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Evolutionary Relationships in the Sand-Dwelling Cichlid Lineage of Lake Tanganyika Suggest Multiple Colonization of Rocky Habitats and Convergent Origin of Biparental Mouthbrooding

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Abstract. The cichlid species flock of Lake Tanganyika is comprised of seven seeding lineages that evolved in step with changes of the lake environment. One seeding lineage diversified into at least six lineages within a short period of time. Our study focuses on the diversification of one of these lineages, the Ectodini, comprising highly specialized, sand- and rock-dwelling species. They display two distinct breeding styles: maternal and biparental mouthbrooding. By analyzing three mtDNA gene segments in 30 species representing all 13 described genera, we show that the Ectodini rapidly diversified into four clades at the onset of their radiation. The monotypic genus *Grammatotria* is likely to represent the most ancestral split, followed by the almost contemporary origin of three additional clades, the first comprising the benthic genus *Callochromis*, the second comprising the benthic genera *Asprotilapia*, *Xenotilapia*, *Enantiopus*, and *Microdontochromis*, and the third comprising the semi-pelagic genera *Ophthalmotilapia*, *Cardiopharynx*, *Cyathopharynx*, *Ectodus*, *Aulonocranus*, *Lestradea*, and *Cunningtonia*. Our study confirms the benthic and sand-dwelling life-style as ancestral. Rocky habitats were colonized independently in the *Xenotilapia*- and *Ophthalmotilapia*-clade. The *Xenotilapia*-clade comprises both maternal and biparental mouthbrooders. Their mode of breeding appears to be highly plastic: biparental mouthbrooding either evolved once in the common ancestor of the clade, to be reverted at least three times, or evolved at least five times independently from a maternally mouthbrooding ancestor. Furthermore, the genera *Xenotilapia*, *Microdontochromis*, *Lestradea*, and *Ophthalmotilapia* appeared paraphyletic in our analyses, suggesting the need of taxonomic revision.

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

Introduction

With an estimated number of 2,000 to 2,500 species, cichlid fishes are the most species rich family of vertebrates. More than 1,500 species live in the Great East African Lakes alone (Turner et al. 2001). The cichlid species flocks of these lakes are the most spectacular examples of explosive speciation, adaptive radiation, and ecological plasticity within a single vertebrate family (Kosswig 1947; Fryer and Iles 1972; McKaye et al. 1984; Greenwood 1984). For this reason Lakes Tanganyika, Malawi, and Victoria represent major model systems for the study of adaptive radiation (Meyer et al. 1990; Nishida 1991; Sturmbauer and Meyer 1992; Meyer 1993; Moran et al. 1994; Sturmbauer and Meyer 1994; Schliewen et al. 1994; Kocher et al. 1995; Rossiter 1995;

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Verheyen et al. 1996; Kornfield and Parker 1997; Mayer et al. 1998; Seehausen and van Alphen 1998; Sturmbauer 1998; Albertson et al. 1999; Rüber et al. 1999; Kornfield and Smith 2000; Nagl et al. 2000; Shaw et al. 2000; Danley and Kocher 2001; Turner et al. 2001; Salzburger et al. 2002a,b).

The evolutionary success of the cichlids is mainly explained by two inherent characteristics of the family, regarded as key innovations. The first lies in the particular anatomy of the pharyngeal apophysis, providing a second set of jaws functionally decoupled from the oral teeth (Liem 1973; Greenwood 1978). Small modifications of this structure allow utilization of new food resources, so that new ecological niches can be rapidly occupied (Meyer 1987; Hunter 1998). The second key innovation promoting adaptive radiation is the highly specialized mating and breeding behavior (Kosswig 1947; Crapon de Caprona 1986; Keenleyside 1991; Barlow 1991; Turner and Burrows 1995; van Alphen and Seehausen 2001). The cichlid species flocks of Lake Victoria and Malawi are exclusively comprised of mouthbrooding species, whereas in Lake Tanganyika about one third of the species are substrate breeders and various modes exist among mouthbrooding species (Sturmbauer et al. 1994; Meyer et al. 1996; Kuwamura 1997). Mouthbrooding is one of the most advanced parental care systems known among fish (Sato 1986). For this reason sexual selection seems to be particularly important for rapid speciation (Dominey 1984; Sturmbauer and Meyer 1992; Seehausen et al. 1997), in addition to selection for ecological divergence (Schliewen et al. 1994).

