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Ancient Divergence in Bathypelagic Lake Tanganyika Deepwater Cichlids: Mitochondrial Phylogeny of the Tribe Bathybatini

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Abstract. The cichlid species flock of Lake Tanganyika represents a polyphyletic assemblage of eight ancestral lineages, which colonized the emerging lake independently. Our study is focused on one of these lineages, the Bathybatini, a tribe of specialized piscivorous cichlids of the deep pelagic zone. By analyzing three mtDNA gene segments of all eight species of the tribe and two species of the closely related Trematocarini, we propose on the basis of a linearized tree analysis that the Bathybatini comprise two distinct lineages, the genera *Hemibates* and *Bathybates*, that seeded the primary lacustrine Tanganyika radiation independently. The genus *Hemibates* is likely to represent a distinct lineage that emerged simultaneously with the tribe Trematocarini and the genus *Bathybates* and should be therefore treated as a distinct tribe. Within the genus *Bathybates*, *B. minor* clearly represents the most ancestral split and is likely to have diverged from the remaining species in the course of the "primary lacustrine Tanganyika radiation" during which also the radiations of the Lamprologini and the H-lineage took place. The remaining "large" *Bathybates* species also diversified almost simultaneously and in step with the diversification of other Tanganyikan lineages—the Limnochromini and Cyprichromini—with *B. graueri* occupying the most ancestral branch, suggesting that these were induced by the same environmental changes. The lack of geographic color morphs suggests that competition and resource partitioning, rather than allopatric speciation, promoted speciation within the genus *Bathybates*.

Key words: Adaptive radiation — mtDNA sequences — Control region — Cytochrome *b* — NADH dehydrogenase subunit 2 — Resource partitioning

Introduction

The Great East African lakes are excellent model systems for the study of adaptive radiation. Each of those lakes is inhabited by flocks of cichlid fishes counting hundreds of endemic species, which evolved independently via intralacustrine speciation (Kocher et al. 1993; Meyer 1993). With an age of 9–12 Myr, Lake Tanganyika is by far the oldest of the African lakes, containing the morphologically, ecologically, and behaviorally most complex species flock of cichlid fishes, with about 200 described species in 54 genera and several more awaiting scientific description (Poll 1986; Snoeks 2000; Turner et al. 2001). The Lake Tanganyika basin has a long and complex geological history. The formation of the lake interrupted the flow of the ancient Proto-Malagarazi-Congo River and progressed in step with the forma-

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tion of the East African rift valley (Tiercelin and Mondeguer 1991). The eight seeding lineages for the Lake Tanganyika cichlid fish radiation (Salzburger et al. 2002) may thus have originated from the Proto-Malagarazi-Congo River and other tributaries of the basin catchment. According to the time estimate of Tiercelin and Mondeguer (1991) for the establishment of deepwater conditions, the primary lacustrine radiation, during which species adapted to the newly available ecological niches, can thus be constrained to about 5–6 MYA. The dynamic basin morphology is characterized by repeated lake level fluctuations caused by changes in precipitation and temperature (Scholz and Rosendahl 1988; Tiercelin and Mondeguer 1992; Cohen et al. 1993, 1997; Lezzar et al. 1996; Scholz et al. 2003). The importance of lake level fluctuations as modulators of gene flow, on the one hand, and diversification, on the other hand, has been demonstrated by a series of recent studies (Sturmbauer and Meyer 1992; Rossiter 1995; Verheyen et al. 1996; Sturmbauer et al. 1997, 2001, 2003; Sturmbauer 1998; Rüber et al. 2001; Baric et al. 2003). The phylogeny of Lake Tanganyika cichlids has been addressed by comparative morphological (Stiassny 1991; Takahashi 2003a,b), lepidological (Lippitsch 1998), and allozyme (Nishida 1991, 1997) analysis and on the basis of mtDNA sequences (Sturmbauer and Meyer 1993; Sturmbauer et al. 1994; Salzburger et al. 2002; Koblmüller et al. 2004) and SINEs (Takahashi et al. 1998, 2001; Terai et al. 2003). These efforts to clarify the phylogenetic relationships of the Lake Tanganyika cichlid species flock have resulted in a detailed phylogenetic hypothesis for the interrelationships of all 12 cichlid tribes in the lake (Salzburger et al. 2002). However, the phylogenetic relationships within and among two of the ancestral seeding lineages, the Bathybatini and Trematocarini, still remain unclear.

The tribe Bathybatini comprises piscivorous deepwater cichlids endemic to Lake Tanganyika. It contains two genera, the monotypic species *Hemibates stenosoma* and seven species of the genus *Bathybates* (Coulter 1991). Very little is known about the biology of the Bathybatini. Except for *Bathybates minor*, the Bathybatini are rather large cichlids, of a maximum size between almost 30 and more than 40 cm. All species are maternal mouthbrooders (Kuwamura 1997) and produce some of the largest cichlid eggs known (7 mm [Konings 1998]). Strong sexual dichromatism is found within the Bathybatini: While females have silvery body coloration, males show a species-specific pattern of dark blotches and stripes. Despite the lakewide distribution of all bathybatine species, distinct color morphs are known only in *Hemibates stenosoma*. For this species two color variants of males have been described which occur in sympatry: Some display distinct dark bars on the anterior part of the flank, while others have irregular black blotches (Konings 1998). Whether these two phenotypes represent morphs of a single polychromatic species or distinct sister species is not known to date.

At present, the phylogenetic relationships of the Bathybatini to other African cichlid lineages are uncertain. Poll (1986) recognized the Bathybatini and the Trematocarini as two distinct tribes. Two more recent works suggest that the genus *Hemibates* is the sister group to both the Trematocarini and the genus *Bathybates* (Stiassny 1981; Takahashi 2003b). Based on allozyme data, Nishida (1997) confirmed Poll's (1986) classification and described a clade consisting of the tribe Trematocarini and the tribe Bathybatini, consisting of the sister genera *Bathybates* and *Hemibates*. In summary, while each of the studies cited above demonstrated the monophyly of the Trematocarini, there is considerable disagreement about the monophyly of the two genera presently included in the tribe Bathybatini. The present study is the first to use mitochondrial DNA sequence data to address the phylogenetic relationships among all species of the tribe Bathybatini and their relationships to the remaining tribes of the Tanganyikan cichlid species flock. In addition, the evolutionary characteristics and phylogenetic performance of three mitochondrial gene segments were evaluated.

Materials and Methods

DNA Extraction, Amplification, and Sequencing

This study is based on a total of 56 individuals, including all 8 described species of the tribe Bathybatini (23 individuals), 2 species of the presumably closely related tribe Trematocarini, and several potential outgroup taxa from elsewhere in Africa published earlier (Table 1, Fig. 1). All taxa of the Bathybatini and Trematocarini were caught in the years 1992 to 2003 during several expeditions to Lake Tanganyika. Voucher specimens are available from the authors.

We analyzed 1047 bp of the entire NADH dehydrogenase subunit 2 gene (ND2) of 51 specimens (including outgroup taxa), a 362-bp segment of the most variable part of the control region (D-loop) of 29 specimens (including outgroup taxa), and 402 bp of the cytochrome *b* gene (cyt *b*) of 27 specimens (including outgroup taxa). When available, previously published sequences were used (Sturmbauer and Meyer 1993; Sturmbauer et al. 1994; Kocher et al. 1995; Kumazawa et al. 1999; Klett and Meyer 2002; Koblmüller et al. 2004) (for accession numbers see Table 1).

Total DNA extraction from ethanol preserved fin-clips or white muscle tissue, polymerase chain reaction (PCR), and chain termination sequencing followed standard protocols (Walsh et al. 1991; Salzburger et al. 2002; Koblmüller et al. 2004). As primers for both amplification and sequencing of the control region we used L-Pro-F (Koblmüller et al. 2004) and TDK-D (Kocher et al. 1989). For cytochrome *b* we used the primers L14724 and H15149 (Kocher et al. 1989), and for ND2 we used the primers MET, ND2.2A, and TRP (Kocher et al. 1995) as well as the newly designed primer ND2.T-R, 5′GGGGCTTTTGTTCAGGATGT3′. Both strands were sequenced and visualized on an ABI 3100 Sequencer (Applied

Table 1. List of samples examined in our analysis, with locality (if known), sequences used, and GenBank accession numbers

