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Evolutionary Relationships of the Limnochromini, a Tribe of Benthic Deepwater Cichlid Fish Endemic to Lake Tanganyika, East Africa

Nina Duftner, Stephan Koblmüller, Christian Sturmbauer

Department of Zoology, Karl-Franzens-University of Graz, Universitätsplatz 2, A-8010 Graz, Austria

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Abstract. Lake Tanganyika harbors an enormous diversity of cichlid fish that stem from eight distinct ancestral lineages, which colonized the lake after its formation 9 to 12 million years ago. Six of twelve currently described tribes are assigned to the "H-lineage," an assemblage of exclusively mouthbrooding cichlids, all of which evolved during a short period of time during the course of the primary radiation of lacustrine species. Our study focuses on the deepwater tribe Limnochromini, comprising bi-parental mouthbrooders, and is based on phylogenetic analysis of two mitochondrial gene segments. We confirm the polyphyletic origin of the Limnochromini as they are defined to date, in that *Gnathochromis pfefferi* is placed among the Tropheini, whereas the genus *Benthochromis* is presented as an independent lineage. The remaining nine species were unambiguously resolved as monophyletic and should be redefined as the tribe Limnochromini. Concerning generic assignments, the genus *Greenwoodochromis* appeared as monophyletic, *Limnochromis* as paraphyletic, and the genera *Reganochromis* and *Baileychromis* as monophyletic sister genera. The linearized tree analysis and the comparison of average sequence divergences to that of the remaining tribes of the H-lineage revealed a relatively recent but simultaneous proliferation of the Limnochromini, suggesting that the same environmental changes triggered the radiation of particular deepwater, benthic, pelagic, and littoral lineages. By using a preliminary calibration of a molecular clock based on gamma-corrected amino acid distances of the *NADH2* gene, the diversification of the Limnochromini could tentatively be dated to 2.9–3.5 MYA, coinciding with a period of aridification in East Africa between 2.5 and 3 MYA. The lack of geographic color morphs and the structural uniformity and resource scarcity of deepwater habitats suggest that competition and resource partitioning leading to differential trophic specialization promoted speciation within the Limnochromini, rather than an allopatric model.

Key words: Adaptive radiation — Control region — NADH dehydrogenase subunit 2 — Explosive speciation — Niche partitioning — Molecular clock

Introduction

Cichlid fish (Perciformes: Teleostei) occur in freshwater systems in Africa, Central and South America, Madagascar, India, Sri Lanka, the Middle East, and Iran. The most spectacular radiation within this family is observed in the three East African Great Lakes Tanganyika, Malawi, and Victoria, together comprising more than half of the estimated total of 2500 species of cichlid fish (Turner et al. 2001). All three cichlid flocks evolved within a relatively short period of time through intralacustrine speciation, thus providing ideal model systems for the study of mechanisms driving explosive speciation and adaptive radiation (Fryer and Iles 1972; Nishida 1991; Rossiter 1995; Sturmbauer 1998; Kornfield and

Correspondence to: Christian Sturmbauer; *email:* christian.sturmbauer@uni-graz.at

Smith 2000; Danley and Kocher 2001; Turner et al. 2001; Takahashi et al. 2001; Verheyen et al. 2003).

With an age of 9 to 12 Myr (Cohen et al. 1993), Lake Tanganyika (Fig. 1) is the oldest of the three lakes and harbors about 200 described cichlid species assigned to 12 tribes (Poll 1986) that are ecomorphologically and behaviorally highly diverse. Takahashi (2003) even proposed the establishment of 16 tribes based on a series of morphological characteristics. In contrast to the cichlids of Lake Malawi and Victoria, those of Lake Tanganyika are known to be of polyphyletic origin, as eight lineages independently colonized the lake from surrounding rivers (Nishida 1991; Salzburger et al. 2002). The current species diversity of the cichlid assemblage of Lake Tanganyika was partly determined by geological activity and lake level fluctuations. The central portion of the lake is the oldest (9–12 MYA), its formation interrupting the ancient Proto–Malagarazi-Congo River, while the northern and southern basins were formed more recently (7–8 and 2–4 MYA, respectively [Cohen et al. 1993]). The three central lake basins were repeatedly split by large fluctuations of the lake level, and the fusion into a single lake with deepwater conditions was gauged to 5–6 MYA (Tiercelin and Mondeguer 1991). The onset of deepwater conditions was taken as prerequisite for the primary lacustrine radiation of five of the eight seeding lineages, as indicated by a burst of cladogenetic events by adaptation to newly available habitats (Sturmbauer 1998; Salzburger et al. 2002). Stable environmental conditions were repeatedly suspended by lake level fluctuations driven by climate changes and geological activity during the Pleistocene and Holocene (Scholz and Rosendahl 1988; Lezzar et al. 1996; Cohen et al. 1997; Scholz et al. 2003). Several studies demonstrated the impact of these lake level changes on the Tanganyika radiation (Sturmbauer and Meyer 1992; Verheyen et al. 1996; Sturmbauer et al. 1997, 2001, 2003; Rüber et al. 1998; Baric et al. 2003).

The objective of our study was to elucidate the evolution of the Limnochromini, a tribe of benthic deepwater invertebrate feeders and mud dwellers. Most of these species are rarely caught, due to their deepwater life style. They encompass a great morphological diversity of adaptations to various benthic niches, ranging from shovel-like to pike-like snouts, exemplified by *Gnathochromis permaxillaris* and *Baileychromis centropomoides*. According to the classification of Poll (1986) the Limnochromini comprise 13 species in eight genera that are all endemic to the lake and belong to an assemblage of six tribes, termed the H-lineage (Nishida 1991; Salzburger et al. 2002). Except for *Gnathochromis pfefferi*, *Benthochromis tricoti*, and *B. melanoides*, all members of this tribe — for which breeding style is known to date — are pair-forming biparental mouthbrooders,

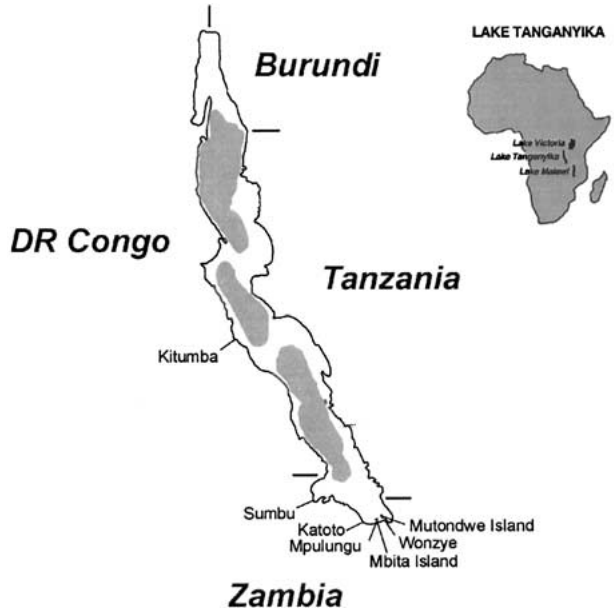

Fig. 1. Map of Lake Tanganyika, Eastern Africa, with emphasis on the sampling localities.

