer and Meyer 1992; Sturmbauer et al. 1997). *Tropheus duboisi* was identified as the most ancestral branch, and after that split seven distinct mtDNA lineages arose almost contemporaneously. Six of these lineages were limited to particular shore regions of the lake, while one lineage secondarily expanded its range of distribution to colonize several rocky habitats throughout the lake. The mtDNA data also showed that—despite similar overall morphology—coloration may vary tremendously among genetically closely related populations, while in other cases it can be very similar among genetically distantly related populations or sister species. This observation was explained in part as the consequence of parallel evolution of similar color patterns within the neutral space of sexual and natural selection or as the result of introgression among two genetically distinct populations after secondary contact and subsequent lineage sorting. The published mtDNA phylogeny is in partial conflict with the existing taxonomy, since some of the species described so far appeared to be paraphyletic (Sturmbauer and Meyer 1992; Sturmbauer et al. 1997).

The study of phylogeographic patterns in species undergoing adaptive radiation provides important information about the driving forces of diversification and speciation. The rapid formation of large species flocks in East African cichlids is currently thought to be triggered by abiotic (physical) factors, such as geological processes and climatic events, as well as by biological characteristics of the radiating organisms. Several studies demonstrated that major fluctuations of the lake level had severe impact on rocky habitats and their species communities in East African rift lakes (Owen et al. 1990; Sturmbauer and Meyer 1992; Johnson et al. 1996; Sturmbauer et al. 1997; Sturmbauer 1998; Rüber et al. 1998; Nagl et al. 2000). Any rise of the lake level will shift the shoreline according to the basin structure of the lake and new rocky habitats are formed. Whenever distances between newly formed habitats exceed the dispersal ability of a species, gene flow will be interrupted and genetic differences between populations will accumulate. A subsequent decrease in the lake level might lead to secondary admixis, causing either an increase in genetic diversity or sympatry of new species. *Tropheus*, with its large number of geographic variants, represents an ideal model organism for tracing such historical processes. The fact that these fish are among the most popular cichlids in aquaristics, and that they are exported throughout the world, makes it possible to obtain samples from remote areas all over Lake Tanganyika.

In this study we present the most comprehensive mtDNA phylogeny of *Tropheus* so far, including 55 geographical variants distributed all over Lake Tanganyika. By applying a new molecular clock for the control region which was independently derived from Lake Malawi cichlids (Sturmbauer et al. 2001), it was possible to gauge the time spans of diversification events within and among *Tropheus* lineages. Patterns of genetic divergences were then related to geological data on historic lake level fluctuations (Lezzar et al. 1996; Cohen et al. 1997), with the goal of reconstructing a hypothetical scenario of the origin and spread of the genus *Tropheus*.

Materials and Methods

Samples and Molecular Techniques

The present study is based upon 243 haplotypes of 365 sequenced specimens of the genus *Tropheus* from 55 localities (45 specimens sequenced earlier and 320 newly sequenced specimens), covering almost the entire shoreline of Lake Tanganyika. Due to computational limitations, subsets of taxa had to be drawn, taking care to represent all genetic subgroups and shore sections. The localities and the exact numbers of individuals analyzed from each locality are reported in Table 1 and Fig. 1. The majority of the *Tropheus* samples was collected during expeditions in 1991, 1992, 1995, and 1999, and some additional specimens from remote areas of the lake in the Congo were provided by importers and aquarists. Voucher specimens of the collected species have been deposited at the Royal Museum of Natural History in Brussels, Belgium, and the Department of Zoology and Limnology of the University of Innsbruck, Austria.

Total DNA was isolated from ethanol-preserved white muscle tissue or fin-clips using Chelex 100 (Walsh et al. 1991) or proteinase K digestion followed by sodium chloride extraction and ethanol precipitation (Bruford et al. 1998). We chose to sequence a 446-bp segment of the mitochondrial control region, including also a part of the threonin tRNA gene, as well as the proline tRNA gene. In addition, from 29 individuals representing all major mtDNA lineages, a 402-bp segment of the cytochrome *b* gene was sequenced. PCR conditions followed standard protocols (Kocher et al. 1989; Lee et al. 1995) and DNA sequencing was carried out using the BigDye Terminator cycle sequencing kit (Applied Biosystems, USA) on an ABI 373A automatic DNA Sequencer (Applied Bio-

Table 1. Localities and number of specimens of *Tropheus* included in the study

Locality name	Locality No.	*N*,[a] mt control region	*N*,[b] cytochrome *b*
Bemba	55	(10) 7/2*	1/1*
Bilila Island	49	(2) 1/1[c]	1/1
Bulu	13	(8) 8/5[d]	
Chaitika	33	(15) 3/2	
Chilanga	35	(2) 2/2	
Chimba	39	(2) 1/1	
Chipimbi	40	(2) 1/1	
Chisiki	38	(1) 1/1	
Funda	31	(30) 3/2	
Ikola	16	(2) 2/1*	1/1*
Inangu	34	(4) 2/1	1/1
Kabezi	1	(1) 1/1*	1/1
Kabimba	51	(2) 2/2*	
Kabwe	11	(2) 2/1	1/1
Kachese	37	(2) 1/1*	
Kala	25	(3) 2/2*	1/1*
Kalambo	27	(2) 1/1*	
Kalanswi	19	(2) 1/1	
Kalemie	47	(1) 1/1*	
Kasanga	26	(2) 1/1	
Katoto	30	(61) 19/6	2/2
Katukula	32	(30) 5/4	
Kavalla	50	(1) 1/1*	
Kibwe	9	(2) 2/2	1/1
Kibwesa	14	(7) 7/6[e]	2/2
Kipampa	48	(7) 2/1	
Kiriza	53	(2) 2/2*	1/1*
Kiti Point	7	(3) 3/3	1/1
Kungwe	12	(12) 12/3[f]	3/3[g]
Kyeso	46	(12) 8/8[c]	2/2
Lupota	43	(1)1/1*	
Manda	20	(3) 1/1	
Masaka	6	(4) 4/1	
Mbita Island	28	(24) 2/2	
Mboko	54	(1) 1/1*	
Minago	3	(1) 1/1*	
Mkagansi	15	(2) 1/1	
Mkombe	17	(2) 2/2	
Mkuyu	8	(2) 2/1	
Moba	45	(1) 1/1*	1/1
Moliro	41	(3) 1/1	
Mpimbwe	18	(1) 1/1*	
Mpulungu	29	(46) 7/3	1/1
Mvua	42	(11) 7/6	2/2
Ngombe	5	(3) 2/2	1/1
Nkondwe Island	21	(1) 1/1	
Nvuna Island	22	(1) 1/1	1/1
Nyanza Lac	4	(1) 1/1*	1/1*
Rutunga	2	(3) 3/2*	1/1*
Segunga	10	(2) 2/2	
Sumbu	36	(2) 1/1	
Ubwari	52	(1) 1/1*	
Wapembe north	23	(3) 3/3*	1/1
Wapembe south	24	(10) 2/1	1/1
Zongwe	44	(4) 4/4[h]	1/1*

Note. *Published sequences.

[a] Total number of sequenced individuals (in parentheses); number of specimens included in study/number of haplotypes.

[b] Number of sequenced specimens/number of haplotypes.

[c] Specimen from a new locality; sequence indentical to a published sequence from Kungwe.

[d] Three sequences published; two new.

[e] Five sequences published; one new.

[f] Four sequences published; two new.

[g] Two sequences published; one new.

[h] Two sequences published; two new.