No.	Species	Sampling locality	GenBank accession No.		
			Control region	Cytochrome *b*	ND2
1	*Heterochromis multidens*	Unknown	—	—	AF398214[d1]
2	*Tylochromis polylepis*[t]	Unknown	—	—	AF398215[d1]
3	*Tylochromis leonensis*	Unknown	—	—	AF317274[c1]
4	*Hemichromis elongatus*	Upper Zambezi	—	—	AY663714
5	*Pelvicachromis pulcher*	Unknown	—	—	AF317271[c1]
6	*Chromidotilapia guentheri*	Unknown	—	—	AF317270[c1]
7	*Thysochromis ansorgii*	Lab breed	—	—	AY663713
8	*Tilapia cessiana*	Cavally River	—	—	AF317253[c1]
9	*Tilapia rendalli*	Kafue River	—	—	AF317259[c1]
10	*Tilapia discolor*	Pra River	—	—	AF317255[c1]
11	*Tilapia busumana*	Bia River	—	—	AF317250[c1]
12	*Tilapia mariae*	Lab breed	—	—	AF317258[c1]
13	*Tilapia cabrae*	Kouilou River	—	—	AF317252[c1]
14	*Tilapia sparrmanii*	Kafue River	—	—	AF317260[c1]
15	*Sarotherodon caudomarginatus*	Sierra Leone	—	—	AF317243[c1]
16	*Sarotherodon galilaeus*	Lab breed	—	—	AF317244[c1]
17	*Sarotherodon occidentalis*	Guinea	—	—	AF317246[c1]
18	*Oreochromis tanganicae*[t]	Unknown	—	—	AF317240[c1]
19	*Oreochromis niloticus vulcani*	Lab breed	—	—	AF317242[c1]
20	*Boulengerochromis microlepis*[t]	Unknown	—	—	AF317229[c1]
21	*Boulengerochromis microlepis*[t]	Unknown	—	—	U07240[b1]
22	*Steatocranus casuarius*	Lab breed	—	—	AF317247[c1]
23	*Steatocranus tinanti*	Lab breed	—	—	AF317248[c1]
24	*Eretmodus cyanostictus*[t]	Unknown	AF400707[d2]	AF428155[d2]	AF398220[d12]
25	*Neolamprologus brichardi*[t]	Unknown	AF400721[d2]	—	AF398227[d12]
26	*Neolamprologus brichardi*[t]	Burundi	—	Z29997[a2]	—
27	*Xenotilapia flavipinnis*[t]	Nkumbula	AY339034[c2]	AY337849[c2]	AY337794[c12]
28	*Cyprichromis leptosoma*[t]	Kitumba	AY339053[c2]	AY337838[c2]	AY337786[c12]
29	*Tropheus moorii*[t]	Unknown	—	—	U07267[b1]
30	*Trematocara unimaculatum*[t]	Zambia	—	AF428168[d2]	AF317268[c12]
31	*Trematocara unimaculatum*[t]	Mpulungu	AY663759	—	—
32	*Telotrematocara macrostoma*[t]	Mpulungu	AY663760	AY663738	AY663715
33	*Hemibates stenosoma*[t]	Sumbu	AY663761	AY663739	AY663716
34	*Hemibates stenosoma*[t]	Mpulungu	AY663762	AY663740	AY663717
35	*Hemibates stenosoma*[t]	Mpulungu	AY663763	AY663741	AY663718
36	*Hemibates stenosoma*[t]	Mpulungu	AY663764	AY663742	AY663719
37	*Bathybates minor*[t]	Ulwile Island	AY663765	AY663743	AY663720
38	*Bathybates minor*[t]	Ulwile Island	AY663766	AY663744	AY663721
39	*Bathybates minor*[t]	Ulwile Island	AY663767	AY663745	AY663722
40	*Bathybates graueri*[t]	Sumbu	AY663768	AY663746	AY663723
41	*Bathybates graueri*[t]	Ulwile Island	AY663769	AY663747	AY663724
42	*Bathybates graueri*[t]	Ulwile Island	AY663770	—	AY663725
43	*Bathybates graueri*[t]	Mpulungu	AY663771	AY663748	AY663726
44	*Bathybates vittatus*[t]	Mpulungu	AY663772	AY663749	AY663727
45	*Bathybates vittatus*[t]	Mpulungu	AY663773	AY663750	AY663728
46	*Bathybates leo*[t]	Mpulungu	AY663774	AY663751	AY663729
47	*Bathybates leo*[t]	Mpulungu	AY663775	AY663752	AY663730
48	*Bathybates leo*[t]	Mpulungu	AY663776	AY663753	AY663731
49	*Bathybates leo*[t]	Mpulungu	AY663777	—	—
50	*Bathybates fasciatus*[t]	Lufubu estuary	AY663778	AY663754	AY663732
51	*Bathybates fasciatus*[t]	Lufubu estuary	AY663779	AY663755	AY663733
52	*Bathybates fasciatus*[t]	Mpulungu	AY663780	AY663756	AY663734
53	*Bathybates horni*[t]	Mpulungu	AY663781	AY663757	AY663735
54	*Bathybates ferox*[t]	Ulwile Island	—	AF428152[d2]	—
55	*Bathybates ferox*[t]	Ulwile Island	AY663782	—	AY663736
56	*Bathybates ferox*[t]	Mpulungu	AY663783	AY663758	AY663737

Note. Representatives of all eight species of the cichlid tribe Bathybatini were analyzed.
Sequences from other sources were obtained from: [a] Sturmbauer et al. (1994), [b] Kocher et al. (1995), [c] Klett and Meyer (2002), [d] Salzburger et al. (2002), and [e] Koblmüller et al. (2004). [t] Species occurs in Lake Tanganyika. [1] Sequence was used for step 1 of our analysis. [2] Sequence was used for step 2 of our analysis. Coordinates of localities where Bathybatini samples were collected: Lufubu estuary, S08°32′, E30°44′; Mpulungu, S08°46′, E31°06′; Sumbu, S08°31′, E30°29′; Ulwile Island, S07°27′, E30°34′.

Fig. 1. Map of Lake Tanganyika, East Africa, showing sample sites of the Bathybatini and the location of its three deep basins at a depth of 600 m.

Biosystems). All new sequences are available from GenBank under the accession numbers given in Table 1.

Phylogenetic Analyses

Alignment of the DNA sequences was performed using Clustal X (Thompson et al. 1997) and improved by eye for the control region. For all data sets a likelihood mapping analysis (Strimmer and von Haeseler 1997) using the program TREE-PUZZLE 5.1 (Schmidt et al. 2001) was performed to visualize the strength of the overall phylogenetic signal. For phylogenetic reconstruction we applied maximum parsimony (MP), neighbor joining (NJ), and maximum likelihood (ML) in parallel using the PAUP* program package (version 4.0b5; Swofford 2002). To evaluate an appropriate substitution model for NJ and ML analysis, we calculated hierarchical likelihood ratio test statistics (Huelsenbeck and Crandall 1997) using the program Modeltest 3.06 (Posada and Crandall 1998). For obtaining MP and ML topologies we applied heuristic search procedures with random addition of taxa and TBR branch swapping (1000 replicates for MP, 100 replicates for ML). As standard measures of confidence we applied bootstrapping (1000 pseudoreplicates for NJ and MP) and quartet puzzling (Strimmer and von Haeseler 1996; 10,000 random quartets for ML). Phylogenetic relationships were also reconstructed by Bayesian inference using MrBayes 3.0b4 (Huelsenbeck and Ronquist 2001). Posterior probabilities were obtained from a 2-million-generation Metropolis-coupled Markov chain Monte Carlo simulation (10 chains; chain temperature, 0.2; trees sampled every 100 generations), with parameters estimated from the data set. A 50% majority-rule consensus tree was constructed after a 1-million-generation burn-in to allow likelihood values to reach stationarity.

In the first step of analysis we aimed to position the tribe Bathybatini within the phylogenetic framework of the African cichlids and to determine the most appropriate outgroup for a detailed analysis of the Bathybatini. To this end we used 34 ND2 sequences, representing 3 species of the Bathybatini, 2 species of the tribe Trematocarini, 1 species of the tribes Eretmodini, Lamprologini, Cyprichromini, Ectodini, and Tropheini, 15 species of the tribe Tilapiini, 2 species of the tribe Tylochromini, and the West African cichlids *Hemichromis elongatus, Thysochromis ansorgii, Pelvicachromis pulcher*, and *Chromidotilapia guentheri*. As outgroup we used the West African cichlid species *Heterochromis multidens*. To evaluate the degree of saturation of transition and transversion mutations for each codon postition of the ND2 gene, we plotted the absolute number of the two types of mutations against the percentage of sequence divergence of 528 pairwise distances (uncorrected p-distances; data not shown), excluding the outgroup taxon *Heterochromis multidens*. A weighted MP analysis was performed for this ND2 data set, in which transitions at the third codon position were weighted 1:8 with respect to transversions, based on the estimated transition/transversion ratio (TI/TV ratio) of 7.8554. Transitions in third codon positions of fourfold degenerate amino acid sites and synonymous transitions at first positions of leucine codons were excluded because of the demonstrated effect of saturation. The hierarchical likelihood ratio test (Huelsenbeck and Crandall 1997) justified the use of the GTR model of molecular evolution (Yang 1994), including invariable sites (I) and a gamma correction (Γ) for NJ and ML analysis. We applied the proportion of invariable sites of 0.3733, the gamma shape parameter 0.8925, and the following base frequencies: A, 0.3073; C, 0.3665; G, 0.1001; and T, 0.2259. To depict a clock-constrained tree showing major diversification events among the major lineages of African cichlids, we applied the linearized tree method, as described by Takezaki et al. (1995), implemented in the program LINTRE (Takezaki et al. 1995). We excluded all taxa with a significantly deviating rate of base substitution after carrying out the branch length test, implemented in the program LINTRE. To find the most appropriate outgroup we performed weighted MP analyses using selected taxa of distinct monophyletic groups, based on the previous analysis, and compared the resulting topologies. Outgroup set 1 consisted of *Tilapia cessiana* and *T. rendalli*, outgroup 2 contained *Boulengerochromis microlepis*, outgroup 3 comprised *Sarotherodon caudomarginatus, S. galilaeus*, and *Oreochromis tanganicae*, and outgroup 4 included *Eretmodus cyanostictus, Neolamprologus brichardi, Xenotilapia flavipinnis*, and *Cyprichromis leptosoma*. Since the topologies did not change with respect to the chosen outgroup taxa (trees not shown) we decided to use *Eretmodus cyanostictus, Neolamprologus brichardi, Xenotilapia flavipinnis*, and *Cyprichromis leptosoma*, four representatives of another monophyletic assemblage of the Tanganyika radiation, as outgroup taxa for all further analyses.

In the second step of analysis we focused on the phylogeny within the tribe Bathybatini and its relationship to the closely related tribe Trematocarini. To this end we analyzed each of the three mitochondrial genes separately, as well as a data set combining all three genes. Based on the results of the first step we chose *Eretmodus cyanostictus, Neolamprologus brichardi, Cyprichromis leptosoma*, and *Xenotilapia flavipinnis* as outgroup taxa. The most appropriate substitution model for each data set, estimated using the program Modeltest 3.06 (Posada and Crandall 1998), was TrN+I+Γ (Tamura and Nei 1993) for the ND2 data set, K81uf+Γ (Kimura 1981) for the cyt *b* data set, HKY+Γ (Hasegawa et al. 1985) for the control region, and HKY+I+Γ for the combined data set. The estimated likelihood parameters were as follows: For ND2 we obtained the following base frequencies: A, 0.2629; C, 0.3533; G, 0.1153; and T, 0.2685. The proportion of invariable sites (I) was 0.4838, and the gamma shape parameter α was estimated as 1.4782. For cyt *b* we estimated A, 0.2573; C, 0.3307; G, 0.1427; T, 0.2693; and α, 0.1812, for the control region A, 0.3752; C, 0.2040; G, 0.1222; T, 0.2986; and α, 0.3164. The base frequencies for the combined data set were A, 0.2904; C, 0.3022; G, 0.1326; and T, 0.2748. The proportion of invariable sites amounted to 0.4613 and α was estimated as 0.9871. For MP analysis transitions and transversions at third codon positions were weighted 1:6

for ND2 and 1:12 for cyt *b*, based on the estimated TI/TV ratio of 5.9882 for ND2 and 12.0462 for cyt *b*. Transversions at third codon positions of fourfold degenerate amino acids were down-weighted two times more than transversions at third codon positions of twofold degenerate amino acids according to the estimated TI/TV ratio of 3.0382 for ND2 and 5.8187 for cyt *b*. C/T substitutions at the first codon position of leucine were treated as a fifth base and down-weighted to the same weight as transitions at the third codon positions. To evaluate if the two protein coding genes ND2 and cyt *b* are affected by different evolutionary constraints, we estimated the numbers of synonymous (K_s) and nonsynonymous (K_a) substitutions per site for all members of the tribe Bathybatini using the computer program DnaSP version 4.0 (Rozas et al. 2003). This comparative analysis was based on a 24-taxon set for ND2 and a 23 taxon set for cyt *b*, including all available bathybatine sequences plus two representatives of the tribe Trematocarini (*Trematocara unimaculatum, Telotrematocara macrostoma*).