It is now clear that the species flocks in Lakes Tanganyika, Malawi, and Victoria evolved independently via intralacustrine speciation (Mayr 1984), though the ancestors of the haplochromine species flocks in Lake Victoria and Malawi are likely to have originated during the Lake Tanganyika radiation (Salzburger et al. 2002a). The main differences between the three major species flocks are their age and their complexity in terms of species number and degree of eco-morphological divergence. With an age of 9 to 12 MY (Cohen et al. 1993) Lake Tanganyika (Fig. 1) is by far the oldest of the three Great East African Lakes and harbors the eco-morphologically and behaviorally most diverse assemblage of about 250 recognized species (Fryer and Iies 1972; Greenwood 1984; Poll 1986; Turner et al. 2001). The lake has a complex geological history, characterized by periods of geological activity and extended intervals of substantially lower lake level (Lezzar et al. 1996; Cohen et al. 1997). The lake was severely affected by the change to a drier climate about 1.1 MYA, resulting in a drop of the lake level by about 650 to 700 m below its present level. Afterward the lake continuously rose until 550 KYA (Cohen et al. 1997) and subsequently underwent repeated lake level fluctuations up until recent times (Scholz and Rosendahl 1988; Gasse et al. 1989; Potts and Behrensmeyer 1992; Lezzar et al. 1996; Cohen et al. 1997).

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

Unlike the monophyletic species flocks of the Lakes Malawi (disregarding five endemic tilapiine species) and Victoria (Meyer et al. 1990), the cichlid fauna of Lake Tanganyika has been shown to be polyphyletic with several ancestral lineages colonizing the lake after its formation (Nishida, 1991; Kocher et al. 1995; Salzburger et al. 2002a). The Lake Tanganyika cichlids are grouped into 12 tribes (Poll 1986), 8 of which are endemic to the lake. At least seven tribes already populated the emerging lake: the Tylochromini, Trematocarini, Bathybatini, two lineages of the Tilapiini, Eretmodini, the ancestor of the Lamprologini, and the ancestor of another mouthbrooding lineage, the "H-lineage," as defined by Nishida (1991), but excluding the Eretmodini (Salzburger et al. 2002a; see also Kocher et al. 1995; Lippitsch 1998). The H-lineage comprises the tribes Tropheini, Haplochromini, Cyprichromini, Limnochromini, Perissodini, and Ectodini, all of which evolved within a very short time during the primary lacustrine radiation of the Lake Tanganyika cichlid flock (Salzburger et al. 2002a). These tribes are clearly distinct, morphologically as well as ecologically and behaviorally. Each tribe inhabits characteristic habitat types and ecological niches therein.

In our study we focus on the tribe Ectodini, which is endemic to Lake Tanganyika and part of the H-lineage. Their monophyly is supported by several anatomical features (Liem 1981; Poll 1986; Takahashi 2003a, b), as well as DNA sequence data (Sturmbauer and Meyer 1993; Takahashi et al. 1998). However, the delineation of species is sometimes problematic, due to the occurrence of morphological and color plasticity and the existence of morphologically intermediate populations among geographically separated species. For example, this is the case for *Ophthalmotilapia ventralis* and *O. heterodonta*, which are connected by morphologically intermediate populations at Mtoto at the west coast of the lake. The same pattern was observed along the Tanzanian coast, from Kigoma to Kipili (Hanssens et al. 1999). Moreover, the sympatry of distinct color morphs has been reported for *Cyathopharynx furcifer* in the south of the lake (Konings 1998; Snoeks, Aibara, Hanssens, and Neat, pers. comm.), which might turn out to be separate species in the future.