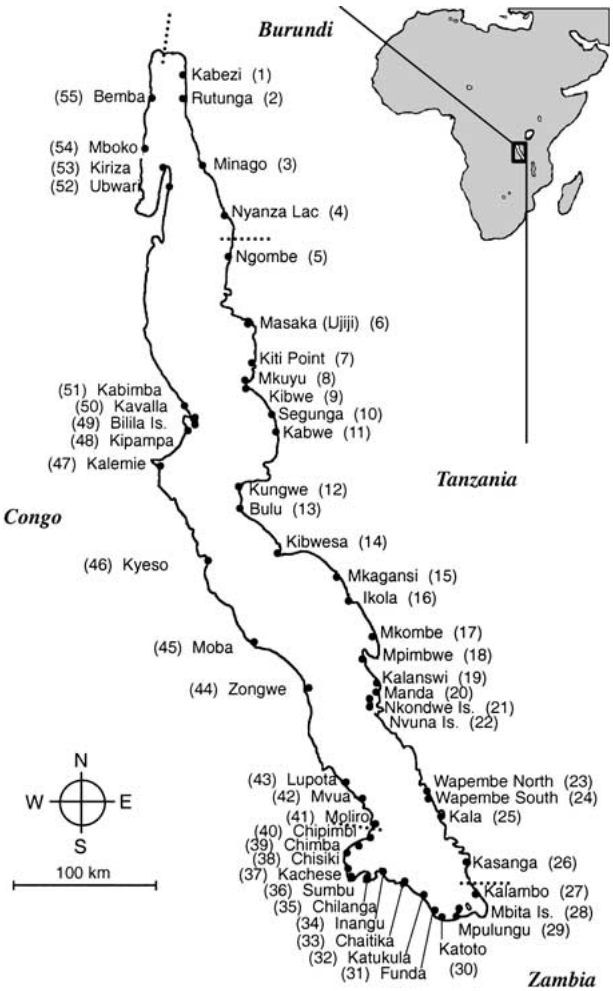


Fig. 1. Location of 55 *Tropheus* populations from Lake Tanganyika included in the study. Populations were named after the nearest village and numbered in clockwise orientation.

systems) for visualization. All samples were sequenced with the forward primer and 53 specimens were also sequenced in the opposite direction, so that each major mtDNA lineage was confirmed by both complementary strands. The nucleotide sequences are available from GenBank under the following accession numbers: Z12047–Z12100, Z75694–Z75709, AJ295902–AJ295924, AJ489622–AJ489743, and AJ491975–AJ492145 for the mtDNA control region and Z12030–Z12044 and AJ487688–AJ487701 for cytochrome *b*.

Phylogenetic Analyses

Sequences were aligned by eye and gaps had to be inferred for the segment of the control region only. The phylogenetic analyses were performed in three steps. In a first neighbor-joining analysis all major clusters of taxa were identified for all 243 haplotypes with the aim of selecting two representative subsets of taxa for more computation-intensive analyses. A first subset with 29 haplotypes (data subset I) was chosen to determine the branching order among the major mtDNA lineages. A second subset with 97 haplotypes (data subset II) was used for the analysis of the geographic distribution of closely related genotypes within each mtDNA lineage. Both data subsets were first tested for their overall phylogenetic content by applying the likelihood mapping analysis [PUZZLE Version 4.0 (Strimmer and von Haeseler 1997)].

The first analysis was based on 446 bp of the mtDNA control region and included all 243 haplotypes of 365 sequenced specimens from 55 localities and two outgroup taxa (*T. duboisi*). After analyzing these data by means of neighbor-joining, using Kimura two-parameter distances, all major clades were identified. This analysis formed the basis for an adequate choice of the two data sets with a reduced number of taxa. Up to three taxa were selected from each clade, taking care to pick specimens and haplotypes over the entire distribution range of each mtDNA lineage. In this way a data set including 29 representative haplotypes (reduced data set I) was generated. To increase the power of resolution a 402-bp segment of the cytochrome *b* gene was added for each of those 29 taxa. Data subset I was analyzed by applying the three most commonly used approaches for tree reconstruction in parallel—maximum parsimony, neighbor-joining, and maximum likelihood—using the computer program PAUP* [β-version 4.0 for Macintosh (Swofford 2000)]. *T. duboisi* was used as outgroup in all analyses (Sturmbauer and Meyer 1992). Parsimony analyses were performed using the options heuristic search with random stepwise addition of taxa and 50 replicated searches. Transversion mutations were weighted nine times over transitions, according to the empirically estimated frequency. Neighbor-joining was carried out with Kimura two-parameter distances. For both approaches the robustness of the inferred trees was tested by bootstrapping with 1000 pseudoreplicates. Maximum likelihood analyses were performed by quartet puzzling based on the HKY85 model with a transition–transversion ratio of nine and empirical base frequencies. A series of four-cluster likelihood mapping analyses (Strimmer and von Haeseler 1997) was performed to estimate the supports for distinct internal branches that were critical for the interpretation of the evolutionary pathways. In this method four clusters of taxa were tested for the relative frequency of the three possible bifurcating trees. In this way whether one of the three alternative topologies was clearly favored over the others was tested. Frequency values for branches of interest were estimated with 10,000 puzzling steps.

For our third analysis, focusing on the phylogeographic distribution of closely related haplotypes within each lineage, we selected a second data subset (data subset II) including 97 haplotypes comprising 6–21 haplotypes of each major mtDNA lineage and including at least one haplotype from each locality (see Fig. 2). This data set was used to construct minimum spanning trees for all major mtDNA lineages, since they best visualize clusters of closely related haplotypes. The trees were generated by means of parsimony analysis, with equal weights for transition and transversion mutations, and were unrooted. In those cases, in which more than one most parsimonious tree was found, the phylogeny was chosen that was most similar to the neighbor-joining tree. On the basis of the minimum spanning trees we derived monophyletic clusters of closely related haplotypes and determined the extension of shoreline colonized by each genetic group. Clusters of closely related haplotypes were defined by the criterion of minimum evolution: any individual assigned to a particular cluster had to differ from any other member of the cluster by a smaller number of mutations than it differed from any member of another cluster.

Estimates of Divergence Time Within and Among the Major Tropheus *Lineages*

To assess the relative time scales of colonization events we calculated genetic distances within and among the major mitochondrial lineages and gauged absolute ages by means of a molecular clock. Since a major precondition for such estimates is a constant rate of nucleotide substitutions, we first applied the two-cluster test implemented in the PHYLTEST program (Kumar 1996) to test the relative evolutionary rates of each major mtDNA lineage with *T. duboisi* as outgroup. This was done by using data subset II (97

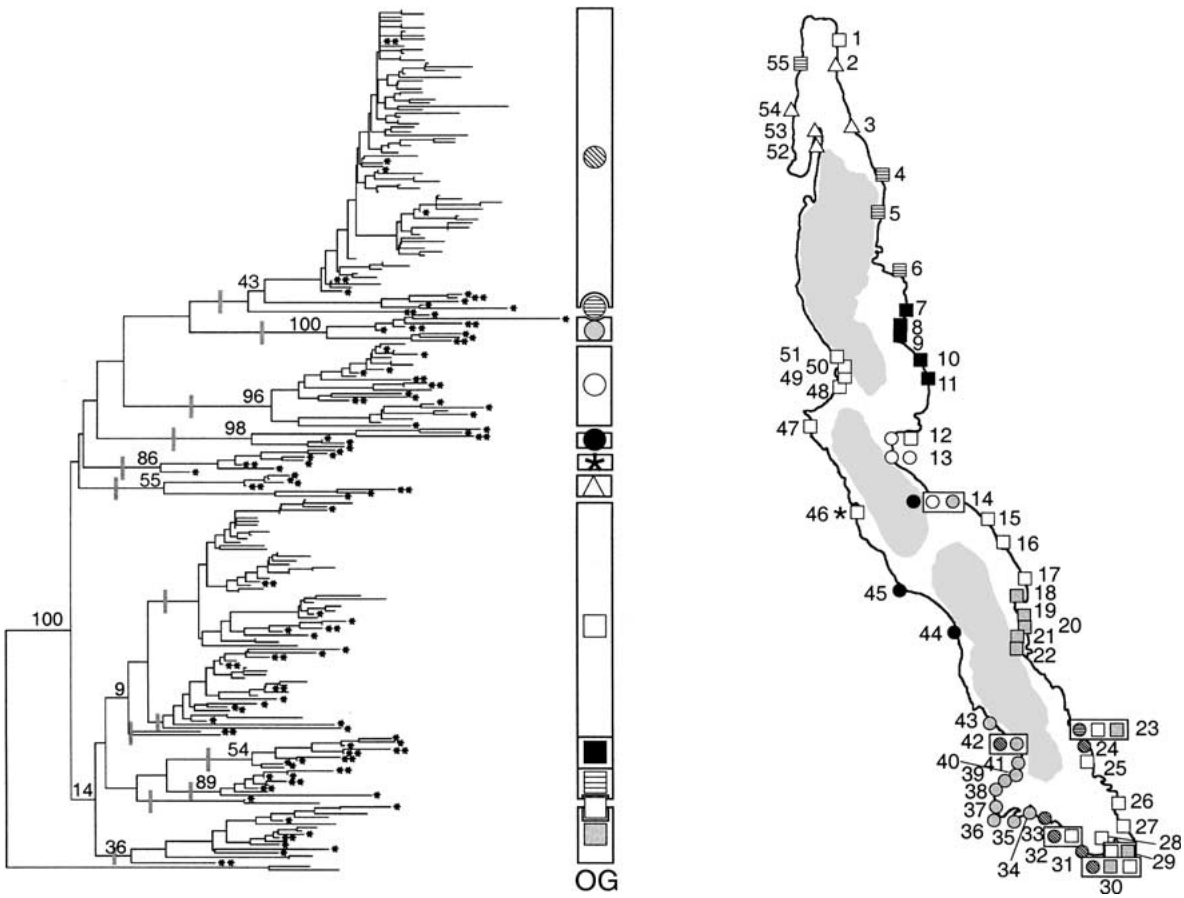


Fig. 2. Neighbor-joining tree (Kimura two-parameter distances) of 243 haplotypes (365 sequenced individuals) of the mitochondrial DNA control region of the genus *Tropheus*. *Asterisks* indicate haplotypes that were selected for further analyses in data subset I (**) and data subset II (*). Bootstrap values obtained in neighbor-joining supporting each major mtDNA lineage or sublineage are shown above the branches. *Vertical bars* on the branches denote the 13 identified major clades. The map shows the distribution patterns of the eight mtDNA lineages all around Lake Tanganyika. Each sampling locality is marked by number.

haplotypes) including 360 bp only covering the first and most variable segment of the mitochondrial control region.