To find the most appropriate weighting for the control region and to investigate the effect of different weightings on the tree topology, we applied four different weighting schemes and compared the resulting MP topologies and consistency indices (Kluge and Farris 1969). First, we did an unweighted MP analysis followed by two analyses in which various weightings were assigned according to regions showing different genetic variation (low-variable, 0–10%; high-variable, 10–20%; hypervariable, >20%). We performed a sliding window analysis (Sturmbauer and Meyer 1992) to define these regions. According to the estimated TI/TV ratios of 4.3952 for the low-variable regions and 1.0521 for the high-variable regions, transitions were weighted 1:4 relative to transversions in low-variable regions and equally weighted as transversions in high-variable regions. In one case transitions in hypervariable regions were weighted 1:4 with respect to transversions according to the estimated TI/TV of 3.8622 and excluded due to their degree of saturation in the second case. The fourth MP analysis of the control region was based on transversions only. The combined data set, including all three genes, was weighted according to the appropriate weighting scheme for each gene. A four-cluster likelihood mapping analysis (Strimmer and von Haeseler 1997) using the program TREE-PUZZLE 5.1 (Schmidt et al. 2001) was applied on the combined data set to evaluate the support of distinct internal branches that are critical for the interpretation of the evolutionary pathways.

In the third step we applied the linearized tree method as described in Takezaki et al. (1995) on the ND2 data set to derive a relative dating of major diversification events. As outgroup taxa we used *Eretmodus cyanostictus, Neolamprologus brichardi, Cyprichromis leptosoma*, and *Xenotilapia flavipinnis*. Constancy of the rate of base substitution among all taxa was tested using the branch length test implemented in the program LINTRE (Takezaki et al. 1995) based on TrN+Γ ($\alpha = 0.2868$) distances. Those taxa that showed significantly different substitution rates at the 1% level were excluded from further analyses. The remaining taxa were used to calculate a clock-constrained tree based on TrN+Γ distances using the program LINTRE (Takezaki et al. 1995). For a relative dating of the major diversification events during the evolution of the Bathybatini, the mean and standard deviation of average pairwise TrN+Γ distances were calculated among the Trematocarini and the Bathybatini and among the major lineages of this tribe.

Results

Positioning of the Bathybatini Within a Representative Subset of African Cichlids

Likelihood mapping yielded 73.7% fully resolved quartets (Fig. 2a), indicating a partially starlike phylogeny. Pairwise distances (uncorrected p-distances) varied from 1.7 to 24.6%. The internal branches interrelating major African cichlid lineages were short, and consequently, the tree topologies were rather inconsistent with respect to the tree building algorithm used (trees not shown), suggesting that several African cichlid lineages originated in a major cladogenic event, as also evident from the likelihood mapping analysis. A strict consensus tree of the MP trees (two most parsimonious trees; tree length, 8927 steps; consistency index [CI] excluding uninformative characters, 0.39; retention index [RI], 0.47; rescaled consistency index [RC], 0.23), NJ, ML, and Bayesian tree is shown in Fig. 3a. In all algorithms the genus *Tylochromis* branched as most ancestral split after the outgroup *Heterochromis multidens*, followed by *Hemichromis elongatus* and a clade containing *Pelvicachromis pulcher, Thysochromis ansorgii*, and *Chromidotilapia guentheri*. Also, the following lineages could be identified by all algorithms, albeit with somewhat differing branching order: *Tilapia discolor* plus *Tilapia busumana*, *T. cessiana* plus *T. rendalli*, *Steatocranus tinanti* plus *S. casuarius, Sarotherodon occidentalis* plus *S. galilaeus* and *Oreochromis* (represented by *O. niloticus vulcani* and *O. tanganicae*), *Eretmodus cyanostictus* plus *Neolamprologus brichardi* and the H-lineage clade (*Xenotilapia flavipinnis, Cyprichromis leptosoma, Tropheus moorii*), and the Trematocarini–Bathybatini clade. For *Tilapia cabrae, T. sparrmanii, T. mariae, Sarotherodon caudomarginatus*, and *Boulengerochromis microlepis*, no unequivocal sister group relationship could be identified. The strict consensus tree of MP, ML, NJ, and Bayesian inference (Fig. 3a) and the linearized tree (Fig. 3b) reflect the observed conflicts in the ancestral branches.

Phylogenetic Relationships Within the Bathybatini and Among the Bathybatini and Trematocarini

In the second step of analysis we focused on the tribe Bathybatini itself and its phylogenetic relationships to the closely related tribe Trematocarini. Likelihood mapping analysis (Fig. 2) demonstrated strong phylogenetic signal for the ND2 data set (97.2% fully resolved quartets) and the control region (93.8% fully resolved quartets). Only the cyt *b* data set showed a considerably lower phylogenetic content (74.9% fully resolved quartets). The data set combining ND2, cyt *b*, and the control region showed the highest phylogenetic content (98.2% fully resolved quartets). Pairwise sequence divergence (uncorrected p-distance) within and among the Bathybatini and Trematocarini ranged from 0.0 to 16.5% in ND2, from 0.0 to 13.2% in cyt *b*, and from 0.0 to 16.0% in the control region. In the combined data set pairwise differences varied from 0.3 to 14.8%.

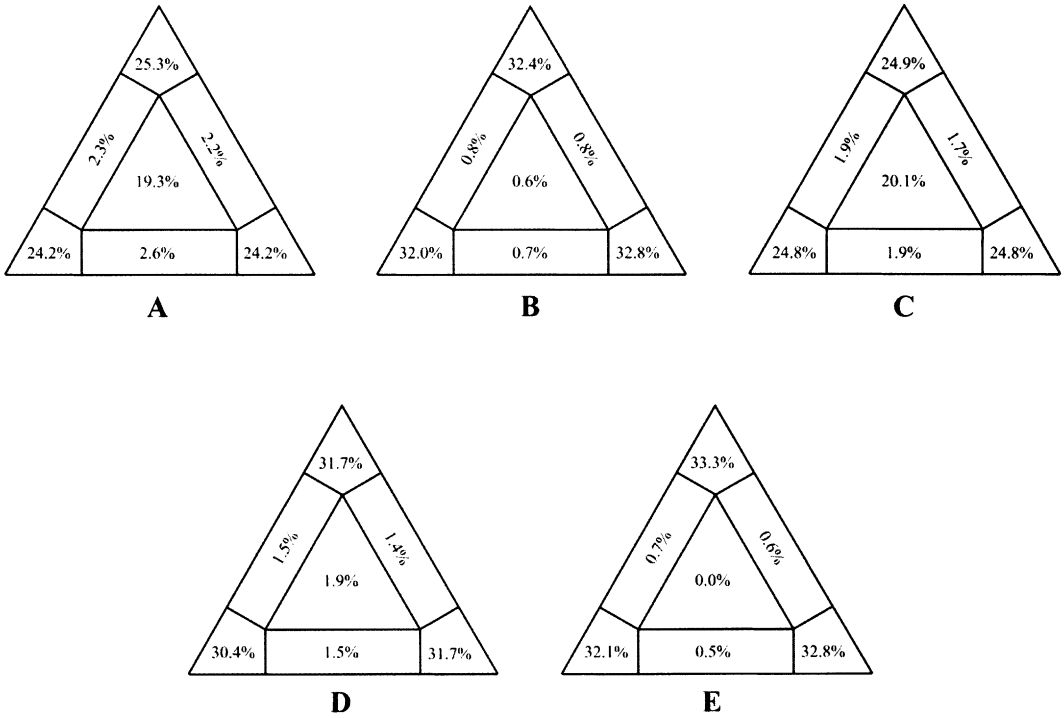


Fig. 2. Results of the likelihood mapping analysis (Strimmer and von Haeseler 1997) of (**A**) the ND2 data set including the major African cichlid lineages, (**B**) the ND2 of the Bathybatini and Trematocarini, (**C**) the cyt *b* of the Bathybatini and Trematocarini, (**D**) the control region of the Bathybatini and Trematocarini, and (**E**) the combined data set of all three genes of the Bathybatini and Trematocarini, presented as barycentric triangles. Values at the corners indicate the percentage of fully resolved quartet topologies, numbers in the rectangular sections give the percentage of partially resolved topologies, and the value in the center of the triangle represents the percentage of unresolved topologies.

Our most inclusive analysis, based on the combined data set (ND2, cyt *b*, control region), yielded highly consistent results. Only slight differences were observed with regard to the tree building algorithm (the ML tree is shown in Fig. 4). In MP transitions were excluded in the D-loop part, justified by the higher consistency index compared to other weighting schemes, by which a singlemost parsimonious tree was found (tree length, 7173 steps; CI excluding uninformative characters, 0.58; RI, 0.77; RC, 0.50; tree not shown). Four major lineages were identified: The two representatives of the tribe Trematocarini were consistently placed as the most ancestral split, followed by *Hemibates stenosoma, Bathybates minor*, and the remaining "large" *Bathybates* species. This partitioning into four distinct lineages was also supported by high bootstrap, quartet puzzling, and posterior probability values. Within the clade of the "large" *Bathybates* species, *B. graueri* clearly resulted as the most ancestral branch. Whereas *B. ferox* was placed as sister group to *B. horni* and *B. fasciatus* in MP, it was sister group to *B. leo* and *B. vittatus* in NJ, ML, and the tree obtained by Bayesian inference.