All Ectodini are mouthbrooders but they consist of both maternal and biparental mouthbrooding species (Poll 1986; Yanagisawa 1986; Kuwamura 1997; Konings 1998) and the number of evolutionary transitions from maternal to biparental mouthbrooding, and vice versa, is not known to date. The mostly sand-dwelling tribe Ectodini contains a few species that prefer rocky substrate. Of 12 genera, *Aulonocranus*, *Callochromis*, *Cardiopharynx*, *Ectodus*, *Grammatotria*, *Lestradea*, and *Microdontochromis* prefer sandy or muddy bottom, whereas *Asprotilapia*, *Cunningtonia*, *Cyathopharynx*, and *Ophthalmotilapia* prefer rocky shores. The genus *Xenotilapia* contains both sand- and rock-dwelling species (Konings 1998). The direction of this ecological transition was argued in the past, with morphology-based results suggesting a transition from a rock- to a sand-dwelling life history (Liem 1981) and with molecular results suggesting the contrary (Sturmbauer and Meyer 1993). However, the published molecular phylogeny did not contain a sufficient representation of sand-dwelling species, so that the number of ecological transitions from maternal to biparental mouthbrooding and from sand to rock dwelling remained phylogenetically unresolved. To adequately address these questions by means of molecular phylogenetic methods, we collected 30 species of Ectodini, so that all but 5 of the described species are represented.

Materials and Methods

Taxonomic Sampling and Molecular Biological Methods

Our study is based on a total of 95 individuals, including 92 Ectodini taxa (28 described species representing all 13 genera and two yet undescribed species: *Xenotilapia papilio* "sunflower," *Ectodus* cf. *descampsii* "north"). Most fish were caught during several expeditions to Lake Tanganyika, and additional samples were obtained from the aquarium trade (see Table 1). Voucher specimens are deposited at the Royal Africa Museum in Tervuren, Belgium under the accession numbers given in Table 1. Additional voucher specimens are available from the authors. As outgroup taxa we used two species of the tribe Limnochromini, *Limnochromis auritus* and *Triglachromis otostigma*, and one species of the tribe Cyprichromini, *Cyprichromis leptosoma*, based on a recent phylogenetic study on Lake Tanganyika cichlid fishes (Salzburger et al. 2002a).

A 365 bp segment of the most variable part of the control region (D-loop) was sequenced in 90 specimens and a 402 bp seqment of cytochrome *b* (cyt *b*) in 65 specimens. Additionally, a 1,047 bp segment of the NADH dehydrogenase subunit 2 gene (ND2) was sequenced for 28 specimens, so that each genus and each of the four major clades was represented. We used previously published sequences when available (Sturmbauer and Meyer 1993; Salzburger et al. 2002a) (for accession numbers see Table 1).

Total DNA was extracted from ethanol preserved fin-clips or white muscle tissue using the Chelex-method (Walsh et al. 1991) or proteinase K digestion followed by sodium chloride extraction and ethanol precipitation (Bruford et al. 1998). DNA amplification via polymerase chain reaction (PCR) was performed with a total volume of 17 μl using an Air-Thermo-Cycler (Idaho Technologies, Inc.). The reaction mix per sample contained 5.5 μl of deionized water, 1.7 μl of dNTP-mix(10×; Idaho Technology), 1.7 μl of a Mg^{2+} buffer (20 m*M*), 1.7 μl of each primer (1 μ*M*), 1.62 μl of enzyme diluent (Idaho Technology), 0.085 μl of *Taq* polymerase (GeneCraft), and 3 μl of the DNA-extract. The Thermo-Cycler program consisted of a denaturation phase of 15 s at 94°C, followed by five cycles of 0 s at 94°C, 5 s at 48°C and 20 s at 70°C, and 35 cycles with 0 s at 94°C, 0 s at 52°C, and 15 s at 72°C. With an aliquot of 2 μl of the PCR products, a minigel electrophoresis was carried out, using an ethidium bromide stained gel of 2% SeaKem agarose in Tris–borate–EDTA buffer (0.1 M, pH 7.2). To purify the PCR-products we used the PCR purification kit NucleoSpin Extract 2 in 1 (Machery-Nagel). Chain termination sequencing was conducted for 27 cycles (0 s at 94°C, 0 s at 52°C, and 45 s at 60°C). The Sequencing cocktail contained 2.8 μl of Big Dye Termination Reaction Mix (Applied Biosystems), 0.7 μl of the primer, 0.7 μl of bovine serum albumin (1 μ*M*; Idaho Technology), and 2.8 μl of a mixture of a.d. and DNA (depending on the DNA concentration of the sequencing template). The primers used for both amplification and sequencing of cytochrome *b* were L14724, 5′ CGAAGCTTGATATGAAAAACCATCGTTC, and H15149, 5′ AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA (Kocher et al. 1989); for the control region the primers L-Pro-F, 5′ AACTCTCACCCCTAGCTCCCAAAG, and TDK-D, 5′ CCTG AAGTAGGAACCAGATG (Kocher et al. 1989), were applied for PCR and sequencing. For the amplification of ND2 we used the primers MET, 5′ CATACCCCAACATGTTGGT, and TRP, 5′ GAGATTTTCACTCCCGCTTA. For the sequencing of ND2 we additionally applied the primer ND2.2A, 5′ CTGACAAAAACTTGCCTT (Kocher et al. 1995). The single-stranded cycle sequencing products were precipitated with sodium acetate and sequenced in both directions on an ABI 373 automatic sequencer (Applied Biosystems).