In the next step average Kimura two-parameter distances among all pairs of sequences between the eight major mtDNA lineages and between clusters of closely related haplotypes within each lineage were calculated. Finally, the average genetic divergence within each cluster of closely related haplotypes was computed. For the more slowly evolving segment of the cytochrome *b* gene, only average Kimura two-parameter distances between all pairs of major mitochondrial lineages were inferred.

We applied a molecular clock for the first segment of the mitochondrial control region that was derived from the average observed divergence among the *Mbuna* and the *Utaka* cichlid lineages in Lake Malawi (Sturmbauer et al. 2001) and a dating of the refilling of Lake Malawi after a period of (almost-) complete desiccation established by Delvaux (1995). According to this clock estimate the evolutionary rate of base substitution for the most variable section of the control region ranged from 6.5 to 8.8% per Ma, defined by the time span for the onset of the refilling of Lake Malawi.

Results

Phylogenetic Analyses

Of a total of 365 individuals of which 446 bp of the mtDNA control region were sequenced, 243 mtDNA haplotypes were found. Neighbor-joining identified 13 major clades, as shown in Fig. 2. Our choice of the 29 haplotypes for data subset I and of the 97 haplotypes (representing 157 sequenced specimens) for data subset II is highlighted in Fig. 2. The likelihood mapping analyses demonstrated a strong phylogenetic signal in both data subsets: data subset I yielded 95.2% of fully resolved quartets, and data subset II 90.2% (Figs. 3a and b). The estimated ratio between transition and transversion mutations in data subset I was 8.82 (SD, 3.93). The levels of genetic divergence determined from data subset II were generally found to be below the saturation level (Fig. 3c). The maximum observed Kimura two-parameter distance amounted to 11.1% in the segment of the mtDNA control region.

The phylogenetic analysis using both gene segments (data subset I) was based on 142 variable characters, of which 90 were phylogenetically informative in parsimony. The three approaches of phylogenetic reconstruction resulted in similar topologies and consistently identified eight major mtDNA lineages (A to H in Fig. 4). Parsimony yielded 28 most parsimonious trees with a score of 553 evolutionary

steps (unweighted tree length, 270 mutations; consistency index excluding uninformative sites, 0.60; retention index, 0.81). Figure 4a shows the strict consensus of the 28 most parsimonious trees, the neighbor-joining tree, and the maximum likelihood tree. In addition to the seven major mtDNA lineages found by Sturmbauer et al. (1997), our study identified one new major mtDNA lineage (lineage C in Fig. 4). This lineage was found at only one locality (Kyeso) at the central western shore of Lake Tanganyika in sympatry with individuals of a different major mtDNA lineage (lineage A2; Figs. 4a and b). While all three approaches clearly supported the monophyly of each of the eight major mtDNA lineages (bootstrap/likelihood values >80% for lineages B, D, E, and F in all three approaches; bootstrap/likelihood values for lineage A >70% in parsimony and maximum likelihood and 68% in neighbor-joining; bootstrap values for lineage G 45 and 69% in parsimony and neighbor-joining, respectively, and likelihood value of 96%), there was conflict in the branching order among them. Nor could the branching order among the major mtDNA lineages be resolved by the four-cluster likelihood analysis, suggesting their almost-contemporaneous origin (see Fig. 4c). Though none of the three algorithms of phylogenetic reconstruction supported the placement of lineage C as sister group of lineages D to H (circles in Fig. 4; see also Fig. 4d), this branching order was favored in 76% of 10,000 puzzling steps in the four-cluster likelihood analysis.

While the sample size in earlier studies was not sufficient to discriminate clades within lineage A, we could identify four distinct sublineages (A1–A4), of which only sublineage A2 covers a wide distribution area, ranging from the very north to the very south of Lake Tanganyika. Sublineages A1 and A3 were found exclusively in individuals from the northeastern part of the lake, and sublineage A4 comprised exclusively individuals from the southeastern coast (see Figs. 4a and b and 6). Again, the branching order between the sublineages could not be unambiguously resolved. Only parsimony supported sublineage A1 as the most ancestral branch of lineage A with a bootstrap value of 68%, while the four-cluster likelihood mapping analysis did not support this topology (73.3% of unresolved quartets; see Fig. 4e). This four-cluster likelihood mapping analysis was performed with the data set of the control region including 44 taxa of lineage A.

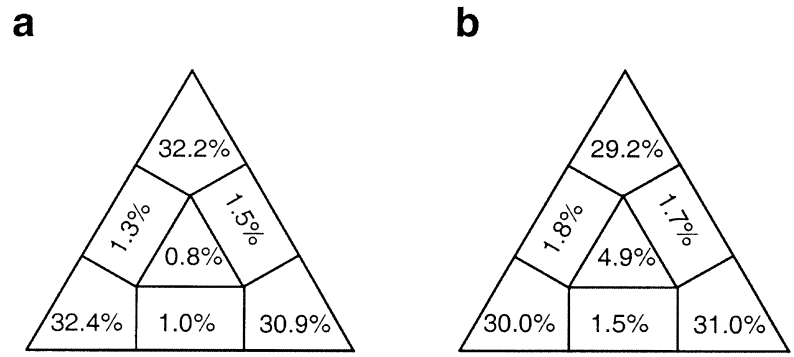
Phylogeographic Patterns in Tropheus

To analyze the geographic distribution of closely related haplotype clusters within each of the eight major mitochondrial lineages, 10 minimum spanning trees were constructed (Figs. 5 and 6). This analysis included 96 haplotypes. Since lineage H was represented only by a single individual sampled near Wapembe (Figs. 4a and b; Wapembe "nortti," circle with horizontal bars), no minimum spanning tree could be created for this lineage. For each sublineage of lineage A a separate minimum spanning tree was constructed. The haplotypes within each monophyletic cluster differed by zero to six mutations, while individuals assigned to different clusters always differed by a higher number of mutations. Within sublineage A2 it was difficult to define clusters, because of the complexity of the minimum spanning tree (see Fig. 6). In general, the haplotype distributions showed a strong geographic structuring on the lineage level, and each lineage contained more than one monophyletic haplotype cluster. However, in some cases no strong phylogeographic pattern was observed within the clusters of closely related haplotypes since some populations contained individuals of different monophyletic haplotype clusters (see, e.g., minimum spanning trees of lineages B and F and sublineages A1, A2, and A4 in Figs. 5 and 6). In contrast, within lineages D and G, as well as in sublineage A3, clusters of closely related haplotypes clearly segregated individuals from different localities. Even though distribution patterns within lineage E did not reveal a clear phylogeographic structure along the Kungwe mountain range, the minimum spanning tree segregated the haplotypes of *T.* "Kirschfleck" from those of *T.polli*.

Estimates of Divergence Time Among and Within the Major Tropheus *Lineages*

The constancy of nucleotide substitution rates among lineages was tested with the two-cluster test implemented in the PHYLTEST program (Kumar 1996). In this test all major lineages were surveyed in 28 pairwise permutations using *T. duboisi* as outgroup. The constancy of the evolutionary rate was rejected at the 5% significance level in only one permutation ($Z = 2.07$), in which lineage F was shown to have a significantly faster evolutionary rate than lineage A. In consequence, pairwise comparisons among these two lineages were ignored for further calculations. Among the remaining pairwise lineage comparisons, Z values ranged from 0.05 to 1.77 (see Table 2).