We further evaluated the branching order among the four major lineages (Trematocarini, *Hemibates stenosoma, Bathybates minor*, "large" *Bathybates* species) and the branching pattern within the "large" *Bathybates* species based on the combined data set (ND2, cyt *b*, control region) by means of the four cluster likelihood mapping method (Strimmer and von Haeseler 1997). With 52.5% fully resolved quartets versus 15.9 and 24.8%, four-cluster likelihood mapping favored the topology with the Trematocarini resolved as sister group to the tribe Bathybatini, with *Hemibates stenosoma* as most ancestral split followed by the genus *Bathybates* (Fig. 5a). Concerning the internal grouping among the "large" *Bathybates* species, the topology with *B. graueri* as most ancestral split, followed by the species pair *B. horni–B. fasciatus, B. ferox*, and the species pair *B. vittatus–B. leo*, gained the strongest support (64.0 versus 23.6 and 0.0%; see Fig. 5b).

Evolutionary Characteristics and Phylogenetic Performance of the Three mtDNA Gene Segments

ND2. Concerning the separate analysis of the three gene segments, the ND2 data set yielded the most stable topology with respect to the tree building algorithm. The results of MP (five most parsimonious trees; tree length, 2131 steps; CI excluding uninformative characters, 0.68; RI, 0.79; RC, 0.53), ML, NJ, and Bayesian analysis were widely congruent (trees not shown). In contrast to the consensus topology of the combined data set, the branch comprising *Hemibates stenosoma* was consistently placed ancestral to

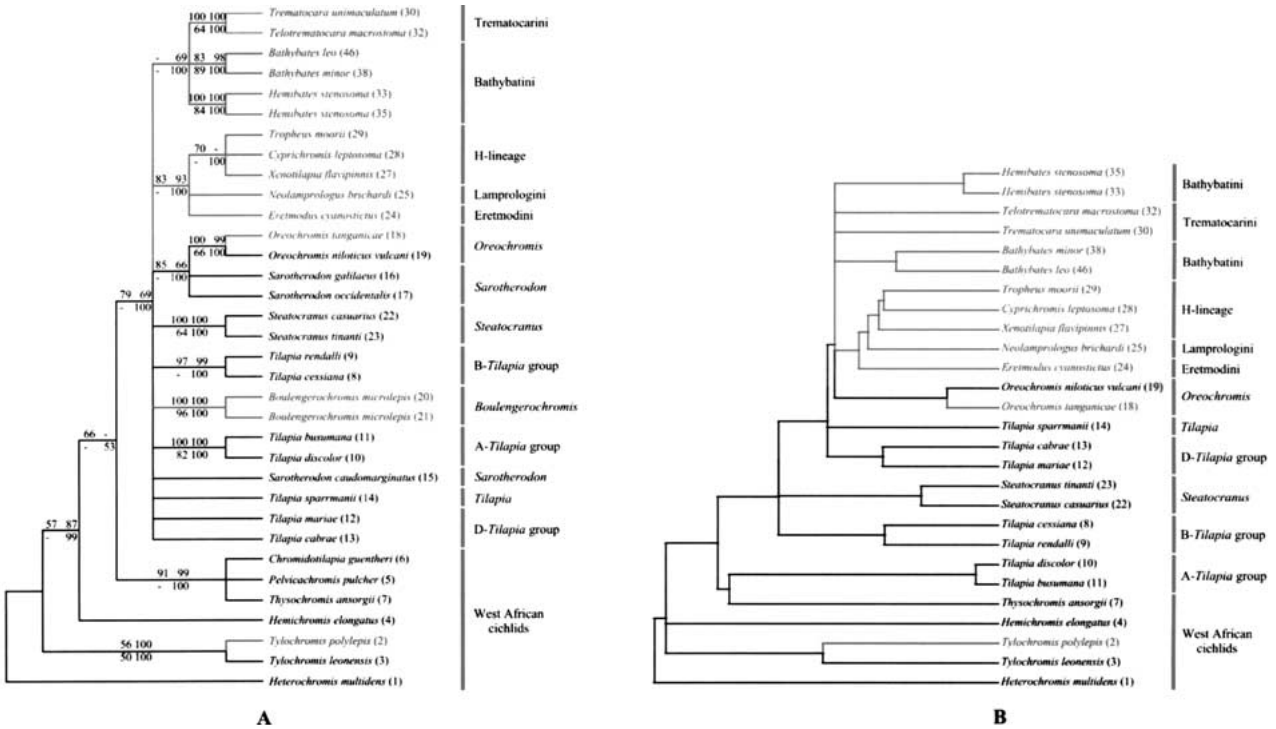


Fig. 3. **A** Strict consensus tree of two most parsimonious trees, the NJ, ML, and Bayesian tree of 54 taxa, representing the major African cichlid lineages, based on analysis of the ND2 gene (1047 bp). Bootstrap values obtained from MP and NJ are depicted above the branches. Quartet puzzling values and posterior probability values are shown below the branches. Only bootstrap, quartet puzzling, and posterior probability values higher than 50 are shown. **B** Linearized tree based on a 1047-bp segment of ND2, compiled with the computer program LINTRE (Takezaki et al. 1995), after performing a branch length test (Takezaki et al. 1995) to test for differences in base substitution rates, using the substitution model TrN + Γ (Tamura and Nei 1993). *Boulengerochromis microlepis*, *Sarotherodon galilaeus*, *S. occidentalis*, *S. caudomarginatus*, *Chromidotilapia guentheri*, and *Pelvicachromis pulcher* were excluded due to a significantly different rate of base substitution. Designation of the major lineages was modified after Klett and Meyer (2002). Taxa shaded in gray belong to the polyphyletic Tanganyika cichlid species flock. Numbers following the species name correspond to the sample list (Table 1).

the Trematocarini and the genus *Bathybates*, although bootstrap and quartet puzzling supports were relatively low. Within the genus *Bathybates*, the topology was similar to that obtained for the combined data set, with slight differences in the branching order with respect to the tree building algorithm. *B. minor* unambiguously formed the most ancestral split, followed by the "large" *Bathybates* species. Within this clade *B. graueri* was consistently placed as the most ancestral branch. In MP, ML, and the topology obtained by Bayesian inference, the sister species *B. vittatus* and *B. leo* were resolved as sister group to the remaining three species of *Bathybates*, with *B. ferox* ancestral to *B. horni* and *B. fasciatus*, whereas in NJ *B. horni* and *B. fasciatus* were sister group to a clade with *B. ferox* as the ancestral branch followed by *B. vittatus* and *B. leo*.

Cytochrome b. As already indicated by the likelihood mapping analysis for cytochrome *b* (see above), which did not reveal strong phylogenetic signal, the results of the analyses of the cyt *b* data set were not as clear as that of ND2, leading to substantial inconsistencies concerning the branching order among the three consistent clades (Trematocarini, *Hemibates*, and *Bathybates*; trees not shown). Only in NJ were the Bathybatini grouped as monophyletic assemblage, sister to the Trematocarini. In ML and the tree based on Bayesian inference, *Hemibates* branched first, followed by the Trematocarini and the genus *Bathybates*. In MP (84 most parsimonious trees; tree length, 603 steps; CI excluding uninformative characters, 0.61; RI, 0.79; RC, 0.53) *Hemibates* branched first, followed by a clade containing *B. minor* and the Trematocarini, sister to the remaining "large" *Bathybates*.

Different Evolutionary Constraints Affecting ND2 and cyt b. As evident from Fig. 6, the relationship of K_a versus K_s varies among the two protein coding genes, ND2 and cyt *b*. In ND2 the rate of K_a is clearly positively correlated with the rate of K_s (linear regression, $y = 0.0865x + 0.0012$; coefficient of determination R^2, 0.7310), whereas there is no positive correlation between K_a and K_s in cyt *b* ($y = 0.0081x + 0.0037$; R^2, 0.1107). The main difference between the two genes concerning the pathway of molecular evolution is the number of

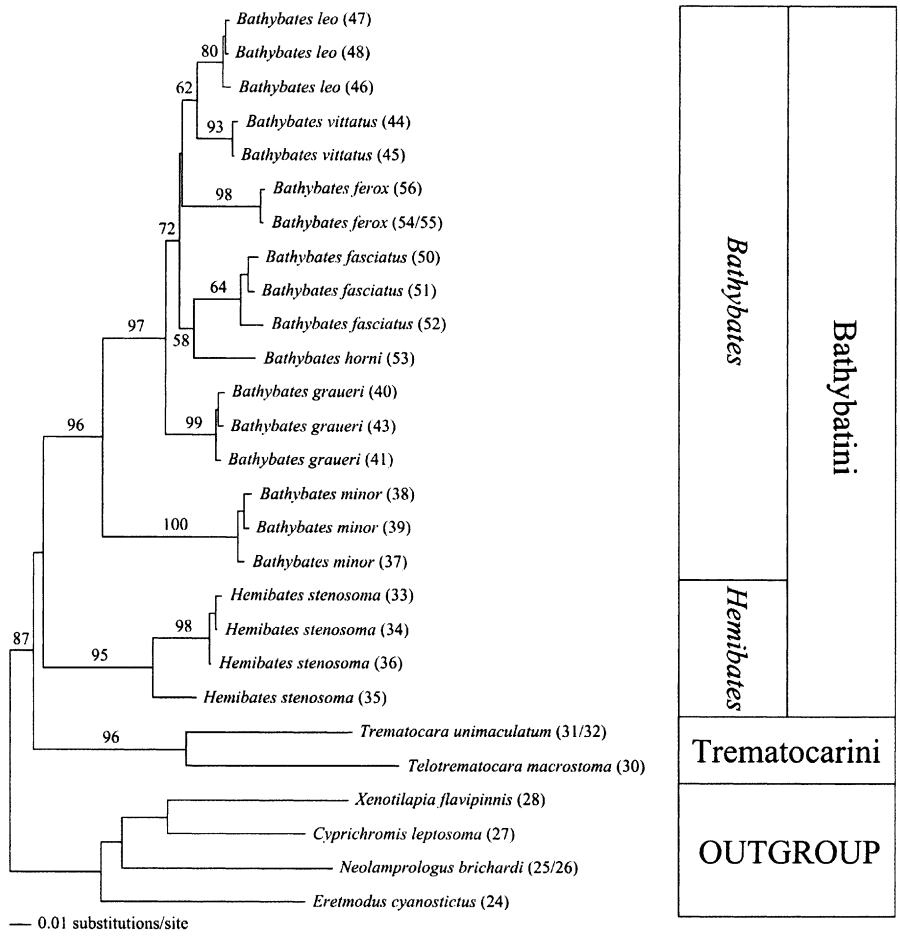


Fig. 4. ML tree obtained from analysis of a combined data set including 1047 bp of the ND2, 402 bp of the cyt *b*, and 362 bp of the most variable part of the mitochondrial control region, comprising 21 taxa (8 species) of the Tanganyikan cichlid tribe Bathybatini, 2 taxa (2 species) of the tribe Trematocarini, and 4 outgroup taxa, using the substitution model HKY + I + Γ (Hasegawa et al. 1985). Quartet puzzling values are shown above the branches. Numbers following the species name correspond to the sample list (Table 1).