in which both males and females incubate up to 250 eggs and/or wrigglers in their buccal cavities and protect their fry against predators. Some species such as *Triglachromis otostigma* dig mud-burrows (Konings 1998). The monophyly of the Limnochromini sensu Poll (1986) was repeatedly questioned in morphological (Takahashi 2003) and molecular (Kocher et al. 1995; Nishida 1997; Salzburger et al. 2002; Sturmbauer et al. 2003) studies. *Benthochromis tricoti* was placed outside the Limnochromini, whereby the exact positioning within the H-lineage remains controversial (Salzburger et al. 2002; Takahashi 2003). *Gnathochromis pfefferi* is a maternal mouthbrooder and was assigned to the tribe Tropheini (Kocher et al. 1995; Salzburger et al. 2002; Sturmbauer et al. 2003), with which it shares several morphological characteristics (Lippitsch 1998; Takahashi 2003). Its congener *G. permaxillaris* took an ancestral position to the Ectodini in a phylogeny derived from 21 allozyme loci (Nishida 1997) but was placed as sister taxon to *Limnochromis auritus* by Salzburger et al. (2002). Takahashi (2003) suggested establishing a new tribe for the genera *Greenwoodochromis* and *Benthochromis* based on morphological features. We use DNA sequences of two mitochondrial gene segments to construct a mitochondrial phylogeny for the tribe Limnochromini, from which we infer major diversification events and discuss factors promoting speciation in benthic deepwater cichlids. Moreover, we present a preliminary calibration of the molecular clock for the *NADH2* gene of cichlid fish, derived from gamma-corrected amino acid distances, by using two geological calibration points. The first calibration point marks the onset of lacustrine

deepwater conditions in the history of Lake Tanganyika (Tiercelin and Mondeguer 1991) and the observed protein distances among subgroups of the H-lineage and the Lamprologini, which are assumed to have radiated at this time (Salzburger et al. 2002). The second calibration point refers to the lacustrine radiation of cichlid fish in Lake Malawi, dated to 0.57–1.0 MYA which is assumed to have taken place when the lake reached a first highstand at about 0.7 MYA after the period of aridification 1.6–1.0 MYA (Delvaux 1995; see also Sturmbauer et al. 2001).

Materials and Methods

Taxonomic Sampling and Molecular Biological Methods

Our study includes 12 of the 13 described species (*Tangachromis dhansi* could not be obtained for this study) currently assigned to the tribe Limnochromini (Poll 1986) and representatives of the Cyprichromini, Ectodini, Haplochromini, Perissodini, and Tropheini, as well as three members of the Lamprologini (*Lepidiolamprologus attenuatus*, *Palaeolamprologus toae*, and *Telmatochromis vittatus*) that were used as outgroup taxa (Salzburger et al. 2002). The complete NADH dehydrogenase subunit 2 gene (1047 bp; *NADH2*) and a 364-bp segment of the most variable part of the control region (D-loop) was obtained from 30 individuals of the Limnochromini (Poll 1986) and 8 individuals of the tribes Ectodini and Lamprologini. We also used previously published *NADH2* and partial control region sequences of 21 individuals representing all tribes of the H-lineage (Table 1). For the calibration of a molecular clock based on the *NADH2* gene we sequenced 12 additional specimens and obtained 15 published sequences from GenBank of cichlids inhabiting Lakes Malawi and Tanganyika (Table 1).

Whole genomic DNA was extracted from fin clips or muscle tissue preserved in 96% ethanol applying proteinase K digestion followed by protein precipitation with ammonium acetate. PCR amplification followed the protocol of Koblmüller et al. (2004). The PCR products were purified with ExoSAP-IT, and chain termination sequencing was carried out in 10-µl reaction volumes in a GeneAmp PCR System 9700 (Applied Biosystems) using 4 µl purified DNA template, 0.8 µl BigDye Termination Reaction Mix (Applied Biosystems), and 0.5 µl primer (0.5 µM). The sequencing program consisted of an initial denaturation phase of 4 min at 94°C, followed by 32 cycles of 30 s at 94°C, 20 s at 50°C, and 4 min at 60°C. The primers used for both amplification and sequencing of the control region were L-Pro-F, 5′ AACTCTCACCCCTAGCTCCCAAAG 3′ (Meyer et al. 1994), and TDK-D, 5′ CCTGAAGTAGGAACCAGATG 3′ (Kocher et al. 1989). The entire *NADH2* gene was amplified using primers binding to the flanking tRNAs methionine (MET), 5′ CATACCCCAACATGTTGGT 3′, and tryptophan (TRP), 5′ GA GATTTTCACTCCCGCTTA 3′ (Kocher et al. 1995). For sequencing we additionally employed two internal primers, NADH2.2A, 5′ CTGACAAAAACTTGCCTT 3′ (Kocher et al. 1995) and the newly designed NADH2.T-R 5′ GGGGCTTTTGTTCAG GATGT 3′. DNA sequences of both light and heavy strand were visualized on a 3100 capillary sequencer (Applied Biosystems).

Phylogenetic Analysis

Alignment of DNA sequences was carried out using CLUSTAL W (Thompson et al. 1994) and was improved by eye for the control region. To assess the overall phylogenetic signal we applied likelihood-mapping analysis for each data set, using PUZZLE 4.0 (Strimmer and von Haeseler 1997) (Fig. 2). For phylogenetic inference maximum parsimony (MP), neighbor joining (NJ), maximum likelihood (ML), and Bayesian inference (BI) were applied using PAUP* 4.0b6 (Swofford 2000) and MrBayes 3.0b4 (Huelsenbeck and Ronquist 2001).

Phylogenetic analysis was based on two mitochondrial DNA segments, the *NADH2* gene (1047 bp) and a 364-bp segment of the control region. Congruence among the two data sets was assessed under the MP criterion using the partition homogeneity test implemented in PAUP* (Swofford 2000) with 1000 replications. Since there was no conflict in the species assignment of the taxa analyzed in the *NADH2* and control region data sets, we selected one taxon per species for the combined data set to reduce the computation time (1411 bp, 35 taxa; see Fig. 3). To assess the degree of saturation of transition (Ti) and transversion (Tv) mutations in each codon position of *NADH2*, we plotted the number of mutations against the percentage of sequence divergence of 1081 pairwise comparisons (not shown). Due to a Ti–Tv ratio of 4.5 inferred from these pairwise comparisons in the third codon position, transversions were weighted 4.5 times over transitions in MP. For the control region we used Tv only in MP to minimize the impact of multiple hits while still having sufficient data to discriminate clades down to the species level. MP topologies were obtained by applying the heuristic search option with stepwise addition of taxa and 1000 replicates. To select the appropriate model of sequence evolution for ML and NJ we applied the hierarchical likelihood ratio test implemented in the computer program Modeltest version 3.06 (Posada and Crandall 1998), which chooses the best-fitting DNA substitution model for the data among a series of increasingly complex assumptions of sequence evolution (Huelsenbeck and Crandall 1997). The chosen substitution models were TrN + I + Γ (Tamura and Nei 1993) for *NADH2* and the control region and GTR +I + Γ (Yang 1994) for the combined data set. The appropriate likelihood parameters for the combined data set were $g_A = 0.2832$, $g_C = 0.3231$, $g_G = 0.1175$, and $g_T = 0.2762$, $\Gamma = 0.8208$, and $I = 0.4133$. The BI was run with 10 separate Markov chains (chain temperature 0.2) for 2 million generations, with a tree saved each 100th generation. The first 10,000 trees were discarded, which poses a highly conservative "burn-in" value for likelihood scores to reach stationary. The remaining 10,000 trees were used to calculate posterior probabilities for the branches of the resulting tree topology. Nodal support in MP and NJ was evaluated by 1000 bootstrap pseudoreplications, whereas the topologies of the ML trees were evaluated by quartet puzzling (QP) in PAUP* and by calculating posterior probabilities in the Bayesian analysis.