From the divergence patterns observed within and among the major mtDNA lineages, three distinct diversification events could be delineated. The first diversification event concerns the formation of the eight major mtDNA lineages. It was already termed the "primary radiation" of *Tropheus* by Sturmbauer and Meyer (1992). In the control region Kimura two-parameter distances among the eight major mitochondrial lineages ranged between 4.6 and 7.9% (mean, 6.14%; SD, 1.37; 2906 pairwise comparisons; see



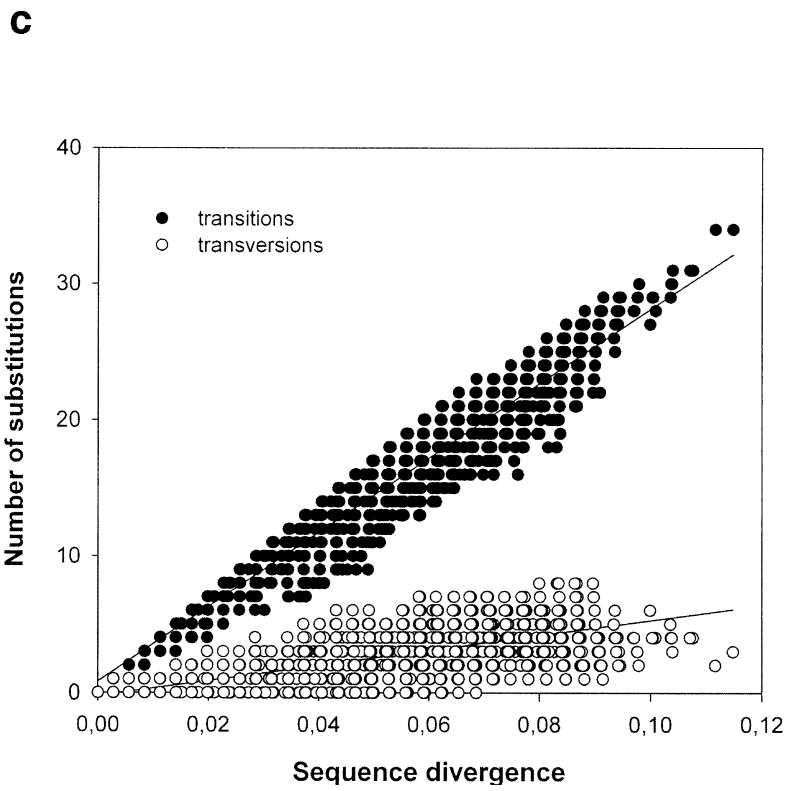
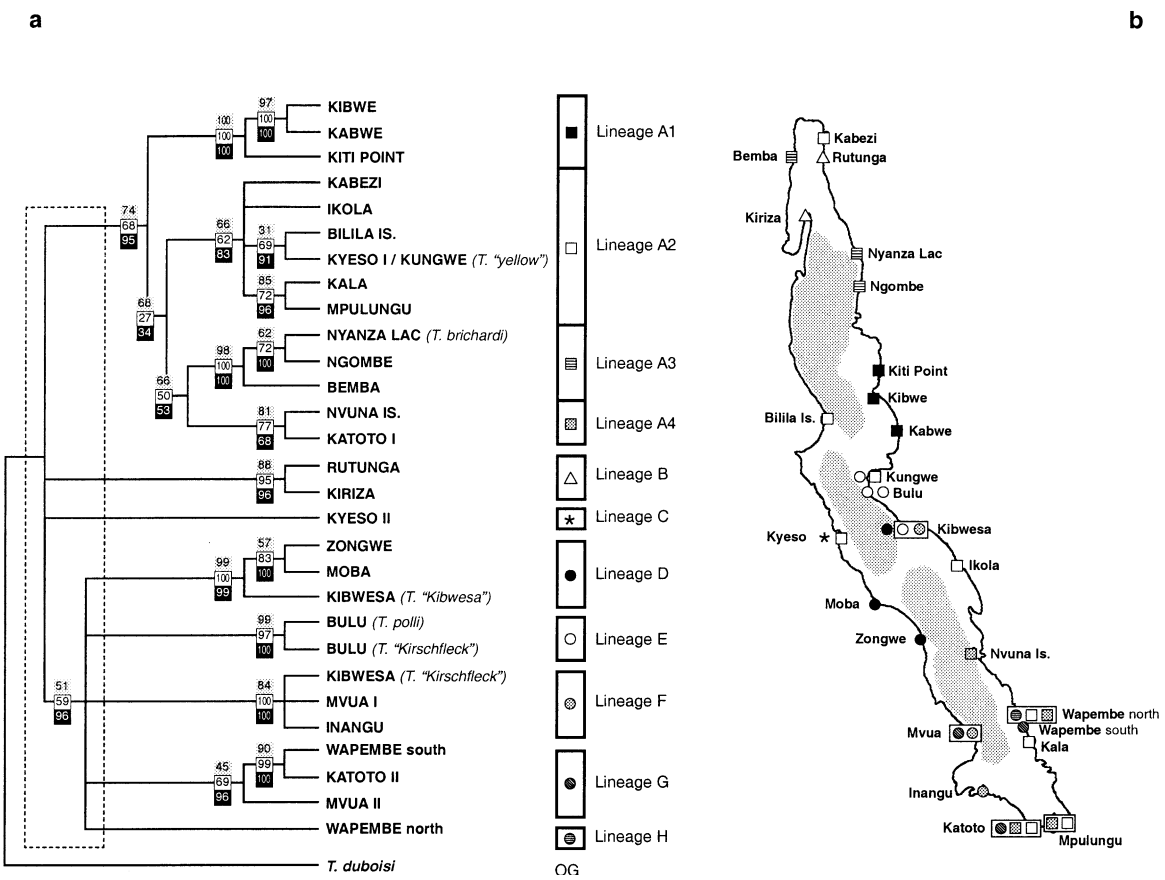
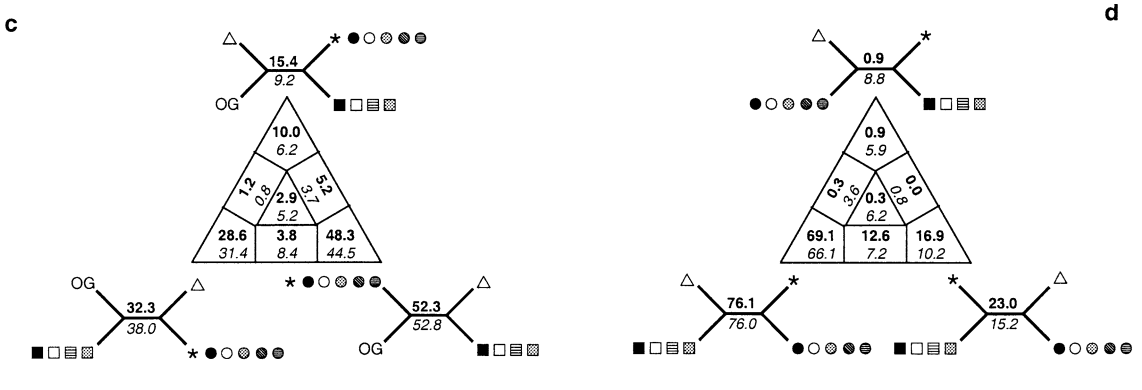


Fig. 3. Results of the likelihood mapping analysis of (**a**) the combined data set of the mtDNA control region and the cytochrome *b* gene, comprising 29 taxa plus outgroup, and (**b**) the most variable part of the mitochondrial control region only, comprising 97 taxa plus 2 outgroup taxa. Values at the corners of the triangle represent percentages of fully resolved quartets; values in the lateral areas, percentages of partially resolved quartets; and values in the central area, the percentage of unresolved quartets. **c** Saturation plot of transition and transversion mutations (*Y* axis) against corrected sequence divergence (Kimura two-parameter; *X* axis) of 4656 pairwise comparisons of taxa calculated from 365 bp of the most variable part of the mitochondrial control region.