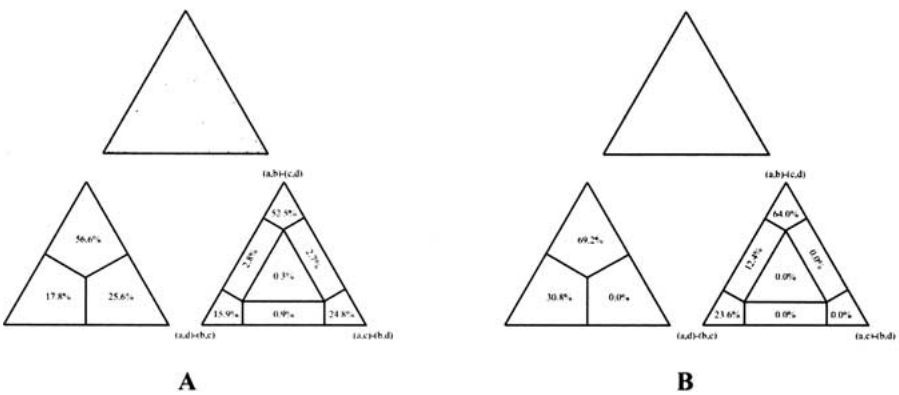


Fig. 5. Results of the four-cluster likelihood mapping (Strimmer and von Haeseler 1997) to evaluate the support of the three alternative branching orders of the four predefined groups. **A** a, outroup; b, Trematocarini; c, *Hemibates*; d, *Bathybates*. **B** a, *Bathybates graueri*; b, *B. fasciatus* and *B. horni*; c, *B. ferox*; d, *B. leo* and *B. vittatus*.

nonsynonymous substitutions, which is more than three times higher in the ND2 gene, while the number of synonymous substitutions is similar.

Control Region. The weighting of the control region was based on a sliding window analysis (Fig. 7a). It demonstrated a high degree of genetic variation within the Bathybatini and Trematocarini—in some regions up to almost 50%—suggesting onset of saturation of transition mutations in regions of high variation. To test for the effect of the weighting regime, we conducted four separate MP analyses as depicted in Figs. 7b–d. The branching order differed clearly with respect to the weighting used. Unweighted MP analysis yielded 11 most parsimonious trees of a length of 377 mutation steps (CI excluding uninformative characters, 0.56; RI, 0.72; RC, 0.44). The branching order conflicted substantially among the 11 most parsimonious trees. The strict consensus identified five distinct lineages, *Trematocara unimaculatum, Telotrematocara macrostoma, Bathybates minor, Hemibates stenosoma*, and a

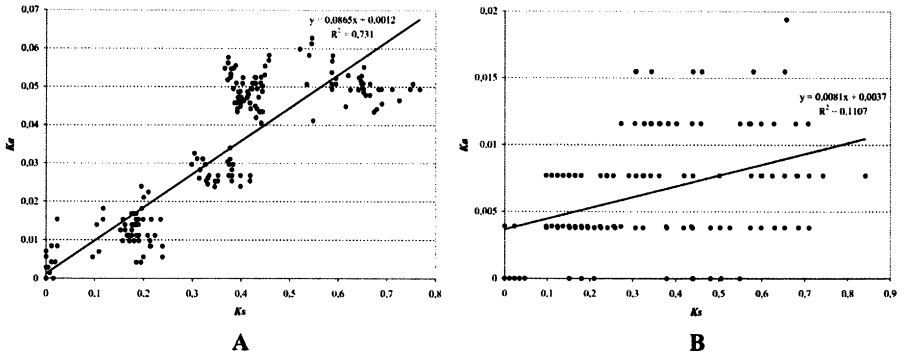
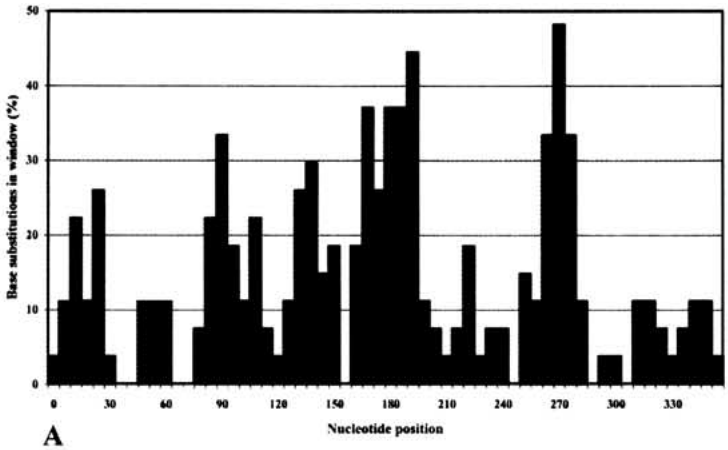


Fig. 6. Relationship between synonymous (K_s) and nonsynonymous (K_a) substitutions for all pairwise comparisons of (**A**) 22 taxa of the tribe Bathybatini plus 2 taxa of the tribe Trematocarini based on the ND2 gene and (**B**) 21 taxa of the tribe Bathybatini plus 2 taxa of the tribe Trematocarini based on the cyt *b* gene. Trend line and coefficient of determination (R^2) are shown for both genes.

clade composed of the remaining "large" *Bathybates* species. Among the "large" *Bathybates* species, *B. graueri* represented the most ancestral split followed by an unresolved split into four lineages represented by *B. ferox, B. vittatus, B. leo*, and the species pair *B. horni–B. fasciatus*. The second MP analysis with weightings according to the calculated TI/TV ratios for regions of the same degree of variation resulted in three most parsimonious trees of a length of 1006 evolutionary steps (CI excluding uninformative characters, 0.54; RI, 0.72; RC, 0.42). The branching pattern was widely congruent with the results of the most inclusive analysis combining all three gene segments. Within the Bathybatini the genus *Hemibates* was placed as most ancestral split, followed by *B. minor*, *B. graueri*, and the remaining "large" *Bathybates* species, with a trifurcation leading to *B. vittatus*, *B. leo*, and a clade consisting of *B. ferox*, followed by *B. horni* plus *B. fasciatus*, which appeared as paraphyletic. The third MP analysis, with identical weighting as in the previous analysis, except for regions of more than 20% genetic variation, where transitions were excluded, resulted in a single most parsimonious tree with a length of 1010 evolutionary steps (CI excluding uninformative characters, 0.54; RI, 0.71; RC, 0.43). The representatives of the tribe Trematocarini were placed as the most ancestral split, followed by *Hemibates stenosoma, Bathybates minor*, and the clade of the large *Bathybates*. In this clade *B. graueri* branched first, followed by a clade consisting of *B. ferox* as the most ancestral branch, and *B. horni* sister to *B. fasciatus*, again resulting as paraphyletic, and a clade comprising *B. leo* and *B. vittatus*, which also appeared as paraphyletic. The fourth MP analysis of the control region, considering transversions only, led to 36 most parsimonious trees with a score of 644 evolutionary steps (CI excluding uninformative characters, 0.50; RI, 0.75; RC, 0.46). Again, the Trematocarini were placed as the most ancestral lineage followed by *Hemibates stenosoma, Bathybates minor*, and the "large" *Bathybates* species. Their branching order was not further resolved. ML and NJ analyses of the control region yielded widely congruent topologies, with only one difference concerning the branching of *B. graueri*. In NJ it was placed as ancestral to the remaining "large" *Bathybates* species, whereas in ML and the topology obtained by Bayesian inference it was placed as sister to *B. leo*. The positioning of *B. graueri* in ML was not supported by quartet puzzling.

Relative Dating of Diversification Events

The branch length test of the 1047-bp segment of the ND2 showed that not a single taxon fell out of the 99% confidence interval surrounding the average root-to-tip distance (Table 2), suggesting an almost-constant rate of molecular evolution. Three major events of diversification can be derived from the linearized tree (Fig. 8). The first concerned the primary radiation of the Bathybatini and the Trematocarini, leading to the origin of the genera *Hemibates* and *Bathybates* as well as to the origin of the two investigated species of the tribe Trematocarini. For this first major split we obtained an average TrN + Γ distance of 25.8% (± 5.3%). The second split concerned the branching of *Bathybates minor* (TrN + Γ distance, 15.5 ± 1.5%). The third and most vigorous cladogenesis event led to the diversification of the "large" *Bathybates* species (TrN + Γ distance of 7.1 ± 0.9%). The split of *B. horni* and *B. fasciatus* (TrN + Γ distance, 6.3 ± 0.1%) and the split between the ancestor of *B. ferox* and that of *B. vittatus* plus *B. leo* happened almost simultaneously (TrN + Γ distance, 6.0 ± 0.2%). The most recent split in the genus *Bathybates* concerned the diversification between *B. vittatus* and *B. leo* (TrN + Γ distance, 3.2 ± 0.2%). Interestingly, in *Hemibates stenosoma* one individual is very divergent from the three remaining specimens (TrN + Γ distance, 4.8 ± 0.4%), indicating that the scope of genetic divergence within *Hemibates stenosoma* is greater than that between some species of the genus *Bathybates*.