Phylogenetic Analyses

DNA sequences were aligned using Clustal W (Thompson et al. 1994). The alignment was improved by eye for the control region. Prior to phylogenetic analyses, each data set was tested for its overall phylogenetic content by applying likelihood mapping analysis, using PUZZLE 4.0 (Strimmer and von Haeseler 1996). For phylogenetic reconstruction the three most commonly used approaches, maximum parsimony (MP), neighbor joining (NJ) and

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

					GenBank accession No.		
No.	Extract.[a]	Species	Locality[b]	Sequence[c]	Control region	Cytochrome *b*	ND2
1	52	*Grammatotria lemairii*	?	+/+/–	Z21743	Z21766	–
2	71	*Grammatotria lemairii*	?	+/+/–	Z21744	Z21767	–
3	1693[G]	*Grammatotria lemairii*	Mpulungu	+/+/+	AY339018	AY337840	AY337787
4	221	*Callochromis pleurospilus*[d]	?	+/+/+	Z21735	Z21760	AY337771
5	1224[G]	*Callochromis stappersii*[d]	?	+/+/+	AY339048	AY337807	AY337775
6	1225[G]	*Callochromis stappersii*[d]	?	+/+/–	AY339049	AY337808	–
7	210	*Callochromis melanostigma*[d]	?	+/+/–	AY339046	AY337797	–
8	225	*Callochromis melanostigma*[d]	?	+/+/–	AY339047	AY337800	–
9	1585[G]	*Callochromis macrops*	Funda Village	+/+/–	AY339050	AY337822	–
10	1854[T1]	*Callochromis macrops*	Chisanza	+/+/+	AY339051	AY337851	AY337795
11	1856[T2]	*Callochromis macrops*	Muzumwa Bay	+/–/–	AY339052	–	–
12	1570	*Xenotilapia caudafasciata*	Lufubu estuary	+/+/+	AY339035	AY337815	AY337777
13	1571	*Xenotilapia caudafasciata*	Lufubu estuary	+/+/–	AY339036	AY337816	–
14	1572	*Xenotilapia longispinis*	Lufubu estuary	–/–/+	–	–	AY337778
15	1573	*Xenotilapia longispinis*	Lufubu estuary	+/+/+	AY339037	AY337817	AY337779
16	60	*Xenotilapia ochrogenys*[d]	Ndole Bay	+/+/+	Z21750	Z21772	AY337767
17	1566[G]	*Xenotilapia spiloptera*	Cape Kachese	+/+/–	AY339040	AY337814	–
18	1582[T3]	*Xenotilapia spiloptera*	Chisanza	+/+/–	AY339041	AY337821	–
19	1584	*Xenotilapia spiloptera*	Chisanza	+/+/–	AY339042	AY33783	–
20	1694[T4]	*Xenotilapia spiloptera*	Kasakalawe	+/+/+	AY339043	AY337841	AY337788
21	1586[T5]	*Xenotilapia boulengeri*	Funda Village	+/+/–	AY339029	AY337823	–
22	1697[T6]	*Xenotilapia bathyphila*	Mpulungu	+/+/–	AY339027	AY337843	–
23	1698[T7]	*Xenotilapia bathyphila*	Mpulungu	+/+/+	AY339028	AY337844	AY337789
24	72	*Xenotilapia* cf. *bathyphila*	Isanga	+/+/–	AY339026	AY337796	AY337768
25	1555[T8]	*Xenotilapia papilio* "sunflower"	Chituta Bay	+/+/–	AY339044	AY337809	–
26	1556[T9]	*Xenotilapia papilio* "sunflower"	Chituta Bay	+/–/+	AY339045	–	AY337776