Fig. 4. **a** Molecular phylogeny of the genus *Tropheus*. Strict consensus of 28 maximum parsimony trees, a neighbor-joining tree (Kimura two-parameter distances), and a maximum likelihood tree based on 845 bp of the mtDNA control region and the cytochrome *b* gene. Transversion mutations were weighted nine times over transitions in parsimony and maximum likelihood. Bootstrap values obtained in parsimony are shown in *shaded boxes* above the branches, while numbers in *white boxes* represent neighbor-joining bootstrap values. Likelihood values obtained by quartet puzzling are shown in *black boxes* below the branches. The "primary radiation," in which the eight major mtDNA lineages arose, is indicated by a *dotted frame*. **b** Map of Lake Tanganyika with localities of 29 *Tropheus* populations included in the second step of analysis. *Gray areas* represent the paleoshoreline at a depth of 600 m. *Boxed symbols* indicate populations having haplotypes assigned to two or more major mtDNA lineages. **c–e** Results of the four-cluster likelihood mapping analysis based on the combined data set of the mtDNA control region and the cytochrome *b* gene (values in *bold face*), comprising 29 taxa plus outgroup, and of an additional four-cluster likelihood analysis using the mtDNA control region only (values in *italics*), comprising 99 taxa including 2 outgroup taxa. Lineages were classified into four groups for determining the support for certain internal branches according to the phylogenetic tree shown in a. Values at the corners of the triangle represent percentages of fully resolved quartets; values in the lateral areas, percentages of partially resolved quartets; and values in the central area, the percentage of unresolved quartets. Overall percentages of support for each of the three possible topologies are given on the branches of the trees. In e, only the data set of the mtDNA control region was included in the analysis.


a
b
KIBWE
KABWE
KITI POINT
KABEZI
IKOLA
BILILA IS.
KYESO I / KUNGWE (T. "yellow")
KALA
MPULUNGU
NYANZA LAC (T. brichardi)
NGOMBE
BEMBA
NVUNA IS.
KATOTO I
RUTUNGA
KIRIZA
KYESO II
ZONGWE
MOBA
KIBWESA (T. "Kibwesa")
BULU (T. polli)
BULU (T. "Kirschfleck")
KIBWESA (T. "Kirschfleck")
MVUA I
INANGU
WAPEMBE south
KATOTO II
MVUA II
WAPEMBE north
T. duboisi
Lineage A1
Lineage A2
Lineage A3
Lineage A4
Lineage B
Lineage C
Lineage D
Lineage E
Lineage F
Lineage G
Lineage H
OG
Kabezi
Rutunga
Bemba
Kiriza
Nyanza Lac
Ngombe
Kiti Point
Kibwe
Bilila Is.
Kabwe
Kungwe
Bulu
Kibwesa
Kyeso
Ikola
Moba
Zongwe
Nvuna Is.
Wapembe north
Wapembe south
Mvua
Kala
Inangu
Katoto
Mpulungu


c
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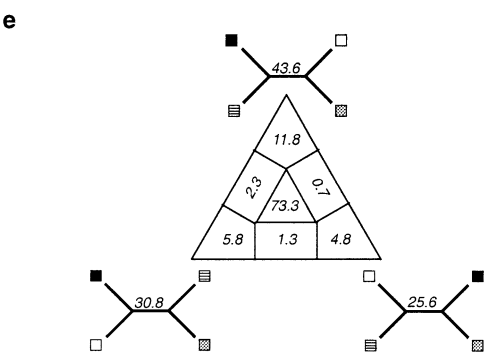
15.2


e
43.6
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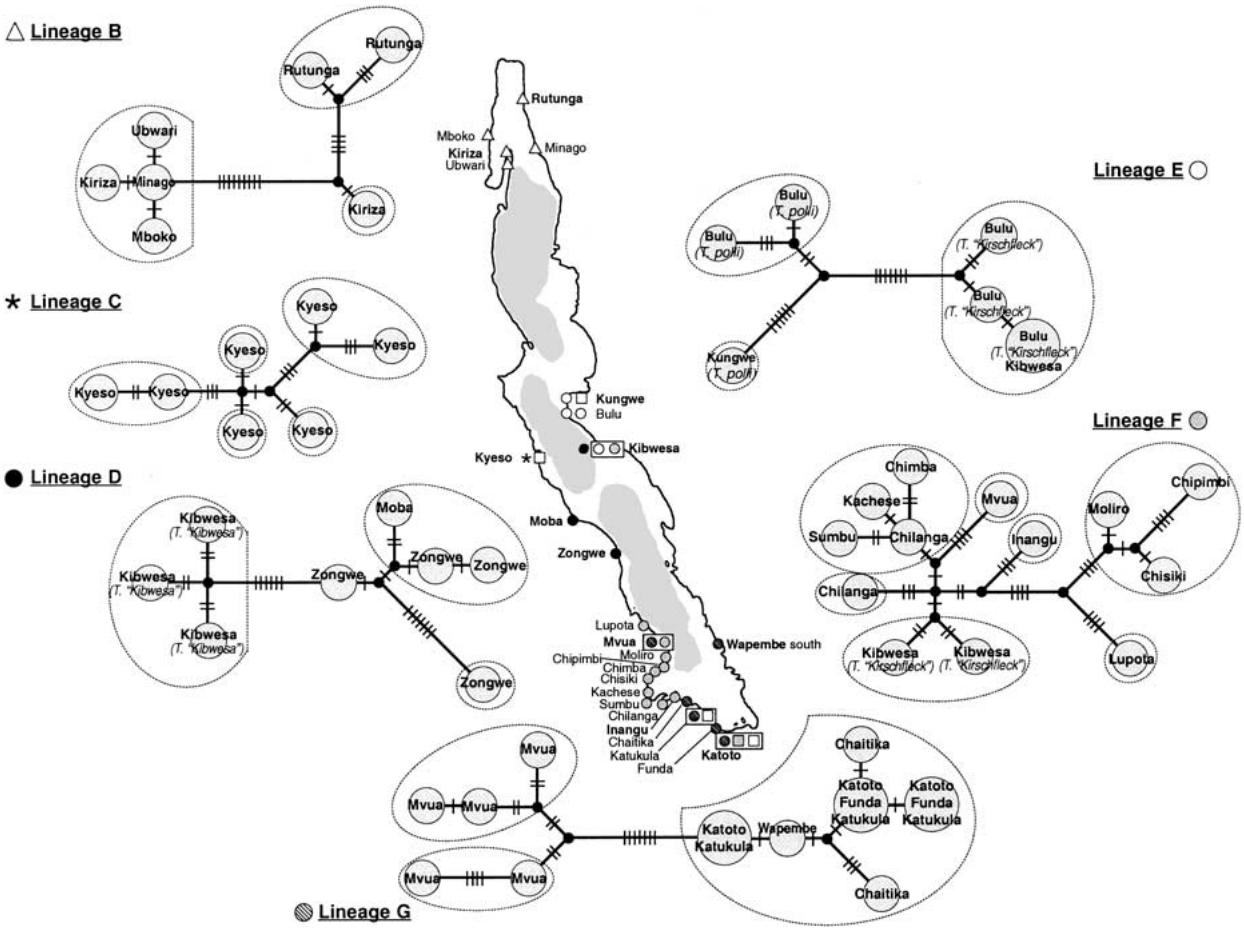

Fig. 5. Minimum spanning trees of lineages B, C, D, E, F, and G. Each *shaded circle* represents a mtDNA haplotype. *Black circles* represent intermediate haplotypes that were not found. Each *crossbar* indicates one mutation step. Clusters of closely related haplotypes are *encircled by dotted lines*. Distribution ranges of each lineage are shown on the map of Lake Tanganyika using symbols assigned to each lineage. All minimum spanning trees are unrooted and obtained in parsimony by using the complete control-region data set (446 bp). If there was more than one most parsimonious tree; the tree that was most similar to the neighbor-joining tree was chosen. **Lineage B**: One most parsimonious tree. Tree length, 21; consistency index excluding uninformative characters, 1.00; retention index, 1.00; rescaled consistency index, 1.00. **Lineage C**: Three most parsimonious trees. Tree length, 17; consistency index excluding uninformative characters, 0.88; retention index, 0.75; rescaled consistency index, 0.66. **Lineage D**: Eight most parsimonious trees. Tree length, 25; consistency index excluding uninformative characters, 0.90; retention index, 0.93; rescaled consistency index, 0.89. **Lineage E**: One most parsimonious tree. Tree length, 23; consistency index excluding uninformative character, 0.92; retention index, 0.94; rescaled consistency index, 0.90. **Lineage F**: Eleven most parsimonious trees. Tree length, 42; consistency index excluding uninformative character, 0.70; retention index, 0.83; rescaled consistency index, 0.71. **Lineage G**: One most parsimonious tree. Tree length, 28; consistency index excluding uninformative character, 0.84; retention index, 0.94; rescaled consistency index, 0.84.

Table 2). In cytochrome *b* an average of 2.2% was found (SD, 0.7; 218 pairwise comparisons).

A second diversification event concerns the ''secondary radiation'' within six major mtDNA lineages: A, B, D, E, F, and G (see Figs. 5 and 6). Similar genetic distances signal that the same change of the lake habitat is likely to have triggered the contemporary emergence of the four sublineages of lineage A, as well as the subdivision of lineages B, D, E, and F into clusters of closely related haplotypes (these were not named as separate sublineages in Fig. 5). Average genetic distances among these sublineages ranged between 2.7 and 3.9% (mean, 3.51%; SD, 0.89; 759 pairwise comparisons; see Table 3).