Base substitutions in window (%)
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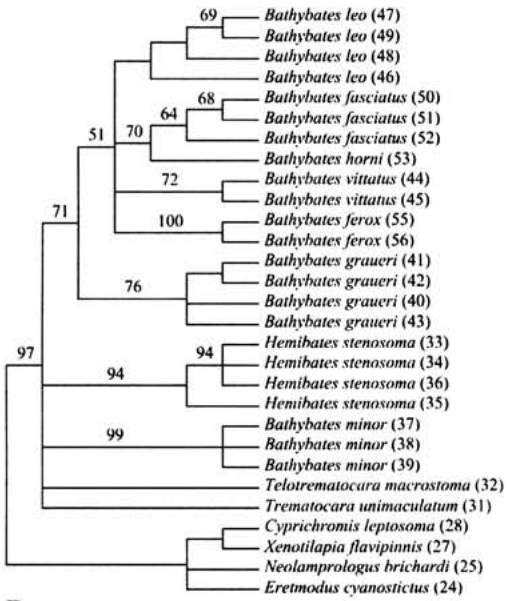
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Telotrematocara macrostoma (32)
Trematocara unimaculatum (31)
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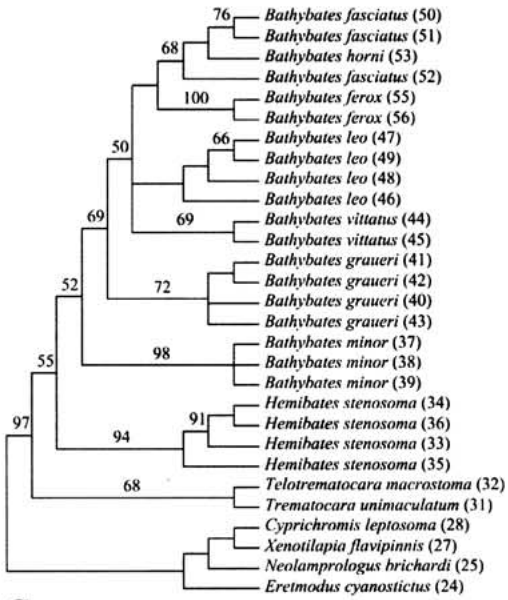
Xenotilapia flavipinnis (27)
Neolamprologus brichardi (25)
Eretmodus cyanostictus (24)

B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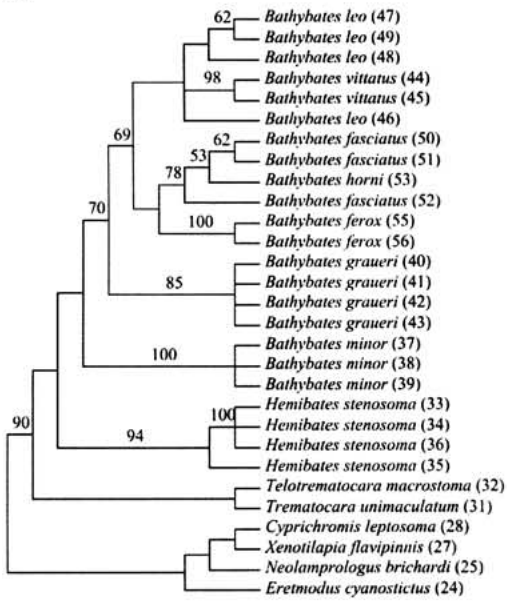
Trematocara unimaculatum (31)
Cyprichromis leptosoma (28)
Xenotilapia flavipinnis (27)
Neolamprologus brichardi (25)
Eretmodus cyanostictus (24)

C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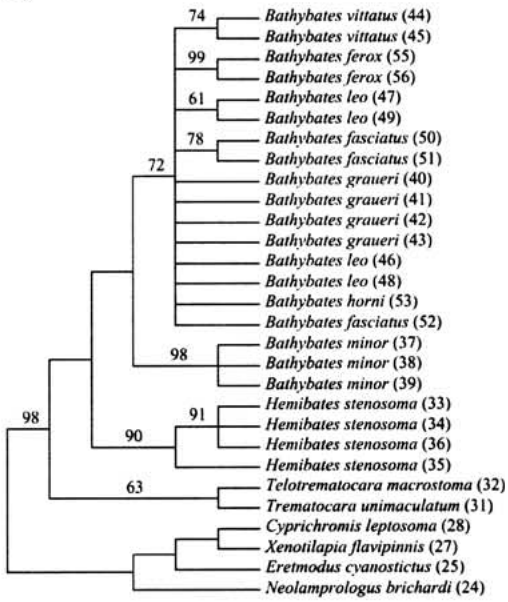
D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Trematocara unimaculatum (31)
Cyprichromis leptosoma (28)
Xenotilapia flavipinnis (27)
Eretmodus cyanostictus (25)
Neolamprologus brichardi (24)

E

Table 2. Branch length test for the ND2 gene of two specimens of the Trematocarini and 22 specimens of the Bathybatini

No.[a]	Species	δ	SE	*Z*
30	*Trematocara unimaculatum*	0.0801	0.0352	2.2742
32	*Telotrematocara macrostoma*	0.0701	0.0344	2.0353
33	*Hemibates stenosoma*	0.0289	0.0242	1.1943
34	*Hemibates stenosoma*	0.0316	0.0236	1.3355
35	*Hemibates stenosoma*	0.0351	0.0226	1.5558
36	*Hemibates stenosoma*	0.0388	0.0230	1.6913
37	*Bathybates minor*	0.0094	0.0183	0.5117
38	*Bathybates minor*	0.0009	0.0195	0.0460
39	*Bathybates minor*	0.0016	0.0190	0.0857
40	*Bathybates graueri*	0.0055	0.0132	0.4139
41	*Bathybates graueri*	0.0016	0.0127	0.1290
42	*Bathybates graueri*	0.0046	0.0128	0.3611
43	*Bathybates graueri*	0.0038	0.0130	0.2966
44	*Bathybates vittatus*	0.0072	0.0119	0.6000
45	*Bathybates vittatus*	0.0072	0.0119	0.6000
46	*Bathybates leo*	0.0150	0.0096	1.5644
47	*Bathybates leo*	0.0185	0.0095	1.9563
48	*Bathybates leo*	0.0185	0.0095	1.9563
50	*Bathybates fasciatus*	0.0086	0.0121	0.7069
51	*Bathybates fasciatus*	0.0076	0.0118	0.6419
52	*Bathybates fasciatus*	0.0196	0.0141	1.3939
53	*Bathybates horni*	0.0044	0.0116	0.3783
55	*Bathybates ferox*	0.0020	0.0121	0.1610
56	*Bathybates ferox*	0.0029	0.0124	0.2339

Note. Substitution model TrN+Γ; average root-to-tip distance = 0.126195. No taxon shows a significant deviation at the 1% level.
[a]Numbers correspond to the sample list (Table 1).

Discussion

Methodological Aspects and Evolutionary Characteristics of the Control Region, ND2 and cyt b

The control region is widely regarded to be the most variable part of the mitochondrial genome, due to its rapid rate of molecular evolution (Hoelzel et al. 1991) caused by reduced functional constraints (Brown 1985). The highest rate of base substitution and insertion/deletion events is observed directly adjacent to the flanking tRNAs (Saccone et al. 1987). The most useful part for phylogenetic analysis is the first half between the tRNA-Pro and the central conserved region because of considerable length variation due to several small indels and high levels of base substitution (Lee et al. 1995). It is regarded to be most suitable for studies of intraspecific variation or phylogenetic analyses of closely related species (Lee et al. 1995) and for rapid diversification events (Sturmbauer and Meyer 1992). Since the Bathybatini represent a relatively old cichlid lineage, we observed several regions in the first section of the control region with varying rates of base substitution, up to almost 50% according to the sliding window analysis (Fig. 7a) and, thus, a high degree of saturation of transition mutations (data not shown). Interestingly, the mode of nucleotide substitutions in the control region seems to vary considerably among different cichlid lineages. For example, the TI/TV ratio of the first half of the control region is 1.50 in the Ectodini (using the dataset of Koblmüller et al. 2004), whereas it is 1.91 in the Bathybatini. A higher TI/TV ratio would also imply more rapid saturation of transition mutations, which is the case in the Bathybatini, in which saturation of transitions starts at about 8% sequence divergence of the control region (data not shown), whereas transitions are not saturated within all pairwise comparisons in the Ectodini (maximum sequence divergence of 13.80%), which is concordant with the findings of Salzburger et al. (2002), who showed that there is no obvious saturation of transitions evident in the clade containing the Eretmodini, the Lamprologini, and the H-lineage. Consequently, it is important to evaluate the characteristics of the control region and to find an

Fig. 7. **A** Sliding window analysis of 362 bp of the mitochondrial control region (window width, 9 bp; overlap, 3 bp) of eight species of the tribe Bathybatini and two species of the tribe Trematocarini. The genetic variation is given as a percentage of the 27 possible (window width, 9 bp) base substitutions from a consensus sequence. MP topologies are depicted for the mitochondrial control region using different weighting schemes: **B** unweighted MP analysis; **C** weighted MP analysis using the appropriate weightings according to the estimated transition/transversion ratio for regions of different genetic variation (low variable, < 10%; high variable, 10–20%; hypervariable, > 20%); **D** weighted MP analysis using the appropriate weightings according to the estimated transition/transverion ratio for low- and high-variable regions, and excluding transitions in hypervariable regions; **E** unweighted MP analysis using transversions only. Bootstrap values are shown above the branches. Only values higher than 50 are presented. Numbers following the species name correspond to the sample list (Table 1).