27	370	*Xenotilapia sima*	?	+/+/–	AY339038	AY337802	–
28	1682[G]	*Xenotilapia sima*[d]	Utinta Bay	+/+/+	AY339039	AY337837	AY337785
29	1561[T10]	*Xenotilapia flavipinnis*	Sumbu	+/+/–	AY339030	AY337811	–
30	1587[T1]	*Xenotilapia flavipinnis*	Funda Village	+/+/–	AY339031	AY337824	–
31	1589[T12]	*Xenotilapia flavipinnis*	Funda Village	+/+/–	AY339032	AY337825	–
32	1590[T13]	*Xenotilapia flavipinnis*	Funda Village	+/–/–	AY339033	–	–
33	1849[G]	*Xenotilapia flavipinnis*	Mbita Island	+/+/+	AY339034	AY337849	AY337794
34	325	*Asprotilapia leptura*	Tanzania	+/+/+	Z21732	Z21758	AY337772
35	367	*Asprotilapia leptura*	?	–/+/–	–	AY337801	–
36	214	*Enantiopus melanogenys*[d]	?	+/+/+	AY339022	AY337798	AY337770
37	222	*Enantiopus melanogenys*[d]	?	+/+/–	AY339023	AY337799	–
38	1563[G]	*Enantiopus melanogenys*	Sumbu	+/+/–	AY339024	AY337813	–
39	1855[T14]	*Enantiopus melanogenys*	Chisanza	+/–/–	AY339025	–	–
40	151	*Microdontochromis tenuidentata*	?	–/+/–	–	Z21769	–
41	1671	*Microdontochromis tenuidentata*	Katoto	+/+/+	AY339019	AY337835	AY337784
42	1672	*Microdontochromis tenuidentata*	Katoto	+/–/–	AY339020	–	–
43	1848[T15]	*Microdontochromis rotundiventralis*	Mbita Island	+/+/+	AY339021	AY337848	AY337793
44	213	*Cardiopharynx schoutedeni*	?	+/+/–	Z21736	Z21761	–
45	1703[G]	*Cardiopharynx schoutedeni*	Mpulungu	+/+/+	AY339000	AY337846	AY337791
46	1850[T16]	*Cardiopharynx schoutedeni*	Kasakalawe	+/–/–	AY339001	–	–
47	1851[T17]	*Cardiopharynx schoutedeni*	Kasakalawe	+/+/–	AY339002	AY337850	–
48	937	*Ophthalmotilapia boops*[d]	?	+/+/+	AY338987	AY337803	AY337773
49	938	*Ophthalmotilapia boops*[d]	?	+/+/–	AY338988	AY337804	–
50	1665	*Ophthalmotilapia nasuta*	Chimba	+/+/+	AY338989	AY337833	AY337783
51	1667	*Ophthalmotilapia nasuta*	Bilila Island	+/+/–	AY338990	AY337834	–
52	1669	*Ophthalmotilapia nasuta*	Chimba	+/+/–	AY338991	–	–
53	73	*Ophthalmotilapia ventralis*	Kalambo	+/+/–	Z21748	Z21771	–
54	74	*Ophthalmotilapia ventralis*	Sumbu	+/+/–	Z21749	Z21798	–
55	1200	*Ophthalmotilapia ventralis*	Funda Village	+/+/+	AY338993	AY337805	AY337774
56	1201	*Ophthalmotilapia ventralis*	Funda Village	+/+/–	AY338994	AY337806	–
57	1593	*Ophthalmotilapia ventralis*	Mpulungu	+/+/–	AY338992	AY337826	–
58	1700[G]	*Ophthalmotilapia ventralis*	Mpulungu	+/–/–	AY338995	–	–
59	—	*Ophthalmotilapia heterodonta*	?	+/–/–	Z96001	–	–
60	—	*Ophthalmotilapia heterodonta*	?	+/–/–	Z96000	–	–
61	191	*Cyathopharynx furcifer*	Zambia	+/+/–	Z21741	Z21764	–
62	192	*Cyathopharynx furcifer*	Zambia	+/+/–	Z21740	Z21763	–