The ''tertiary radiation'' can be defined as the diversification within the clusters of closely related haplotypes. Average genetic distances found among clusters of closely related haplotpes within sublineages A1, A3, and A4 ranged between 1.6 and 2.0% (mean, 1.76%; SD, 0.51; 58 pairwise comparisons; Table 3). Average genetic distances found within the clusters of closely related haplotypes within lineages B, D, E, and F were mostly below 1%, down to a minimum of 0.4%. The maximum divergence of 1.3% was observed in a cluster of lineage F among individuals from Moliro, Chisiki, and Chipimbi. The observed variation in the average divergences may suggest that the ''tertiary radiation'' did not always

Table 2. Pairwise genetic distances among and within major mitochondrial lineages of *Tropheus*

	A	B	C	D	E	F	G	H
Lineage A (n = 44)	—[a]	Z = 1.20	Z = 0.25	Z = 1.77	Z = 0.97	Z = 2.07[b]	Z = 0.69	Z = 0.40
Lineage B (n = 7)	4.75 ± 0.77	*3.34 ± 0.66*	Z = 0.70	Z = 0.91	Z = 0.05	Z = 1.08	Z = 0.23	Z = 0.52
Lineage C (n = 7)	4.58 ± 0.88	5.29 ± 0.66	*1.27 ± 0.47*	Z = 1.55	Z = 0.77	Z = 1.75	Z = 0.45	Z = 0.13
Lineage D (n = 8)	7.64 ± 0.98	7.64 ± 1.05	6.83 ± 0.77	*2.90 ± 0.82*	Z = 0.90	Z = 0.13	Z = 1.45	Z = 1.41
Lineage E (n = 6)	6.36 ± 1.14	6.93 ± 0.48	5.50 ± 0.99	7.88 ± 1.45	*3.22 ± 0.48*	Z = 1.11	Z = 0.27	Z = 0.50
Lineage F (n = 13)	—	7.70 ± 0.64	6.29 ± 0.62	6.87 ± 0.92	6.66 ± 1.12	*2.65 ± 0.97*	Z = 1.51	Z = 1.69
Lineage G (n = 11)	6.17 ± 0.79	6.45 ± 1.05	5.47 ± 0.54	5.31 ± 0.99	7.23 ± 0.90	6.31 ± 0.72	*3.16 ± 0.80*	Z = 0.33
Lineage H (n = 1)	4.47 ± 0.84	5.20 ± 0.66	4.92 ± 0.52	6.24 ± 0.76	6.89 ± 1.12	5.80 ± 0.39	4.05 ± 0.90	—

Note. Below the diagonal, percentages of Kimura two-parameter distances among major mitochondrial lineages in the most variable segment of the mt control region, with standard deviations, are given. Above the diagonal are Z values resulting from the two-cluster test for constancy of evolutionary rates among major mitochondrial lineages (Kumar 1996). Genetic distances among clusters within each lineage are given on the diagonal, in *italics*.

[a] Genetics distances among sublineages of lineage A are given in Table 3.

[b] Significant at the 5% level; not used for inferring age estimates.

Table 3. Pairwise genetic distances among sublineages of lineage A

	A1	A2	A3	A4
A1 (n = 9)	*1.61 ± 0.38*	n = 189	n = 54	n = 72
A2 (n = 21)	3.74 ± 0.94	*2.28 ± 0.93*[a]	n = 126	n = 168
A3 (n = 6)	3.35 ± 0.60	3.43 ± 0.90	*2.04 ± 0.32*	n = 48
A4 (n = 8)	3.93 ± 0.74	3.74 ± 0.94	3.88 ± 0.79	*1.84 ± 0.60*

Note. Below the diagonal, percentages of Kimura two-parameter distances, with standard deviations, are given. Number of pairwise comparisons is given above the diagonal. Genetic distances among clusters within each clade are given in *italics* on the diagonal.

[a] For sublineage A2 average genetic divergence among all haplotypes is given, since it was not possible to define clusters.

proceed synchronously due to the influence of local factors such as basin morphology.

The average genetic distances assigned to each of the three radiations were then translated into absolute time estimates for each diversification event, to compare them with datings of geological events (see Fig. 7). This was done by applying an evolutionary rate of 6.5 to 8.8% per Ma for the most variable section of the control region (see Sturmbauer et al. 2001). The slower rate estimate of 6.5% per Ma would date the "primary radiation" to about 945 Ka; the faster rate of 8.8% per Ma would place this event at about 698 Ka. The slow rate would date the "secondary radiation" to 540 Ka; the fast rate, to 399 Ka. The "tertiary radiation" would be dated to between 270 Ka (slow rate) and 200 Ka (fast rate; see Fig. 7).

A second independent age estimate for the "primary radiation" was obtained from the analyzed segment of the cytochrome *b* gene by applying a divergence rate of 2.5% per Ma (Meyer et al. 1990; Irwin et al. 1991). This would date the primary radiation of *Tropheus* to 868 Ka, well within the boundaries of the D-loop estimate.

Discussion

Phylogenetic and Phylogeographic Patterns

Lake Tanganyika has a complex geological history, characterized by extended time periods of a substantially lower lake level (Lezzar et al. 1996; Cohen et al. 1997). The lake was severely affected by the change to a drier climate at about 1.1 Ma, resulting in a drop in the lake level to about 650 to 700 m below its present level. After that the lake rose continuously until 550 Ka, although the precise magnitude of this rise is unknown (Cohen et al. 1997). More recent drops in the lake level were found between 390 and 360 Ka, by 350 m; between 290 and 260 Ka, by 350 m; and between 190 and 170 Ka, by 250 m. In the most recent history, the lake was lower during the late Pleistocene ice ages, when the climate in wide areas of Africa became more arid (Potts and Behrensmeyer 1992). These lowstands were dated to between 40 and 35 Ka (−160 m) and between 23 Ka and 18 Ka [most probably 600 m (see Scholz and Rosendahl 1988; Gasse et al. 1989; Lezzar et al. 1996; Cohen et al. 1997)].

The calibration of the molecular clock for East African cichlid fish remains difficult due to the absence of a reliable fossil record. Thus, age estimates can be derived only from comparisons of genetic divergences to datings of geological events. To estimate the time spans of diversification in *Tropheus*, we used a new molecular clock estimate (Sturmbauer et al. 2001) that was calibrated on the basis of the average molecular divergence among two major lineages of the Lake Malawi cichlid species flock (Meyer et al. 1990) and a reconstruction of the history of Lake Malawi (Delvaux 1995). Since the rise of Lake Ma-