The phylogenetic positioning of the two species of *Benthochromis* that were considered to form an independent tribe within the H-lineage by Takahashi (2003) was tested by a comparison of constrained tree topologies with monophyletic clades of *Benthochromis*–Cyprichromini (A) and *Benthochromis*–Perissodini (B) applying the Swofford, Olsen, Waddell, and Hillis (SOWH) test (Goldman et al. 2000). While the most commonly used likelihood-based Kishino–Hasegawa (KH) (1989) test of competing evolutionary hypotheses is restricted to testing topologies that were selected independently of the data, the SOWH test allows the inclusion of the ML tree derived from the data (Goldman et al. 2000).

Calibration of the Molecular Clock

For our — so far preliminary — calibration of a molecular clock based on the *NADH2* gene only, we followed the procedure described by Kumazawa and Nishida (2000), who calibrated a molecular clock for bony fish (including six cichlid species) using gamma-corrected ML distances of *NADH2* and cytochrome *b*

Table 1. List of samples examined in this study, with classification, locality data, and GenBank accession number

No.	Sample ID[a]	Species	Class.	Geogr. coord.	Locality	GenBank accession no.	
						NADH2	Control region
1	2313[M]	*Baileychromis centropomoides*	LIM	8°45′S 31°06′E	Mpulungu	AY682509	AY682472
2	2314[H]	*Baileychromis centropomoides*	LIM	8°45′S 31°06′E	Mpulungu	AY682511	AY682474
3	2371[H]	*Baileychromis centropomoides*	LIM	8°45′S 31°06′E	Mpulungu	AY682510	AY682473
4	1838	*Benthochromis melanoides*	LIM	8°31′S 30°28′E	N-Sumbu	AY682512	AY682475
5	1839	*Benthochromis melanoides*	LIM	8°31′S 30°28′E	N-Sumbu	AY682513	AY682476
6	2315	*Benthochromis tricoti*	LIM	8°45′S 31°06′E	Mpulungu	AY682514	AY682477
7	2316	*Benthochromis tricoti*	LIM	8°45′S 31°06′E	Mpulungu	AY682515	AY682478
8	2378	*Cunningtonia longiventralis*[b]	ECT	8°45′S 31°06′E	Mpulungu	AY682516	AY682479
9	1844	*Enantiopus melanogenys*[b]	ECT	8°47′S 31°01′E	Katoto	AY682517	AY682480
10	2310	*Gnathochromis pfefferi*	TRO*	8°45′S 31°05′E	Mbita Island	AY682518	AY682481
11	1611[G]	*Gnathochromis permaxillaris*	LIM	8°45′S 31°06′E	Mpulungu	AY682519	AY682482
12	1612[G]	*Gnathochromis permaxillaris*	LIM	8°45′S 31°06′E	Mpulungu	AY682520	AY682483
13	2311	*Gnathochromis permaxillaris*	LIM	8°45′S 31°06′E	Mpulungu	AY682521	AY682484
14	2312[M]	*Gnathochromis permaxillaris*	LIM	8°45′S 31°06′E	Mpulungu	AY682522	AY682485
15	2323[M]	*Greenwoodochromis bellcrossi*	LIM	8°45′S 31°06′E	Mpulungu	AY682523	AY682486
16	2486[M]	*Greenwoodochromis bellcrossi*	LIM	8°45′S 31°06′E	Mpulungu	AY682524	AY682487
17	2325[M]	*Greenwoodochromis christyi*	LIM	8°45′S 31°06′E	Mpulungu	AY682525	AY682488
18	2326[M]	*Greenwoodochromis christyi*	LIM	8°45′S 31°06′E	Mpulungu	AY682526	AY682489
19	2678	*Greenwoodochromis christyi*	LIM	8°45′S 31°06′E	Mpulungu	AY682527	AY682490
20	2679	*Greenwoodochromis christyi*	LIM	8°45′S 31°06′E	Mpulungu	AY682528	AY682491
21	2328	*Haplotaxodon microlepis*[b]	PER	8°45′S 31°06′E	Mpulungu	AY682529	AY682492
22	2025[T]	*Haplotaxodon* sp.[b]	PER	8°45′S 31°06′E	Mpulungu	AY682530	AY682493
23	2585[G]	*Haplotaxodon trifasciatus*[b]	PER	8°45′S 31°06′E	Mpulungu	AY682531	AY682494
24	2526	*Lepidiolamprologus attenuatus*[b]	LAM	8°43′S 31°08′E	Wonzye	AY682532	AY682495
25	2327	*Limnochromis abeelei*	LIM	8°45′S 31°06′E	Mpulungu	AY682533	AY682496
26	2388[M]	*Limnochromis abeelei*	LIM	8°45′S 31°06′E	Mpulungu	AY682534	AY682497
27	2487	*Limnochromis abeelei*	LIM	8°45′S 31°06′E	Mpulungu	AY682535	AY682498
28	2319[M]	*Limnochromis auritus*	LIM	8°45′S 31°06′E	Mpulungu	AY682536	AY682499
29	2320	*Limnochromis auritus*	LIM	8°45′S 31°06′E	Mpulungu	AY682537	AY682500
30	2317[T]	*Limnochromis staneri*	LIM	8°45′S 31°06′E	Mpulungu	AY682538	AY682501
31	2321[T]	*Limnochromis staneri*	LIM	8°45′S 31°06′E	Mpulungu	AY682541	AY682502
32	2383	*Limnochromis staneri*	LIM	8°45′S 31°06′E	Mpulungu	AY682539	AY682504
33	2385[T]	*Limnochromis staneri*	LIM	8°45′S 31°06′E	Mpulungu	AY682542	AY682503
34	2386	*Limnochromis staneri*	LIM	8°45′S 31°06′E	Mpulungu	AY682540	AY682505
35	217	*Palaeolamprologus toae*[b]	LAM	?	?	AY682543	AF400723
36	3492[T]	*Reganochromis calliurus*	LIM	?	?	AY682544	AY682506
37	2119	*Telmatochromis vittatus*[b]	LAM	8°42′S 31°07′E	Mtondwe Island	AY682545	AY682507
38	1840	*Triglachromis otostigma*	LIM	8°42′S 31°07′E	Mtondwe Island	AY682546	AY682508
		Previously published sequences					
39		*Astatoreochromis alluaudi*[b]	HAP			AF398234	AY226786
40		*Astatotilapia burtoni*[b]	HAP			AF317266	AY226787
41		*Benthochromis tricoti*	LIM			AF317264	AF400725
42		*Cyphotialipa frontosa*[b]	TRO			CFU07247	AF400732
43	1684	*Cyprichromis leptosoma*[b]	CYP	6°40′S 29°29′E	Kitumba	AY337786	AY339053
44	1660	*Cyprichromis leptosoma* "gold"[b]	CYP			AY740344	AY740290
45	C27	*Cyprichromis leptosoma*[b]	CYP			AY740379	AY740328
46	1680	*Cyprichromis microlepidotus*[b]	CYP			AY740354	AY740301
47	C04	*Cyprichromis pavo*[b]	CYP			AY740358	AY740307
48	C01	*Cyprichromis* "tricolor jumbo"[b]	CYP			AY740355	AY740304
49	1673	*Cyprichromis zonatus*[b]	CYP			AY740347	AY740295
50		*Gnathochromis pfefferi*	TRO*			U07248	AF400727
51		*Grammatotria lemairii*[b]	ECT			AY337787	AY339018
52	59	*Limnochromis auritus*	LIM			AF398216	AF400728
53		*Limnochromis auritus*	LIM			U07253	Z21746