Continued

Table 1. Continued

No.	Extract.[a]	Species	Locality[b]	Sequence[c]	GenBank accession No.		
					Control region	Cytochrome *b*	ND2
63	1564	*Cyathopharynx furcifer*	Sumbu	+/–/–	AY338979	–	–
64	1574	*Cyathopharynx furcifer*	?	+/+/–	AY338980	AY337818	–
65	1595	*Cyathopharynx furcifer*	Sondwa Village	+/+/+	AY338981	AY337828	AY337781
66	1601	*Cyathopharynx furcifer*	Isanga	+/–/–	AY338982	–	–
67	1603	*Cyathopharynx furcifer*	Isanga	+/–/–	AY338983	–	–
68	1605	*Cyathopharynx furcifer*	Isanga	+/–/–	AY338984	–	–
69	1606	*Cyathopharynx furcifer*	Isanga	+/–/–	AY338985	–	–
70	1857	*Cyathopharynx furcifer*	Muzumwa Bay	+/–/–	AY338986		–
71	117	*Ectodus descampsii*	?	+/+/–	Z21742	Z21765	–
72	1699[G]	*Ectodus descampsii*	Mpulungu	+/–/+	AY339014	–	AY337790
73	1846[T18]	*Ectodus descampsii*	Kasakalawe	+/–/–	AY339015	–	–
74	1847[T19]	*Ectodus descampsii*	Chisanza	+/–/–	AY339016	–	–
75	1852[T20]	*Ectodus descampsii*	Kasakalawe	+/–/–	AY339017	–	–
76	1599[G]	*Ectodus* cf. *descampsii* "north"[d]	?	+/+/–	AY339011	AY337831	–
77	1600	*Ectodus* cf. *descampsii* "north"[d]	?	+/+/–	AY339012	AY337832	–
78	1681[G]	*Ectodus* cf. *descampsii* "north"[d]	?	+/+/–	AY339013	AY337836	–
79	1562[T21]	*Lestradea stappersii*	Sumbu	+/+/–	AY338996	AY337812	–
80	1771[T22]	*Lestradea stappersii*	Chisanza	+/+/+	AY338997	AY337847	AY337792
81	1853[T23]	*Lestradea stappersii*	Chisanza	+/–/–	AY338998	–	–
82	10.1	*Lestradea perspicax*	?	+/+/+	Z21745	Z21768	AY337765
83	212	*Cunningtonia longiventralis*	?	+/+/–	Z21738	Z21762	–
84	1594	*Cunningtonia longiventralis*	Sondwa Village	+/+/+	AY338999	AY337827	AY337780
85	1557	*Aulonocranus dewindti*	Chituta Bay	+/+/–	AY339003	AY337810	–
86	1580	*Aulonocranus dewindti*	Chisanza	+/+/–	AY339004	AY337819	–
87	1581	*Aulonocranus dewindti*	Chisanza	+/+/–	AY339005	AY337820	–
88	1596[G]	*Aulonocranus dewindti*	Mpulungu	+/+/–	AY339006	AY337829	–
89	1597	*Aulonocranus dewindti*	Mpulungu	+/+/+	AY339007	AY337830	AY337782
90	1692	*Aulonocranus dewindti*	Mbita Island	+/+/–	AY339008	AY337839	–
91	1696	*Aulonocranus dewindti*	Kasakalawe	+/+/–	AY339009	AY337842	–
92	1701	*Aulonocranus dewindti*	Mpulungu	+/+/–	AY339010	AY337845	–
93	59	*Limnochromis auritus*[d]	?	+/+/+	Z21746	Z21775	AY337766
94	103	*Triglachromis otostigma*	Burundi	+/+/+	Z30035	Z30004	AY337769
95	1684	*Cyprichromis leptosoma*[d]	Kitumba	+/+/+	AY339053	AY337838	AY337786