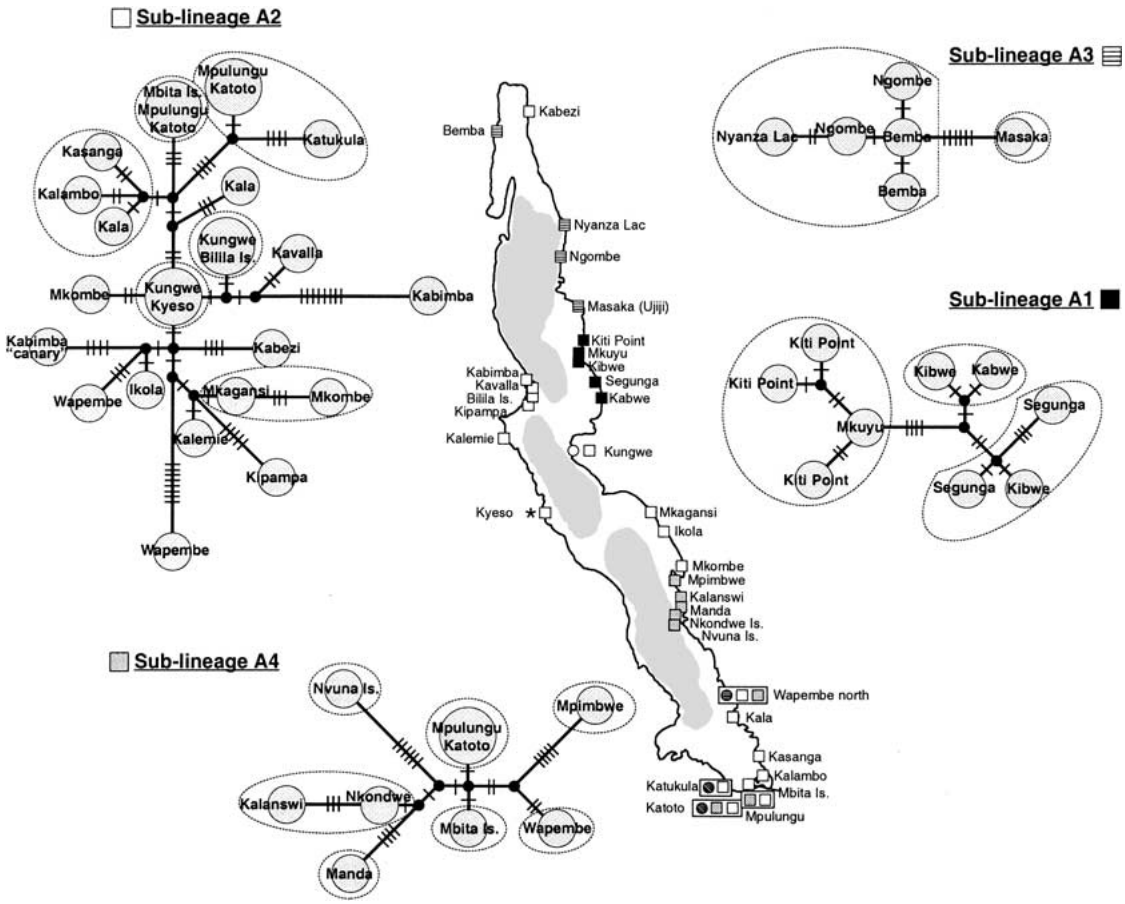

Fig. 6. Minimum spanning trees of each of the four sublineages of lineage A. Distribution ranges of each cluster are shown on the map of Lake Tanganyika using *square symbols* with *different patterns*, which were assigned to each sublineage. All minimum spanning trees are unrooted and obtained in parsimony by using the complete control-region data set (446 bp). If there was more than one most parsimonious tree, the tree that was most similar to the neighbor-joining tree was chosen. **Sublineage A1**: Eight most parsimonious trees. Tree length, 20; consistency index excluding uninformative characters, 0.75; retention index, 0.81; rescaled consistency index, 0.65. **Sublineage A2**: One hundred sixteen most parsimonious trees. Tree length, 73; consistency index excluding uninformative characters, 0.53; retention index, 0.69; rescaled consistency index, 0.55. **Sublineage A3**: One most parsimonious tree. Tree length, 11; consistency index excluding uninformative characters, 1.00; retention index, 1.00; rescaled consistency index, 1.00. **Sublineage A4**: Six most parsimonious trees. Tree length, 27; consistency index excluding uninformative characters, 0.67; retention index, 0.67; rescaled consistency index, 0.59.

lawi after a period of (almost-) complete desiccation could be dated only in the form of a minimum–maximum estimate, a range of an evolutionary rate from 6.5 to 8.8% per Ma was obtained for the most variable segment of the mtDNA control region (for details see Sturmbauer et al. 2001). We are aware that the clock estimate available for East African cichlids is relatively imprecise, so that future studies are likely to provide more accurate estimates. Nevertheless, applying this molecular clock estimate for *Tropheus* seems justified, because similar substitution rates in *Tropheus* and rock-dwelling cichlid fish from Lake Malawi were verified for the most variable segment of the control region by means of a relative rate test (Sturmbauer et al. 2001). Rate constancy was further confirmed among the major lineages of *Tropheus*. Molecular clocks are expected to be rather unsteady, especially when the time of divergence is relatively recent. However, it seems remarkable that both the molecular clock for the control region and that for the cytochrome *b* gene give a highly similar age estimate for the ''primary radiation'' of *Tropheus*. These age estimates even fit well to geological datings for the rise of Lake Tanganyika after a major lowstand between 1.1 Ma and 550 Ka (Lezzar et al. 1996; Cohen et al. 1997). Moreover, our age estimates of diversification events based on the fast substitution rate for the control region (8.8% per Ma) are widely congruent with two more recent datings of geological events (Fig. 7). However, it cannot be expected that diversification events within and among all populations would have occurred exactly at the same time, since the effect of fluctuating lake levels on particular populations will depend strongly on the basin structure of Lake Tanganyika. Therefore, each lineage should and does show its own peculiarities. This

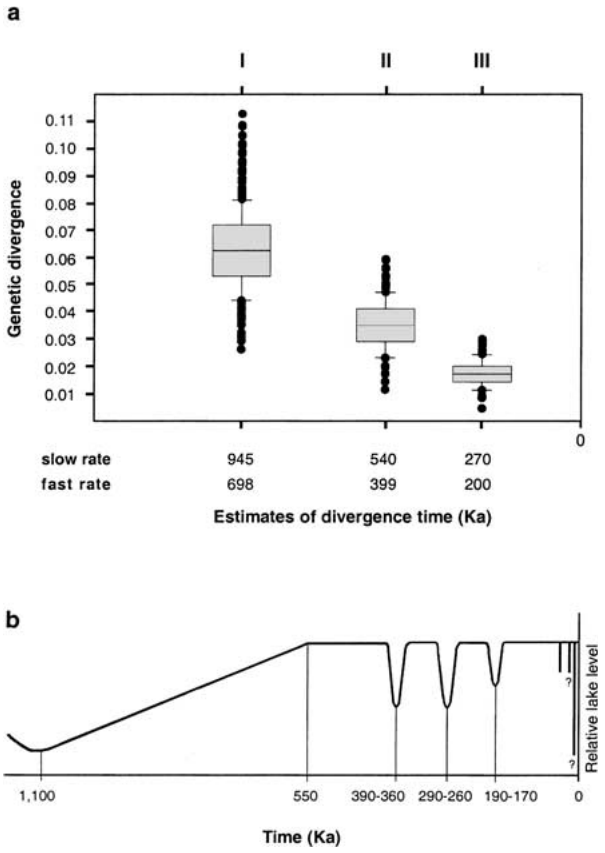

Fig. 7. **a** Hierarchically ordered levels of genetic divergences (I, "primary radiation"; II, "secondary raditation"; III, "tertiary radiation") observed in the genus *Tropheus*. Estimates of divergence time were derived by relating the average genetic distances to an evolutionary rate of 6.5% (slow rate) and 8.8% (fast rate) per Ma, according to the molecular clock derived by Sturmbauer et al. (2001). **b** Reconstruction of lake level lowstands based on Lezzar et al. (1996) and Cohen et al. (1997). The three *vertical lines* on the *right* indicate the most recent lake level lowstands, about 40–35 Ka (by 160 m), 23 Ka, and 18 Ka. *Question marks* denote that the exact magnitude of the lowstand is unknown.

seems to be clearly reflected by the relatively high degree of variation in the observed average genetic divergences within monophyletic haplotype clusters (termed as "tertiary radiation"). Still, it seems quite remarkable that we repeatedly found highly similar patterns of genetic divergence, pointing to a clear reflection of major abiotic events in the observed genetic divergences. In summary, we suggest that the "primary radiation" of *Tropheus* is related to the rise of the lake level between 1.1 Ma and 550 Ka; the "secondary radiation," to the lowstand between 390 and 360 Ka; and the "tertiary radiation," to the lowstand between 190 and 170 Ka. Sturmbauer et al. (2001) found further genetic traces in cichlids for a much more recent climate change (about 17 Ka) resulting in a dramatic drop of the lake level in Lakes Tanganyika and Malawi, and in the desiccation of Lake Victoria. This event was suggested to have synchronized the most recent diversification events of cichlid fish in all three lakes.

The most plausible explanation for the observed genetic patterns in *Tropheus* are three periods of low lake level, each retreating to at least 550 m below the present level, so that the lake was (almost) split into three lakes. Such a decrease in lake level is necessary to allow for a crossing of the lake at the borders of the lake basins as suggested by our genetic data. This would imply that the retreats of the lake level between 390 and 360 Ka and between 170 and 190 Ka were underestimated by geological evidence. It might be possible that the period of minimum lake level was too short to leave sufficient traces in the sedimentation pattern. Since the period of low lake level at 290–260 Ka did not leave clear genetic traces in *Tropheus*, it may have been less severe than the two older lake level changes.

Although the application of the slower evolutionary rate would result in a time estimate more similar to the previously published age estimate of 1.25 Ma for the primary radiation of *Tropheus*, by Sturmbauer and Meyer (1992), we now tend to favor the faster evolutionary rate of 8.8% per Ma for *Tropheus*. Our view is supported by the better match of the three observed levels of genetic divergence in *Tropheus* (the "primary," "secondary," and "tertiary radiation") with the geology-based datings. A second argument in favor of the faster evolutionary rate may be that the slower rate of 6.5% is outside the range of molecular clocks for the control region reported for several other organisms, ranging from 8 to 20% per Ma (Vigilant et al. 1991; Brown et al. 1993; Stewart and Baker 1994; Bowen and Grant 1997).