(Continued)

Table 1. Continued

No.	Sample ID[a]	Species	Class.	Geogr. coord.	Locality	GenBank accession no. *NADH2*	GenBank accession no. Control region
54		*Paracyprichromis brieni*[b]	CYP			AF398223	AF400700
55		*Perissodus microlepis*[b]	PER			AF317265	AF400730
56		*Petrochromis orthognatus*[b]	TRO			POU07262	POU12549
57		*Plecodus straeleni*[b]	PER			AF398221	AF400731
58	103	*Triglachromis otostigma*	LIM	?	Burundi	AY337769	Z30035
59		*Tropheus moorii*[b]	TRO			AB018975	TMO489716
		Additional sequences for the calibration of a molecular clock					
	586	*Altolamprologus compressiceps*[b]	LAM			AF398229	
		Aulonocara sp. "gold"[b]	mb			AF305285	
		Aulonocara sp. "yellow"[b]	mb			AF305284	
		Chromidotilapia guentheri[b]	n-mb			AF317270	
	1699	*Ectodus descampsi*[b]	ECT			AY337790	
	3055	*Labidochromis caeruleus*[b]	n-mb			AY740383	
	577	*Lamprologus callipterus*[b]	LAM			AF398226	
	307	*Lamprologus congoensis*[b]	LAM			AY740385	
	582	*Lamprologus lemairii*[b]	LAM			AY740386	
	1827	*Lepidiolamprologus attenuates*[b]	LAM			AY740387	
	208	*Neolamprologus caudopunctatus*[b]	LAM			AY740388	
	297	*Neolamprologus christyi*[b]	LAM			AY740389	
	618	*Neolamprologus marunguensis*[b]	LAM			AY740390	
	315	*Neolamprologus niger*[b]	LAM			AY740391	
	1227	*Neolamprologus nigriventris*[b]	LAM			AY740392	
	631	*Neolamprologus olivaceous*[b]	LAM			AY740393	
	1834	*Neolamprologus palmeri*[b]	LAM			AY740394	
	481	*Neolamprologus pulcher*[b]	LAM			AY740395	
	1200	*Ophthalmotilapia ventralis*[b]	ECT			AY337774	
		Pallidochromis tokolosh[b]	n-mb			AF305276	
	3058	*Pseudotropheus tropheops*[b]	mb			AY740384	
	106	*Telmatochromis bifrenatus*[b]	LAM			AF398228	
	583	*Telmatochromis vittatus*[b]	LAM			AY740396	
	1570	*Xenotilapia caudafasciata*[b]	ECT			AY337777	
		Xenotilapia flavipinnis[b]	ECT			AY337794	

[a]Extraction number.

[b]Sequences selected for the calibration of a molecular clock; class., classification of the taxa: (G) voucher specimen at the University of Graz, Department of Zoology, Austria; (H) voucher specimen at the Hokkaido University, Graduate School of Fisheries Sciences, Laboratory of Marine Biodiversity, Japan; (M) voucher specimen at the Department of Fisheries at Mpulungu, Ministry of Agriculture and Cooperatives, Zambia; (T) voucher specimen at the Ocean Research Institute, Tokyo; (T) voucher specimen at the Royal Africa Museum in Tervuren, Belgium. ECT, Ectodini; HAP, Haplochromini; LAM, Lamprologini; LIM, Limnochromini; PER, Perissodini; TRO, Tropheini [(TRO*, including *Gnathochromis pfefferi*, a former member of the tribe Limnochromini, which was consistently resolved within the Tropheini in molecular studies (Kocher et al. 1995; Salzburger et al. 2002; Sturmbauer et al. 2003)]; mb, mbuna; n-mb, nonmbuna cichlids of Lake Malawi.

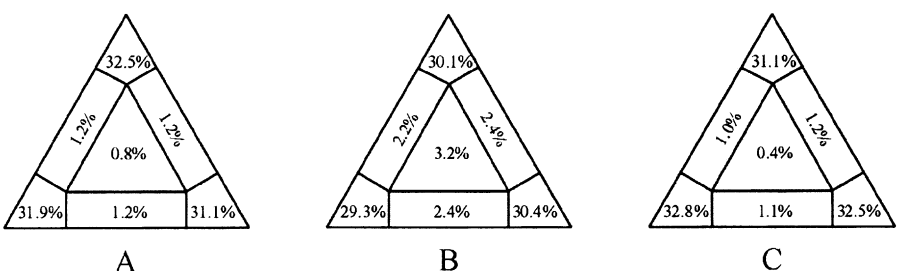

Fig. 2. Likelihood-mapping analysis (Strimmer and von Haeseler 1997) for the three data sets: (**A**) the *NADH2* (44 taxa plus outgroup), (**B**) the control region (49 taxa plus outgroup), and (**C**) the combined (44 taxa plus outgroup) data sets. Values in the corners of the triangles represent the percentage of a well-resolved phylogeny, while the intermediate regions specify difficulties in distinguishing between two of three possible quartet topologies. In the center the percentage of unresolved trees is shown, indicating a star-like evolution.