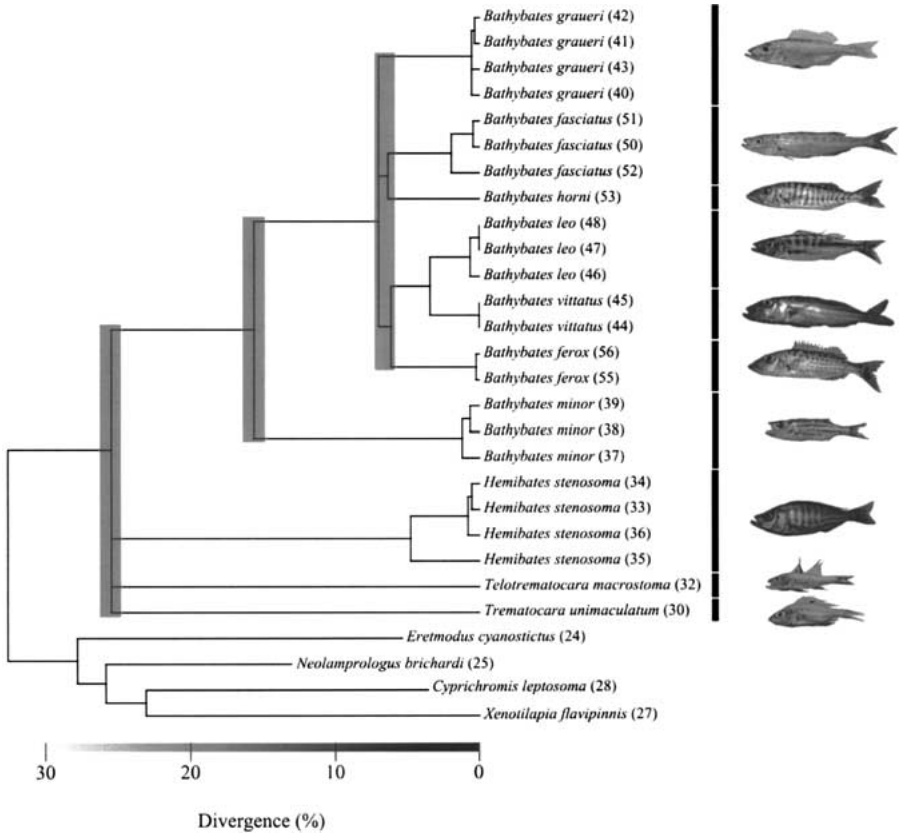

Fig. 8. Linearized tree based on a 1047-bp segment of the ND2 gene. The linearized tree was compiled with the computer program LINTRE (Takezaki et al. 1995) after performing a branch length test (Takezaki et al. 1995) to test for differences in base substitution rates, using the substitution model TrN + Γ (Tamura and Nei 1993). The distance values in the scale below the phylogenetic tree correspond to the observed mean sequence divergence using the substitution model TrN + Γ. Numbers following the species name correspond to the sample list (Table 1).

appropriate weighting scheme for the data set used. Our goal was both to find the most appropriate weighting scheme and to evaluate the effect of the weighting scheme on the tree topology. To this end the resulting MP topologies of the control region data set were compared to that obtained by our combined analysis of all three mtDNA gene segments, and consistency indices resulting from the four distinct weighting schemes were compared to each other (see Materials and Methods; Figs. 7b–d). Interestingly, increasing resolution of the tree topology did not necessarily result in an increase of the tree consistency, albeit relatively low differences were observed. The lowest consistency index was observed for the weighting according to the estimated TI/TV ratio in three regions of different genetic variation, although the topology was stable and most consistent to that found by our combined analysis of all three gene segments, particularly at the ancestral branches. The use of the same weighting scheme plus exclusion of transitions in regions showing more than 20% genetic variation resulted in almost the same topology and consistency index, indicating that exclusion of transition mutations in hypervariable regions does not change the results. The highest consistency index was observed in the analysis using transversions only. In this case the topology was still consistent with the results of the combined analysis of all three gene segments at the ancestral branches constituting the split of major lineages but remained unresolved among evolutionarily younger branches such as the diversification of the "large" *Bathybates* species. In contrast, "unweighted" MP analysis yielded a higher consistency index than weighted MP and only a slightly lower consistency index than MP based on TV only. This analysis differed substantially from the combined analysis at the ancestral branches, but recovered the same tree topology for the young splits. These observations point to a severe effect of multiple hits in transition mutations for the ancestral branches, particularly when transition mutations are weighted equally to transversions and indels. Our comparative analysis thus indicates that despite considerable saturation, transition mutations contain substantial information, necessary to resolve the relationships among evolutionary young splits. When they are down-weighted according to their relative frequency in relation to transversions, they provide resolution for young splits without obscuring the deep splits. It seems best justified to use transversion mutations only when addressing the most ancestral splits constituting the diversification of major lineages. To obtain optimum consistency and resolution among closely related taxa it seems better to apply a weighting scheme based on the TI/TV ratios for precisely defined regions exhibiting different levels of genetic variation. For the protein coding genes ND2 and cyt *b*, previous investigations on cichlids already

showed that the use of distinct weightings for MP, dependent on the codon position, results in better and more consistent resolution (Salzburger et al. 2002). Our comparative evaluation further shows that the ND2 gene seems to be applicable for the analysis within a broad range of taxonomic levels (see Klett and Meyer 2002; Koblmüller et al. 2004), even if it exhibits a mutation rate similar to that of the control region in the Bathybatini and Ectodini (Koblmüller et al. 2004). So far, the control region alone was considered to be best suitable for analysis of closely related taxa (Lee et al. 1995). When analyzing evolutionarily old lineages, protein-coding genes seem to require the exclusion of synonymous substitutions at the first positions of leucine codons and transition mutations in third codon positions of fourfold degenerate amino acids, due to their degree of saturation (Klett and Meyer 2002). However, there is evidence that there are some limits of resolution in cyt *b* for analyzing relationships among closely related species, most likely due to its high degree of conservation at the amino acid level (Farias et al. 2001). Indeed, our analysis demonstrates that ND2 and cyt *b* are affected by different evolutionary constraints. The positive correlation between the rate of nonsynonymous (K_a) and synonymous (K_s) substitutions observed in our analysis of the ND2 gene is in concordance with other studies that were based on different organisms and genes (Graur 1985; Sharp and Li 1987; Wolfe and Sharp 1993; Akashi 1994; Comeron and Aguadé 1996; Comeron and Kreitman 1998). The low number of nonsynonymous substitutions in the cyt *b*—a maximum of five nonsynonymous substitutions was observed—indicates that strong evolutionary constraints are acting on the structure of this gene, hardly allowing any amino acid changes to occur without having a severe effect on the gene's functionality. For ND2 these evolutionary constraints seem to be less tight, allowing amino acid changes without affecting the functionality of the gene. This also explains the low overall substitution rate of cyt *b* compared to ND2 (0.7 times). Despite the relatively old age of the Bathybatini, compared to other Tanganyikan cichlid lineages, and the low substitution rate of cyt *b* compared to both the control region and ND2, there is some lack of resolution even among the most ancestral splits, most likely due to the lack of nonsynonymous substitutions. Nevertheless, combining cyt *b* with ND2 and the control region increases resolution and branch support.

Positioning of the Bathybatini in the Large-Scale Phylogenetic Framework of African Cichlids

Within the polyphyletic flock of cichlid fishes in Lake Tanganyika, the Bathybatini are known to represent one of eight old cichlid lineages that have seeded the radiation. They do not belong to the monophyletic assemblage containing the tribes Eretmodini and Lamprologini and the H-lineage (Nishida 1991, 1997; Sturmbauer and Meyer 1993; Lippitsch 1998; Klett and Meyer 2002; Takahashi et al. 2001; Salzburger et al. 2002). Their relatively close relationship to the Trematocarini, another Tanganyikan deepwater tribe, was demonstrated by several authors by means of morphological (Stiassny 1981; Poll 1986), lepidological (Lippitsch 1998), and molecular genetic approaches (Nishida 1997; Klett and Meyer 2002). Nevertheless, the relationships of these two tribes in relation to other cichlid lineages still remain unclear. Previous molecular genetic investigations were not able to unambiguously identify a sister group to the monophyletic assemblage containing the Bathybatini and Trematocarini. Based on ND2, Klett and Meyer (2002) suggested a sister-group relationship of the Bathybatini and Trematocarini to the monophyletic assemblage containing the tribes Eretmodini and Lamprologini and the H-lineage, and no closer relationship to *Tylochromis polylepis, Oreochromis tanganicae*, and *Boulengerochromis microlepis*. Based on nuclear markers other authors suggested a close relationship of *Boulengerochromis microlepis* to the Bathybatini and Trematocarini (Nishida 1997). Salzburger et al. (2002) suggested that the Bathybatini and Trematocarini are two distinct ancestral lineages that seeded the Lake Tanganyika radiation and originated almost simultaneously with *Boulengerochromis microlepis* and the ancestors of the tribes Eretmodini and Lamprologini and the H-lineage. Our new analysis unambiguously placed the Bathybatini and Trematocarini in a monophyletic assemblage. No unequivocal sister-group relationship of this group to any other lineage could be determined, although it seems most likely that either *Boulengerochromis microlepis* or the monophyletic assemblage consisting of the tribes Eretmodini and Lamprologini and the H-lineage are the sister group. Moreover, our data suggest an ancient major "African" cladogenesis event (prior to the Tanganyika radiation), leading to an almost-simultaneous diversification of several distinct *Tilapia* lineages and to the origin of two species-rich "nontilapiine" lineages, which later also seeded Lake Tanganyika: The first "nontilapiine" lineage includes the deepwater tribes Bathybatini and Trematocarini; the other consists of the tribes Eretmodini and Lamprologini and the H-lineage (Fig. 3). This finding is congruent with the findings of Klett and Meyer (2002) and Salzburger et al. (2002). Since both the Bathybatini and Trematocarini underwent radiation in Lake Tanganyika and no closely related species are known in extant river systems outside the lake, these two tribes might represent the only surviving descendants of an ancient African lineage.