Scenario of the Colonization of Lake Tanganyika by Tropheus

We tried to reconstruct the chronicle of the spread and diversification of the genus *Tropheus* on the basis of hierarchically ordered levels of genetic divergences within and among the major lineages. The "primary radiation" of the genus was likely to have been triggered by a substantial rise in the lake level about 700 Ka. The branching order of the phylogeny and the present distribution of the eight major mtDNA lineages indicate that lineages A and B (squares and triangles in Fig. 4) originated by colonization of the northern basin of Lake Tanganyika, while lineages C and D (asterisks and black circles in Figs. 4 and 5) arose at the western coast of the central basin, and lineage E (white circles in Figs. 4 and 5) arose by colonizing the eastern coast of the central basin. Lineages F, G, and H (gray circles and circles with horizontal and transversal bars; Figs. 4 and 5) are most likely to be indigenous to the southern basin of the lake.

It should be noted that the newly discovered eighth lineage (C) sampled at Kyeso is likely to represent *Tropheus annectens*, because Kyeso is located in close vicinity to the origin of the type specimens described by Boulenger in 1900. These individuals were found to live in sympatry to individuals belonging to lineage A2, which was found at both lake sides of the central basin. A preliminary morphological analysis showed that six of the seven sampled individuals had four anal spines and the seventh individual had five anal spines. The other five individuals collected at Kyeso had six anal spines and also differed in the mouth shape and coloration from *T. annectens*. Interestingly, the individuals collected at Kyeso preliminarily assigned to *T. annectens* belong to a different major mitochondrial lineage (C) than *T. polli* (E) from the opposite shoreline, even if they appear similar in overall morphology, anal spine number, and coloration (see also Poll 1986; Konings 1999).

The minimum spanning trees indicate that most of the major lineages expanded their distribution to neighboring areas during the secondary radiation about 400 Ka and that lineages A and D even managed to cross to the opposite shore regions in the central basin. In particular, lineage A was split into four distinct sublineages after invading the eastern shoreline about 400 Ka (A1 to A4; see Figs. 4 and 6). Sublineages A1 and A3 probably emerged via range expansion within the eastern shore of the northern basin, sublineage A2 originated by colonizing the northwestern shore of the northern and central basin, while sublineage A4 probably originated by colonization of the eastern shores of the southern basin (Fig. 6). Lineage D is likely to have invaded a very short section of the eastern coast of the central basin at Cape Kibwesa from its hypothesized location of origin at the western coast of the southern basin. The crossing of the lake in these areas would imply a retreat of the lake level by a minimum of 550 m about 400 Ka, since *Tropheus* is not able to disperse through open water (Brichard 1978; Sturmbauer and Dallinger 1995). Only a drop over minimally 550 m would be sufficient to shift a continuous band of rocky bottom into the depth limit of *Tropheus* at about 50 m. The magnitude of the level drop would be greater than suggested by geological evidence [about 350 m 390–360 Ka (Lezzar et al. 1996; Cohen et al. 1997)].

The distribution of individuals of *T.* "Kirschfleck" assigned to lineage F at the eastern coast of the central basin in and north of Kibwesa seems quite puzzling, given the present-day distribution of the remaining members of this lineage at the southwestern section of Lake Tanganyika in and around Cameron Bay. At Kibwesa three variants of *Tropheus* live in sympatry [*T. polli*, *T.* "Kibwesa," and *T.* "Kirschfleck," Fig. 5 (Snoeks et al. 1994)] that can be clearly distinguished on basis of their mtDNA haplotypes. However, in specimens of *T.* "Kirschfleck," mtDNA haplotypes of two lineages were found (Sturmbauer et al. 1997), indicating a past hybridization event upon secondary contact, most probably between the indigenous *T. polli* lineage (E) and invading *T.* "Kirschfleck" ancestors of lineage F. Two alternative scenarios may be implied. First, members of lineage F could have moved along the western coast along the southern basin, finally to cross the lake at the border area between the central and the southern basin. Still, it remains unclear how lineage F could move through such a wide section of steeply sloping shore line at the western coast of the southern basin, which is presently occupied by lineage D, without leaving any genetic traces or residual populations living in sympatry with members of lineage F. The alternative explanation—which we favor—would imply that lineage F was originally distributed along the southeastern shoreline from Kibwesa to about Wapembe, to be replaced later by invading members of lineage A, so that the haplotypes of lineage F at Kibwesa are relicts of an originally much wider distributed lineage. This hypothesis would also imply that lineage F secondarily colonized their present center of diversity in and around Cameron Bay in the southwestern section of the lake during the major rise of the lake level about 400 Ma. This scenario would also explain the presence of two distinct mtDNA haplotypes in the population at Mvua (F and G) as being the consequence of hybridization upon secondary contact with invading members of lineage F (Figs. 4 and 5). If our hypothesis is correct, this colonization event might have almost totally replaced the previously indigenous lineage G, which, at the present time, has its center of diversity south of the estuary of the Lufubu River. Taking into account that the Lufubu River, as the third-largest tributary to the lake, represents a highly stable ecological barrier separating the Chaitika mountain shores from those north of the Inangu peninsula, lineage G may well have maintained its original distribution south of the Lufubu, but was replaced by members of lineage F in the Cameron Bay area after a drop in the lake level. Which of the hypotheses is correct may be clarified in the future after additional populations are analyzed.

During the "tertiary radiation" about 200 Ka three sublineages of lineage A spread farther along the coasts where they originated. Sublineage A2 must have crossed the lake on the southern edge of the central basin to enter to the eastern shores of the southern basin of Lake Tanganyika. Sublineages A2 and A4 expanded their range along the southeastern coast toward the most southern part of Lake Tanganyika. Presumably, members of sublineages A2 and A4 outcompeted the indigenous *Tropheus* lineage(s) from this area. The hypothesized replacement of a

previously indigenous lineage by sublineages A2 and A4 is in fact substantiated by the presence of haplotypes assigned to two major mtDNA lineages in three localities in the southern region (Fig. 6). At the locality Wapembe "north" one sequenced individual had a mtDNA haplotype belonging to lineage H, which arose from the primary radiation, and all other individuals had haplotypes belonging to two sublineages of lineage A. Konings (personal communication) reported two different *Tropheus* living in sympatry near Wapembe. At Katoto a major breakpoint between lineage A and lineage G was found: about 50% of the population had haplotypes of lineage G and 50% of sublineages A2 and A4. Lineage A2 was also found at Katukula, but this population was dominated by individuals belonging to lineage G (Fig. 6).

In summary, seven lineages of *Tropheus* show a clear phylogeographic signature. That most lineages did not dramatically alter their ranges of distribution may be due to the stability of their habitats centering along steeply sloping shores. These habitats would not be considerably affected by lake level fluctuations, since they would be only shifted up and down along a continuous slope. In contrast, one sublineage of lineage A (A2) was found almost throughout the entire lake and even individuals from distant populations were closely related to each other. The reason this particular sublineage expanded its distribution so dramatically may in fact lie in differences in ecology and behavior, but also in the location of its origin in a very unstable section of the lake. The original habitat was likely to be located close to the border of the northern and the central basin of Lake Tanganyika, which contains very shallow areas that were most severely affected by changes in the lake level (Sturmbauer and Meyer 1992; Sturmbauer et al. 1997).

The phylogeographic patterns of mtDNA haplotypes in *Tropheus* show striking parallels to that found in the tribe Eretmodini. The average corrected sequence divergence of 4.5 to 7.7% that was found among clades of lineage B of the Eretmodini (Verheyen et al. 1996) almost perfectly overlaps with the observed genetic distances resulting from the "primary radiation" of *Tropheus*. Also, lineage A of the Eretmodini (3.1 to 3.4% Kimura two-parameter distances) seems to have spread to all three basins of the lake at exactly the same time that lineage B of *Tropheus* expanded its range of distribution. Another recent study demonstrated the connection of the radiation of the Tanganyikan tribe Cyprichromini, an open-water lineage of cichlid fish, to the same rise of the lake level that also triggered the primary radiation of *Tropheus* and the Eretmodini (Brandstätter et al. unpublished). Therefore, it seems very likely that the same changes in the lake habitat triggered the diversification of various cichlid groups and that particular geological events might leave similar traces in the genetic population structure of species with similar biological characteristics. Future studies of the phylogeographic history of other cichlid lineages, but also of other organisms in Lake Tanganyika, will be important to gain deeper insights into the connection between changes in the lake and the colonization history of its species.

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