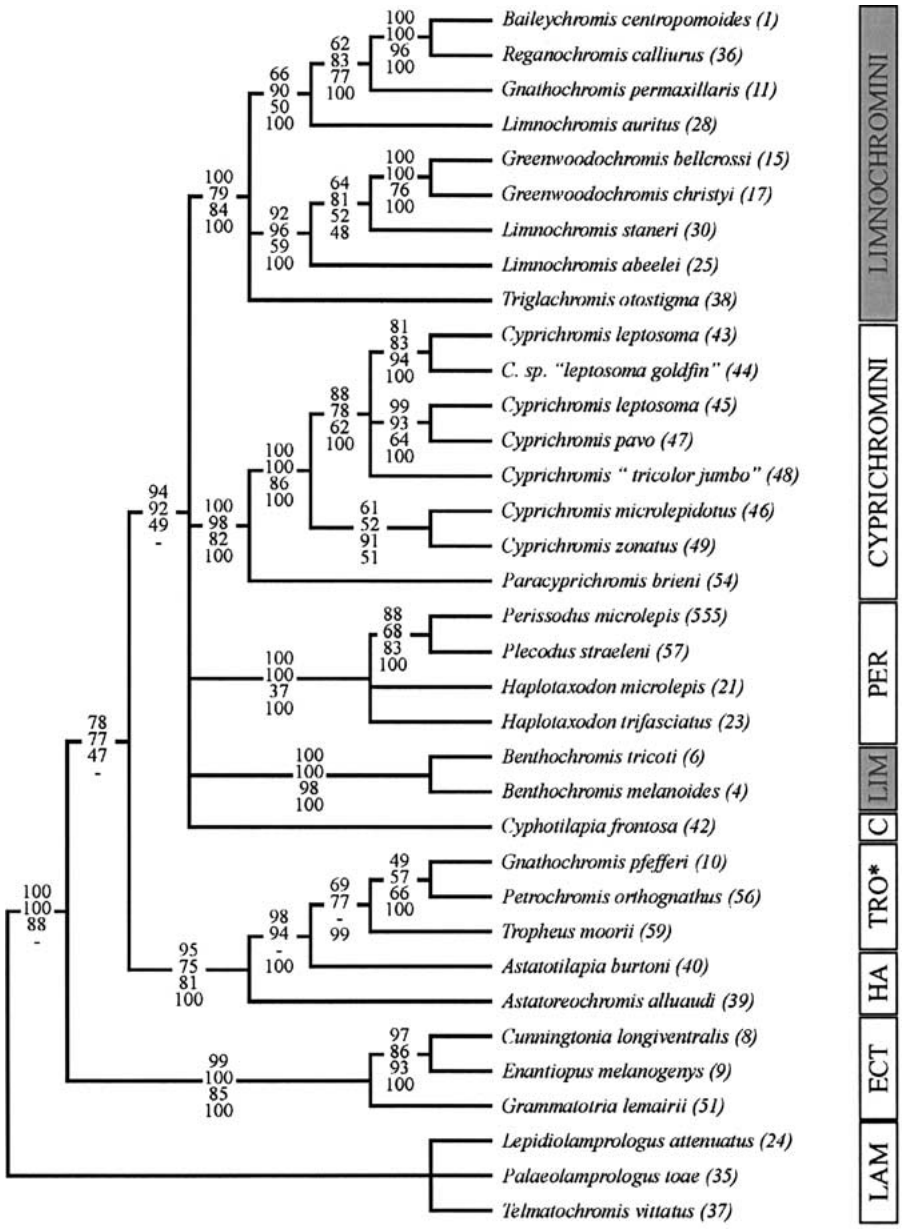

Fig. 3. Strict consensus tree of NJ, MP, and ML based on the combined anaylsis of the two mitochondrial genes, *NADH2* and the control region (1411 bp) comprising 35 taxa representing tribes assigned to the H-lineage as defined by Nishida (1991) but excluding the Eretmodini (Salzburger et al. 2002). BS values are shown for NJ and MP, quartet puzzling values for ML, and Bayesian posterior probabilities based on a 2-million–step MCMC analysis, from top to bottom upon the nodes. Shaded boxes delineate the paraphyly of the tribe Limnochromini. Numbers in parentheses refer to the sample list in Table 1. C, "*Cyphotilapia*" lineage (previously assigned to the Tropheini but shown to represent an independent lineage by Kocher et al. [1995] and Salzburger et al. [2002]); ECT, Ectodini; HA, Haplochromini; LAM, Lamprologini; LIM, Limnochromini; PER, Perissodini; TRO*, Tropheini (including *Gnathochromis pfefferi*, a former member of the tribe Limnochromini, which was consistently resolved within the Tropheini in molecular studies [Kocher et al. 1995; Salzburger et al. 2002; Sturmbauer et al. 2003]).

amino acid sequences. In contrast to this approach, we restricted the clock calibration to the *NADH2* gene of cichlid fish by using two geological calibration points, the first one indicating the onset of lacustrine deepwater conditions in the history of Lake Tanganyika dated to 5–6 MYA (Tiercelin and Mondeguer 1991) and the observed protein distances among subgroups of the H-lineage and the Lamprologini, which are assumed to have radiated at this time (Salzburger et al. 2002), and the second one referring to the diversification among the mbuna and nonmbuna cichlids of Lake Malawi dated to 0.57–1.0 MYA (Sturmbauer et al. 2001) after the dry-up of the lake 1.6–1.0 MYA (Delvaux 1995). The rock-dwelling mbuna and sand-dwelling nonmbuna cichlids represent two major clades, each containing more than 200 species, that are derived from a generalized cichlid, which colonized Lake Malawi (Moran et al. 1994).

For this approach, we selected a total of 51 sequences of cichlid fish from Lakes Malawi and Tanganyika (see Table 1) for which we calculated gamma-corrected ML amino acid distances using TREE PUZZLE, version 5.1 (Schmidt et al. 2002), by applying the mtREV24 (Adachi and Hasegawa 1996) matrix. To obtain protein distances corresponding to the onset of the radiation of the H-lineage, we used six representatives of the Ectodini, four of the Haplochromini/Tropheini clade, four of the Perissodini, and six of the Cyprichromini. To represent the primary lacustrine radiation of the Lamprologini, we used 15 taxa representing diverse clades within this tribe. Protein distances among the mbuna and nonmbuna cichlids of Lake Malawi are based on three taxa each of the mbuna and nonmbuna for which *NADH2* sequences could be obtained from GenBank (Table 1). A linear regression through the origin was derived from these calibration points and used to estimate the age of the Limnochromini.

Estimates of Major Cladogenetic Events Within the Limnochromini

To reconstruct the timing of the divergence of species within the tribe Limnochromini and to relate these cladogenetic events to splits in other Tanganyikan cichlid tribes, we performed a branch

length test (Takezaki et al. 1995), in which the nucleotide sequence data of the selected taxa were tested for rate heterogeneity. All sequences evolving in a significantly faster or slower pace ($p = 0.01$) were iteratively eliminated, and a linearized tree (Takezaki et al. 1995) was computed using the computer program LINTRE. This analysis was based on 1047 bp of the *NADH2* gene due only to the lesser degree of saturation of transition mutations compared to the control region. We used a subset of the available taxa including the tribes Ectodini, Cyprichromini, Perissodini, Limnochromini, and *Cyphotilapia frontosa*. *Lepidiolamprologus attenuatus*, *Palaeolamprologus toae*, and *Telmatochromis vittatus* were used as outgroup. The substitution model applied to construct the linearized tree was TrN+Γ (Γ = 0.66216). Finally, we calculated relative divergence times on the basis of average TrN+Γ distances (arithmetic mean and standard deviation) for the major cladogenetic events and estimated the age of the Limnochromini according to our preliminary clock calibration based on protein gamma-corrected amino acid distances.

Results

Phylogenetic Analyses

Likelihood mapping demonstrated adequate signal for phylogenetic reconstruction in all data sets (Fig. 2), with high levels of fully resolved quartets (89.8 to 96.4%). The combination of both gene segments considerably improved the phylogenetic resolution. MP of the combined data set yielded a single most parsimonious tree (8497 steps; CI excluding uninformative sites, 0.41; retention index [RI], 0.60; rescaled consistency index [RC], 0.29 [Kluge and Farris 1969]; tree not shown). As evident from the strict consensus tree of weighted MP, NJ, ML, and BI shown in Fig. 3, the following relationships were consistently found: the Ectodini occupied the most ancestral split, followed by a clade containing the Haplochromini and Tropheini. The branching order among the remaining clades (the Limnochromini, Cyprichromini, Perissodini, *Benthochromis*, and *Cyphotilapia frontosa*) differed among MP, NJ, ML, and Bayesian inference. While the two species of the genus *Benthochromis* were resolved as a separate lineage sister to the Cyprichromini in MP, NJ, and BI (BS = 54 for both analyses; posterior probability, 45), they were grouped as sister to the Perissodini plus Cyprichromini by ML analysis, albeit without quartet puzzling support. In concordance with previous studies (Salzburger et al. 2002; Takahashi et al. 2003), *Cyphotilapia frontosa* also formed a separate lineage, occupying a branch ancestral to the Perissodini in NJ and MP (BS = 81 and 58, respectively), whereas being resolved as branch ancestral to the Limnochromini in ML and BI (no quartet puzzling support; posterior probability, 82). The two alternative groupings of the genus *Benthochromis*, (A) the *Benthochromis*–Cyprichromini grouping and (B) the *Benthochromis*–Perissodini grouping, were evaluated further by means of the SOWH test. The null hypothesis of no significant difference in the log likelihood scores among the constrained tree versus the best unconstrained tree was rejected for both constrained topologies ($p < 0.01$), so that none of the two groupings was better supported than the topology of the best unconstrained tree. This finding corroborates the establishment of an independent tribe suggested by Takahashi et al. (2003).