Phylogenetic Relationships Within the Bathybatini and the Trematocarini

While it is highly likely that the Bathybatini and Trematocarini constitute a monophyletic lineage, little was known about their precise phylogenetic relationships. Those studies that targeted this topic by means of morphological analyses led to more or less conflicting results (Stiassny 1981; Poll 1986; Takahashi 2003b). According to Poll (1986) the tribe Bathybatini comprises the genera *Bathybates* and *Hemibates*, well separated from the tribe Trematocarini by a number of morphological characters. The Bathybatini can be easily distinguished from the Trematocarini by the larger body size, more than 15 cm; number and shape of teeth; size of scales; number of scales in the longitudinal line; and higher number of fin rays in the dorsal and anal fins. A clear distinction is also supported by lepidological data (Lippitsch 1998) and a previous molecular investigation (Nishida 1997). In contrast, Stiassny (1981) postulated a sister-group relationship of *Hemibates* and the *Trematocarini*, based on the shared and unique condition of the first levator internus, palatine–mesethmoid ligament, and rostral cartilage on the premaxillae. A more recent morphological study focusing on the Trematocarini (Takahashi 2002) unambiguously showed the monophyly of the Trematocarini, which are clearly separated from *Bathybates* and *Hemibates* by a number of autapomorphic characters. In this study no clear sister-group relationship of the Trematocarini to either *Bathybates* or *Hemibates* could be demonstrated. Takahashi (2003a) further analyzed these relationships based on comparative osteology of infraorbitals and showed that the three genera *Bathybates, Hemibates*, and *Trematocara* (including *Telotrematocara*) have different types of infraorbitals. The type expressed in *Hemibates* had been inferred to represent the plesiomorphic condition for African cichlids (Stiassny 1997), also exhibited in many other cichlids, whereas the types expressed in *Bathybates* and the Trematocarini are synapomorphic in these two lineages. Analysis of 37 morphological characters for the whole Tanganyika cichlid species flock placed *Hemibates stenosoma* as ancestral to *Bathybates* and the Trematocarini (Takahashi 2003b). Our analyses of three mitochondrial genes place weak bootstrap support for the monophyly of the Bathybatini (*Hemibates* plus *Bathybates*) as sister group to the Trematocarini. In addition, the results of the linearized tree analysis (Fig. 8) suggest that there might in fact be three distinct old lineages that populated the emerging Lake Tanganyika: the tribe Trematocarini, the genus *Hemibates*, and the genus *Bathybates*. The observed great divergence among the two representatives of the Trematocarini (Fig. 8) might be due to an underrepresentation of the nine species of the tribe Trematocarini in the analysis (see Fig. 4).

Concerning the phylogeny within the genus *Bathybates, Bathybates minor* clearly represents the most ancestral split. This is also indicated by its overall morphology and appearance. *Bathybates minor* is the only smaller *Bathybates* species, barely reaching a total length of 20 cm. The remaining ''large'' *Bathybates* species with a total length of 30 to more than 40 cm are likely to have originated within a very short time span, distinguishing *B. graueri* as most ancestral branch. Among the ''large'' *Bathybates* two species pairs could be identified, one including *B. horni* and *B. fasciatus*, and the other consisting of *B. leo* and *B. vittatus*. *Bathybates ferox* seems to represent a distinct lineage, most likely ancestral to *B. leo* and *B. vittatus*.

Age Estimates for Major Diversification Events

Since it is not possible to use the molecular clock of Sturmbauer et al. (2001) for older lineages (Koblmüller et al. 2004), we applied the linearized tree method on the 1047-bp-long ND2 gene to derive a chronicle of the diversification in the Bathybatini (Fig. 8). These results suggest, that with an average TrN + Γ distance of 25.8% (± 5.3%), the oldest split (among *Hemibates*, *Bathybates*, and the Trematocarini) is at least as old as the divergence between the Lamprologini and the H-lineage (unpublished data). Since the Lamprologini and the H-lineage are likely to represent two distinct lineages that indepently seeded the Tanganyika radiation (Salzburger et al. 2002), this old dating supports the hypothesis that the genera *Hemibates* and *Bathybates* diversified before the primary lacustrine radiation and independently populated the emerging lake. Subsequently, the genus *Bathybates* diversified, first by the branching of *B. minor* (TrN + Γ distance, 15.5 ± 1.5%) and later by the diversification of the six ''large'' *Bathybates* species. The linearized distance observed for the first split fits with the primary diversification within the tribe Ectodini (Koblmüller et al. 2004), which is likely to have happened in the course of the primary lacustrine radiation (Salzburger et al. 2002). The second diversification event within the genus *Bathybates* (TrN + Γ distance, 7.1 ± 0.9%), leading to the current diversity of six ''large'' *Bathybates* species, is likely to have proceeded at the same time as the split between the genera *Cyprichromis* and *Paracyprichromis* (TrN + Γ distance, 7.2 ± 0.6%) in the Cyprichromini, a tribe of pelagic but shore-associated cichlids (Brandstätter et al. 2005), the diversification of some species of the genus *Xenotilapia* in the Ectodini (several distinct lineages with a TrN + Γ distance of 7–8%; Koblmüller et al. 2004), and the

primary radiation of the Limnochromini (TrN + Γ distance, 7.9 ± 1.1%), a tribe of benthic deepwater cichlids (Duftner et al. 2005). The observed concordance of diversification events in independent lineages of the Tanganyika radiation suggests that the radiations of benthic and pelagic deepwater tribes were triggered by the same environmental factors that also induced diversification of littoral species. Most likely lake level fluctuations of several hundred meters, leading to a separation of the lake into its three deep basins, induced rapid speciation in both benthic and pelagic lineages of deepwater cichlids, as it is already known for littoral species (Sturmbauer and Meyer 1992; Rossiter 1995; Verheyen et al. 1996; Sturmbauer et al. 1997, 2001, 2003; Sturmbauer 1998; Rüber et al. 2001; Baric et al. 2003). Moreover, the observed rapid pace of speciation of this pelagic piscivorous cichlid lineage is in contrast to the evolutionary scenario for pelagic cichlids postulated by Coulter (1994), who suggested pelagic taxa to have gradually evolved in the course of their long history. Assuming an age of 5–6 MY for the primary lacustrine radiation (Salzburger et al. 2002) based on the geological dating for the onset of clear and deepwater conditions in Lake Tanganyika (Tiercelin and Mondeguer 1991), the branching of *B. minor* can be tentatively dated to this age. The "large" *Bathybates* species can be constrained to an age of 2.3–2.7 MY. Likewise, the split between Trematocarini, *Hemibates*, and *Bathyates* can be tentatively dated to an age of 8.3–9.9 MY.

Promoters of Speciation in the Bathybatini

All species of the tribe Bathybatini exhibit a striking sexual dichromatism. While the females have a silvery body coloration, the males show a conspicuous pattern of dark blotches and stripes and a series of egg-blotches on the anal fin, similar to haplochromine cichlids (Poll 1986). Despite their lakewide distribution, intraspecific genetic variation is likely to be small (as suggested by our data for *B. graueri* that were almost identical in three locations in the southern basin of the lake), also indicated by the absence of distinct and geographically restricted color morphs. This might be due to the bathypelagic life style of the Bathybatini resulting in great dispersal ability. The only exception seems to be *Hemibates stenosoma*, for which two male color forms are reported, albeit in sympatry (Konings 1998). Some males have distinct vertical bars on the anterior part of the flank; other males show irregular dark blotches. There are three possible explanations for this phenomenon: Males may be polychromatic, male color pattern changes with age (supported by the observation that usually larger individuals exhibit vertical bars), or there are two different species (Konings 1998). In our data set intraspecific genetic variation was rather large in *Hemibates stenosoma* compared to the species of the genus *Bathybates*. Unfortunately, three of four individuals analyzed in this study were females, so that we could not assign them to a distinct color morph and further investigations are needed.

Lake level fluctuations were shown to be major promoters for diversification events in littoral Tanganyikan cichlid lineages (Sturmbauer and Meyer 1992; Rossiter 1995; Verheyen et al. 1996; Sturmbauer et al. 1997, 2001, 2003; Sturmbauer 1998; Rüber et al. 2001; Baric et al. 2003). Here we show that they might also affect radiations in pelagic deepwater cichlids, which are not restricted to a distinct type of substrate. The "large" *Bathybates* species might have evolved in separate sublakes during low-stand epochs, caused by a period of aridification in eastern Africa about 2.5–3 MYA (Cane and Molnar 2001), followed by an adaptation to coexistence in the same habitat when united by rising lake levels (Coulter 1994). Moreover, resource partitioning was suggested to be another driving force of speciation, so that the "large" *Bathybates* might have evolved in sympatry, as suggested for littoral species of Lake Tanganyika (Nakai et al. 1994). The members of the Bathybatini show a clear segregation of prey choice. In agreement to its phylogenetic placement, *Hemibates stenosoma* is clearly differentiated to all members of the genus *Bathybates* in terms of prey choice. With a maximum length of almost 30 cm, this fish is a respectable predator, migrating to shallow water during the night, often found together with members of the tribe Trematocarini, thus it might also prey on these small invertebrate feeders (Konings 1998) as well as on shrimp and clupeids (Coulter 1994). In contrast to *Hemibates stenosoma*, the species of the genus *Bathybates* are more specialized concerning their prey choice. In the genus *Bathybates* three species (*B. minor, B. fasciatus, B. leo*) almost exclusively prey on huge shoals of the Tanganyikan clupeids, *Stolothrissa tanganicae* and *Limnothrissa miodon*. One species—*B. minor*—is a surprise hunter. *Bathybates minor* has a similar body size and coloration as clupeids and is regularly caught as by-catch of clupeids, so that it seems likely to mimic its prey (Konings 1998). Two species—*B. fasciatus* and *B. leo*—are pursuit hunters and live in the open water and at greater depths than *B. minor*, down to a depth of 200 m, just above the anoxic zone of the lake (Poll 1956). The other four *Bathybates* species seem to prefer regions closer to the bottom and seem to feed on cichlids (Konings 1998). It is highly likely that *B. vittatus*, which is found down to depths of more than 200 m over muddy bottom, preys on cichlids rather than clupeids (Konings 1998). Nothing is known about the prey of

B. horni, because it is a rather rare species (Coulter 1991). Due to its streamlined body shape it is believed to behave as a pursuit hunter, but living in different areas or habitats, or preying on different fish, than *B. leo* and *B. fasciatus* (Konings 1998). As indicated by their deeper body, *B. ferox* and *B. graueri* are usually found near the bottom, where they mainly prey on bottom-dwelling cichlids like *Xenotilapia* spp. (Coulter 1991), thus using a different food source compared to the pursuit hunters of the open water. While *B. graueri* is often found in depths of more than 160 m, *B. ferox* has never been caught deeper than 70 m, indicating that some niche segregation concerning the foraging area seems possible. In summary, it is highly likely that resource partitioning concerning foraging area and specialization on distinct prey types played an important role for the diversification into the seven species of the genus *Bathybates*. Especially the case of the two species pairs, *B. horni*/*B. fasciatus and B. leo*/*B. vittatus*, is of interest. In both pairs, one species exclusively preys on clupeids, while the other one seems to prefer cichlids. That means that in both lineages specialization on clupeids is likely to have originated independently. But to confirm this hypothesis, further investigations concerning the feeding ecology of the distinct species comprising the genus *Bathybates*, especially *B. horni*, are needed.

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