Within the clade comprising nine species of the Limnochromini (excluding *Benthochromis* and *Gnathochromis pfefferi*), only *Triglachromis otostigma* changed its position and was resolved as the most ancestral split in MP (BS = 79), while being placed sister to *Limnochromis auritus*, followed by *Gnathochromis permaxillaris*, *Reganochromis calliurus*, and *Baileychromis centropomoides* in NJ, ML, and BI (BS = 69; QP = 57; posterior probability, 75).

In summary, our analyses suggest a redefinition of the tribe Limnochromini, which should contain only the following nine species (not taking into account *Tangachromis dhansi*, which, according to morphological data, also clusters within this group [Takahashi 2003]): *Baileychromis centropomoides*, *Gnathochromis permaxillaris*, *Greenwoodochromis christyi*, *G. bellcrossi*, *Limnochromis abeelei*, *L. auritus*, *L. staneri*, *Reganochromis calliurus*, and *Triglachromis otostigma*. Three species consistently fall outside the Limnochromini: *Benthochromis melanoides*, *B. tricoti*, and *Gnathochromis pfefferi*. *Benthochromis* was placed as a separate lineage sister to the Cyprichromini, to the Cyprichromini plus Perissodini, or to the Haplochromini–Tropheini clade; *Gnathochromis pfefferi* was placed in the tribe Tropheini, corroborating the findings of Kocher et al. (1995), Lippitsch (1998), Salzburger et al. (2002), Sturmbauer et al. (2003), and Takahashi (2003).

Preliminary Clock Calibration for the Cichlid NADH2 *Gene and Estimates of Divergence Time*

The branch length test applied to the gamma-corrected amino acid distances did not detect any rate inconsistencies among taxa so that all pairwise comparisons of taxa could be used for the calibration of the molecular clock. The clock calibration resulted in a linear regression of 0.0117 substitutions/site/Myr ($R^2 = 0.3693$; 95% confidence interval, 0.0068–0.0165) when the lower bound (5 MYA) of the time estimate for the onset of deepwater conditions in Lake Tanganyika was used and 0.0097 substitution/site/Myr ($R^2 = 0.3693$; 95% confidence interval, 0.0057–0.0137) when the upper time estimate (6 MYA) was used (Fig. 4). Gamma-corrected amino acid distances were calculated for the limnochromine taxa, to obtain average protein distances among the three deepest subgroupings of the redefined Limnochromini, demarcating the onset of their radiation

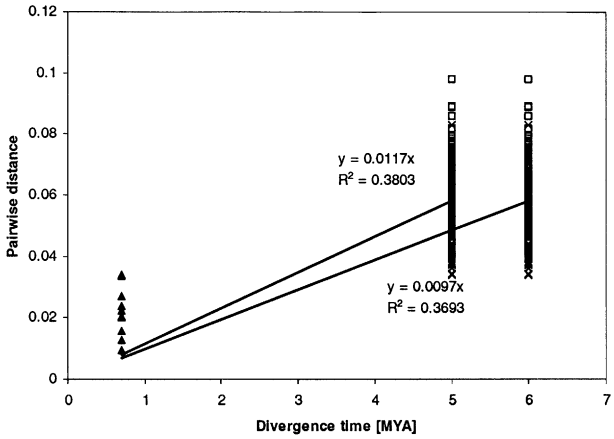

Fig. 4. Calibration of the molecular clock based on gamma-corrected ML amino acid distances among pairwise comparisons of 51 taxa plotted against estimated divergence times. Plotted data represent the divergence of mbuna and nonmbuna cichlids in Lake Malawi (0.7 MYA; triangles), and two alternative datings for the radiation among the members of the H-lineage (open rectangles) and the Lamprologini (crosses) in Lake Tanganyika (between 5 and 6 MYA). Regression lines for the two alternatives through the origin were obtained (R^2 = 0.3803, 0.0117 substitution/site/Myr for 5 MYA; R^2 = 0.3693, 0.0097 substitution/site/Myr for 6 MYA).

(average, 3.36%; SE, 0.35%; 16 pairwise comparisons).

A second branch length test was applied to DNA sequences of the *NADH2* gene and resulted in the exclusion of all specimens of *Limnochromis auritus*, *L. staneri*, *Greenwoodochromis bellcrossi*, and *G. christyi*. The confidence interval surrounding the average root-to-tip distance was set to 99% (Table 2). According to the linearized tree, four cladogenetic events become evident (Fig. 5). The first event represents the split between the Ectodini and all remaining lineages of the H-lineage, for which we calculated an average TrN + Γ distance of 19.5% (±2.1%). The second event refers to the split of the Haplochromini–Tropheini clade from the remaining members of the H-lineage (15.0 ± 1.5%). The third event concerns the almost simultaneous formation of five major lineages at a divergence level of 12.4–13.0%: the Limnochromini clade, the *Cyphotilapia frontosa* clade, the *Benthochromis* clade, the Perissodini clade, and the Cyprichromini clade. The fourth event refers to the radiation of three major clades within the redefined Limnochromini (TrN + Γ distances, 6.9–7.2%), which happened almost simultaneously with the diversification among the two genera *Paracyprichromis* and *Cyprichromis* within the tribe Cyprichromini (TrN + Γ distance, 6.7% ± 1.5%) and among the two species *Tropheus moorii* and *Petrochromis orthognathus* within the tribe Tropheini (TrN + Γ distance, 6.2% ± 1.0%). By applying the molecular clock to the gamma-corrected amino acid distances corresponding to the primary radiation of the Limnochromini, the diversification of this group could tentatively be dated to 2.95–3.5 MYA.

Discussion

The strategy of our phylogenetic analysis was to combine two mtDNA gene segments that proved to be informative for Lake Tanganyika cichlids, albeit on two different levels. The control region is best suited to address splits among closely related taxa, whereas the *NADH2* gene allows us to address deeper phylogenetic splits. Both gene segments were first analyzed separately, and then combined for a comprehensive analysis, the results of which are discussed below (see strict consensus tree in Fig. 3). Among the 12 tribes of Lake Tanganyika cichlid fish (Poll 1986), six are currently included in the H-lineage (*sensu* Nishida [1991] but excluding the Eretmodini [Salzburger et al. 2002]). These six tribes were derived from one seeding lineage of the intralacustrine radiation, presumably a nonmouthbrooding *Lamprologus*-like species, and diversified during the "primary lacustrine radiation" (Salzburger et al. 2002). Monophyly of tribes within the H-lineage has been shown so far for the Tropheini (Sturmbauer and Meyer 1992; Meyer et al. 1996; Takahashi et al. 1998; Sturmbauer et al. 2003), the Ectodini (Sturmbauer and Meyer 1993; Koblmüller et al. 2004), the Cyprichromini (Brandstätter et al. 2005), and the Perissodini (Takahashi et al. 1998). The tribe Haplochromini was suggested to be of polyphyletic origin with a high number of species (about 1700) widespread in freshwater systems across Africa (Klett and Meyer 2002; Salzburger et al. 2002). Several previous studies (Kocher et al. 1995; Nishida 1997; Lippitsch 1998; Salzburger et al. 2002; Sturmbauer et al. 2003; Takahashi 2003) questioned the monophyly of the Limnochromini sensu Poll (1986). Our phylogenetic hypothesis agrees with these studies in that *Gnathochromis pfefferi* and *Benthochromis* fall outside the Limnochromini. *Gnathochromis pfefferi* clustered within the Tropheini in all our analyses. *Benthochromis tricoti* and *B. melanoides* appeared as a separate lineage (NJ) or as the sister group of the Cyprichromini and the Perissodini (MP and ML), congruent with Salzburger et al. (2002). Our results contrast with those based on morphological features (Takahashi 2003), in which *Benthochromis* was resolved as ancestral to the tribes Bathybatini, Trematocarini, Cyprichromini, and Ectodini. Ecologically, *Benthochromis* displays similarities to the Cyprichromini, as *Benthochromis*, *Paracyprichromis*, and *Cyprichromis* are all maternal mouthbrooders that form large schools at depths below 10 m and mainly feed on zooplankton (Konings 1998). Our analyses unambiguously grouped 9 of the 12 analyzed species

Table 2. Results of the branch-length test (Takezaki et al. 1995) computed in the program LINTRE based on *NADH2* nucleotide sequences

Species	δ	SE	*Z*
Astatoreochromis alluaudi (39)	0.037132	0.016619	2.234316
Astatotilapia burtoni (40)	0.001458	0.012647	0.115314
Baileychromis centropomoides (1)	0.010534	0.010535	0.999903
Baileychromis centropomoides (2)	0.011076	0.010515	1.053307
Benthochromis melanoides (4)	0.000523	0.01112	0.047062
Benthochromis melanoides (5)	0.004613	0.010708	0.430764
Benthochromis tricoti (6)	0.00298	0.011025	0.270253
Benthochromis tricoti (7)	0.006615	0.010559	0.626467
Cunningtonia longiventralis (8)	0.050036	0.02214	2.25998
Cyphotialipa frontosa (42)	0.004644	0.012211	0.380285
Cyprichromis "tricolor jumbo" (48)	0.007845	0.010663	0.735734
Cyprichromis leptosoma "gold" (44)	0.017767	0.009129	1.946227
Cyprichromis leptosoma (43)	0.007914	0.010405	0.760611
Cyprichromis leptosoma (45)	0.009444	0.009825	0.961201
Cyprichromis microlepidotus (46)	0.014961	0.009733	1.537024
Cyprichromis pavo (47)	0.006189	0.010203	0.606581
Cyprichromis zonatus (49)	0.005702	0.012229	0.466246
Enantiopus melanogenys (9)	0.032207	0.020704	1.555611
Gnathochromis pfefferi (10)	0.015496	0.013715	1.129877
Gnathochromis pfefferi (50)	0.026731	0.014779	1.808702
Gnathochromis permaxillaris (11)	0.022886	0.009478	2.414564
Gnathochromis permaxillaris (13)	0.021202	0.009518	2.227599
Gnathochromis permaxillaris (14)	0.022618	0.009494	2.382381
Grammatotria lemairii (51)	0.050056	0.022761	2.199193
Haplotaxodon microlepis (21)	0.001109	0.010719	0.103427
Haplotaxodon sp. (22)	0.004376	0.011465	0.381705
Haplotaxodon trifasciatus (23)	0.004376	0.011465	0.381705
Limnochromis abeelei (25)	0.022539	0.009507	2.370841
Limnochromis abeelei (26)	0.022329	0.009508	2.348416
Limnochromis abeelei (27)	0.022345	0.009497	2.352723
Paracyprichromis brieni (54)	0.022075	0.009948	2.219061
Perissodus microlepis (55)	0.039085	0.01461	2.675153
Petrochromis orthognatus (56)	0.001035	0.011762	0.088031
Plecodus straeleni (57)	0.022123	0.013927	1.588523
Reganochromis calliurus (36)	0.00815	0.011249	0.724491
Triglachromis otostigma (38)	0.004257	0.012997	0.32754
Triglachromis otostigma (58)	0.004161	0.013004	0.319997
Tropheus moorii (59)	0.004248	0.012436	0.341632

Notes. Average root-to-tip distance, 0.83521. Numbers in parentheses refer to the sample list in Table 1. δ, difference of the root-to-tip distance from the average distance.

of the Limnochromini (*Limnochromis auritus, L. abeelei*, and *L. staneri, Greenwoodochromis bellcrossi* and G. *christyi, Triglachromis otostigma, Reganochromis calliurus, Baileychromis centropomoides*, and *Gnathochromis permaxillaris*) as a monophyletic assemblage with high nodal support (BS = 100 in NJ, BS = 79 in MP, QP = 84; posterior probability, 100), suggesting the redefinition of the Limnochromini to comprise only these nine species. All members of this group—as known to date—are biparental mouthbrooders bonded in a monogamous relationship during the breeding period (Konings 1998), that incubate and protect large numbers of fry. Three sublineages are suggested within this group: the first branch comprises *Triglachromis*; the second *L. auritus, G. permaxillaris*, and *Reganochromis calliurus* plus *Baileychromis centropomoides*; and the third, *L. abeelei, L. staneri*, and *Greenwoodochromis bellcrossi* plus *G. christyi*. Thus, the genus *Limnochromis* appears to be paraphyletic. In contrast to our results, Takahashi (2003) suggested excluding *G. christyi* (*G. bellcrossi* was not included in this analysis) from the Limnochromini and erecting a new tribe, on the basis of internal and external morphological features.

Age Estimates of Major Cladogenetic Events

Due to the lack of a reliable fossil record the calibration of a molecular clock for East African cichlids remains difficult and can so far only be approximated by comparison of average genetic distances with datings of geological events concerning the lake history. The only molecular clock available to date is based on the most variable segment of the mitochondrial

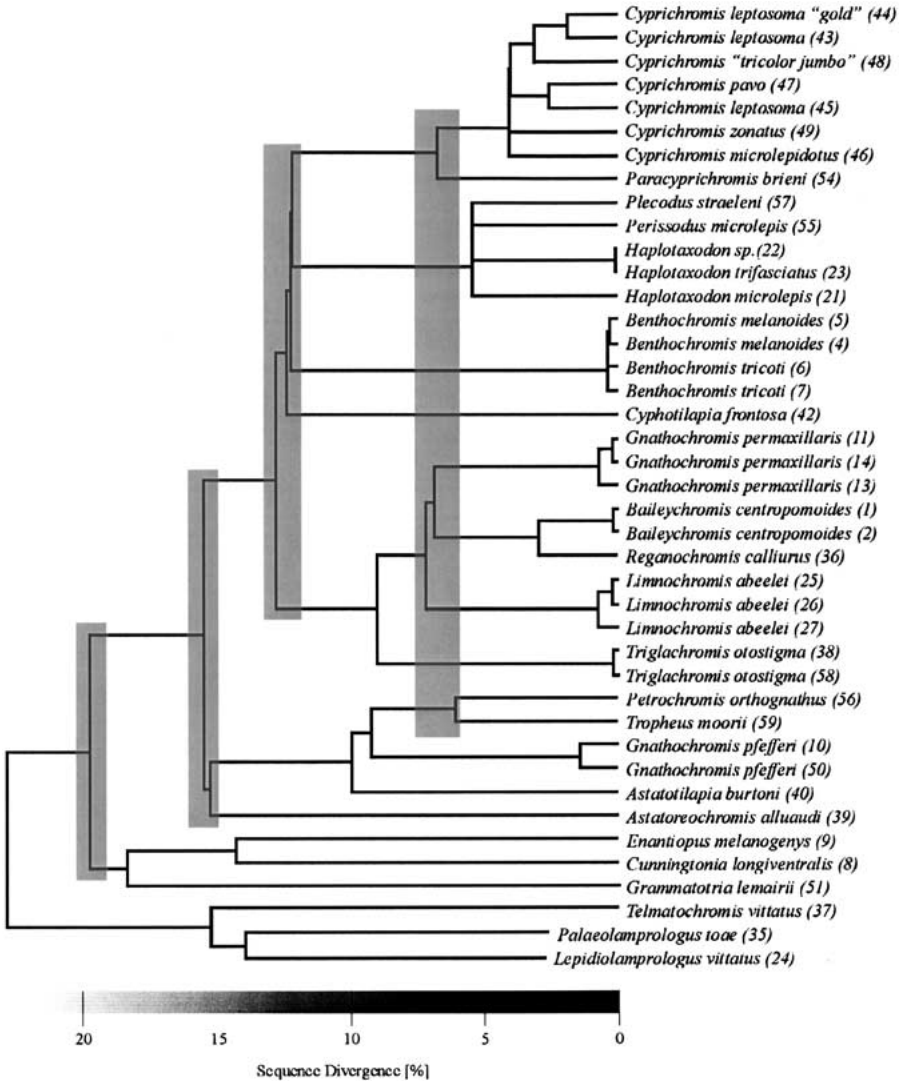

Fig. 5. Linearized tree based on *NADH2* nucleotide sequences (1047 bp), calculated under the assumption of rate constancy with the branch length test implemented in the computer program LINTRE (Takezaki et al. 1995). Following the result of the branch length test all specimens of *Limnochromis auritus*, *L. staneri*, *Greenwoodochromis bellcrossi*, and *G. bellcrossi* were excluded due to inconsistencies in the rates of sequence evolution. The sequence evolution model applied was TrN + Γ (Tamura and Nei 1993). Gray bars emphasize four major diversification events. Sequences that significantly deviated in their branch length from the average root-to-tip distance were excluded from the analysis. The scale below the linearized tree depicts the percentage of sequence divergence calculated by using the appropriate DNA substitution model TrN + Γ.

control region (Sturmbauer et al. 2001). While this clock was employed for the dating of diversification events in young lineages of cichlid fish (Baric et al. 2003; Sturmbauer et al. 2003; Brandstätter et al. 2005), it proved to be unreliable when applied to older lineages due to saturation of transition mutations (Koblmüller et al. 2004). Since our study aimed to elucidate the phylogenetic positioning of the Limnochromini within the H-lineage, an assemblage of relatively distantly related taxa, we established a preliminary clock calibration for the *NADH2* gene (1047 bp). This first approach is based on two clusters of gamma-corrected amino acid distances—marking the onset of the radiation of the H-lineage and the Lamprologini (5–6 MYA)—and one cluster of amino acid distances corresponding to the lacustrine radiation of the Lake Malawi cichlid species flock (0.7 MYA). Despite their reciprocal monophyly (Moran et al. 1994) the divergence of the mbuna and non-mbuna cichlids might have already occurred earlier in the history of Lake Malawi as suggested by the observed gamma-corrected amino acid distances (Fig. 4). Alternatively, the two groups underwent a rapid radiation with an accelerated rate of sequence evolution. By applying the molecular clock the diversification of the Limnochromini could tentatively be dated to 2.9–3.5 MYA. However, as discussed above, absolute time estimates for cladogenetic events in East African cichlids must remain approximative and be treated with caution.

The linearized tree analysis suggests four major diversification events: the first concerning the split of the Ectodini from the remaining members of the H-lineage (TrN + Γ distance, 19.5%); the second, the split of the Haplochromini–Tropheini clade; the third, the radiation within the H-lineage (TrN + Γ distances, 13.4–14.6%); and the fourth, the diversification of three major clades within the redefined Limnochromini (TrN + Γ distances, 6.9–7.2%). The time estimate for the diversification of the Limnochromini thus suggests a very recent diversification of this group. Clearly, this occurred after the "primary

lacustrine radiation'' of the H-lineage and at the same time as the diversification of the Cyprichromini (TrN + Γ distance 7.0% [Brandstätter et al. 2005]) and the Tropheini (TrN + Γ distance, 6.2%), and also several diversification events within the Ectodini (TrN + Γ distances, 7.1–8.8% [Koblmüller et al. 2004]) and the Bathybatini (TrN + Γ distance, 7.1% [Koblmüller et al. 2005]) derived from *NADH2* distances. This series of time coincidence suggests that the same environmental changes affected the diversification of several lineages of cichlids in deepwater (Bathybatini and Limnochromini), pelagic (Cyprichromini), and littoral habitats (Tropheini).

Implications on the Evolution of Benthic Deepwater Cichlids

Clearly, the radiation of the Limnochromini did not reach the complexity and diversity encountered in cichlid fish of the littoral habitat (Coulter 1991). If one considers the mosaic-like structured availability of space for feeding and breeding that is exploited by—often territorial—cichlids, it seems obvious that the littoral habitat provides more opportunity for ecological diversification than the open waters inhabited by the Limnochromini (and also the Bathybatini). Moreover, fish communities in shallow waters were more severely affected by historical lake level fluctuations that led to repeated segregation and amalgamation of populations (Rossiter 1995; Verheyen et al. 1996; Sturmbauer et al. 1997, 2001; Rüber et al. 2001; Baric et al. 2003). According to the age estimate for the radiation within the tribe Limnochromini (excluding *Gnathochromis pfefferi* and the two *Benthochromis* species), this may have been connected to a period of aridification in East Africa between 2.5 and 3 MYA (Cane and Molnar 2001). During that period the lake level is most likely to have decreased by several hundred meters. Given that only the uppermost 250 m of the water layer contain oxygenized water, a decrease of the lake by 200–400 m might in fact have substantially increased the habitat of benthic deepwater cichlids, since shelf areas between the lake basins were oxygenated. Concerning the question whether allopatric or sympatric mechanisms drove the diversification in benthic deepwater cichlids, one must consider the absence of geographical variants in all species known to date. Moreover, deepwater species frequently form shoals outside the breeding period, promoting gene flow among individuals of different populations and inhibiting the divergence of isolated populations and ultimately speciation. In theory, the current diversity within the Limnochromini could have emerged in sympatry via adaptation to different ecological niches in benthic deepwater habitats. Several species of the tribe indeed coexist in this relatively uniform environment by utilizing different food resources. *Triglachromis otostigma* has an intestinal tract reaching 2.5 times its total length of about 12 cm, while the 17-cm-long *Limnochromis auritus* has an intestinal tract measuring only 30% of its total length (Coulter 1991; Konings 1998). Its congenerics *L. staneri* and *L. abeelei* represent the largest members of the Limnochromini, with a total length of 19 and 24 cm, and feed on snails, crustaceans, and fish (Konings 1998). Furthermore, the two closely related species *Reganochromis calliurus* and *Gnathochromis permaxillaris*, with a total length of about 15 cm, avoid competition in food resources in that the former feeds on shrimp, crabs, and small fish, whereas the latter specializes on the uptake of tiny invertebrates close to the bottom (Konings 1998). Unfortunately, there is no information available on the feeding preferences of *Baileychromis centropomoides*, *Greenwoodochromis bellcrossi*, and *Greenwoodochromis christyi*. Nevertheless, the differences of dietary composition known so far mirror the utilization of radically different food sources (Konings 1998). In addition, niche segregation combined with territorialism and homing might have also promoted microallopatric speciation.

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