Representatives of all 13 genera of the cichlid tribe Ectodini were analyzed. Species names were assigned according to fishbase (http://www.fishbase.org). (G) Voucher specimen at the Department of Zoology of the University of Graz, Austria. (T) Voucher specimen at the Royal Africa Museum in Tervuren, Belgium: 1, MRAC 2001.94.P.520; 2, MRAC 2001.94.P.519; 3, MRAC 99.87.P.10; 4, MRAC 2001.94.P.19; 5, MRAC 2001.94.P.14; 6, MRAC 2001.94.P.18; 7, MRAC 99.87.P.2; 8, MRAC 99.87.P.11; 9, MRAC 99.87.P.12; 10, MRAC 99.87.P.9; 11, MRAC 99.87.P.3; 12, MRAC 99.87.P.5; 13, MRAC 99.87.P.6; 14, MRAC 2001.94.P.721; 15; MRAC 2001.04.P.2; 16, MRAC 2001.94.P.784; 17, MRAC 2001.94.P.785; 18, 20, two specimens from lot MRAC 2001.94.P.912–925; 19, one specimen from lot MRAC 2001.94.P.628–630; 21, MRAC 99.87.P.1; 22, MRAC 2001.94.P.634; 23, MRAC 2001.94.P.635.

[a] Numbers correspond to the extraction numbers.

[b] Coastal region where sample was obtained.

[c] Gene sequenced: control region/cytochrome *b*/ND2.

[d] Sample obtained from aquarium trade.

maximum likelihood (ML) were applied. Phylogenetic analyses, quartet puzzling (Strimmer and von Haeseler 1997) and bootstrapping (Felsenstein 1985) were carried out using the PAUP* program package (version 4.0 [Swofford 2000]). Due to the large number of taxa we applied heuristic search procedures with 10 replicates for MP and ML using the PAUP* option "heuristic search with random stepwise addition of taxa" and 1,000 replications for bootstrapping.

The phylogenetic analysis was carried out in three steps. In the first step, focusing on the identification of major groupings, we constructed separate phylogenies for the two protein coding gene segments (data not shown) and for the control region using all available DNA sequences for each gene segment. For the control region, transition mutations (Ti) were weighted 1:2 with respect to transversion mutations (Tv) in maximum parsimony, based on the ML-estimated Ti–Tv ratio of 2.0619. To avoid bias on the estimated Ti–Tv ratio due to saturation, we calculated the Ti–Tv ratio for the *Asprotilapia*- and the *Ophthalmotilapia*-clade separately. The resulting Ti–Tv ratios of 2.1749 for the *Asprotilapia*- and 2.5581 for the *Ophthalmotilapia*-clade justify our weighting scheme for the control region. In MP of the protein-coding DNA-segments (cyt *b* and ND2), weights were assigned according to the ML-estimated six-fold (cyt *b*) or seven-fold (ND2) higher Ti–Tv ratio in third codon positions. The use of ML and NJ required the specification of explicit assumptions. The program Modeltest (version 3.04, Posada and Crandall 1998) was used to test the fit of 56 sequence evolution models on the given data using a likelihood ratio test framework. The program examined the fit of each model independently, as well as with the addition of either a proportion of invariable sites parameter (I), a gamma distribution shape parameter (Γ), or both ($I+\Gamma$). The resulting substitution models were $HKY+\Gamma$ (Hasegawa et al. 1985) for the cyt *b* data set, $TrN + \Gamma$ (Tamura and Nei 1993) for the ND2 data set, $HKY+I+\Gamma$ for the control region, and $GTR+I+\Gamma$ (Yang 1994) for the combined

dataset of all three genes. Accordingly, NJ was carried out using the appropriate algorithm for each data set. For both MP and NJ the robustness of the inferred topologies was evaluated with bootstrap replications (1,000), heuristic search, and the PAUP* option "simple addition of taxa." ML-analyses were performed based on the data set–specific models suggested by the program Modeltest (version 3.04 [Posada and Crandall 1998]). In addition to the ML-analyses the generated topologies were evaluated by quartet puzzling (PAUP*) to check the robustness of the trees inferred by ML-analyses. To reduce calculation time we decided to enforce the following constraints for the ML analysis of the control region data set, based on the NJ topology. Nodes supported by NJ bootstrap values > 80 were constrained. The constrained clades are (presented as Venn diagram; taxon numbers correspond to Table 1) ((((((((((65,68,69),(63,67)),(62,66)),(61,70)),64),((59,60),((((57,58), 56),(53,54)),55)),(((45,46),47),44),((50,52),51),(48,49),((83,84), ((((((90, 91),92),88),(86,87)),85),89)),82,(79,80,81),((76,77,78),71,72, 74,(73, 75))),(18,19),17,20,(41,42),(29,30,31,32,33),((36,37),38,39), 24,23,22, 21,((12,13),15),(27,28),(25,26),43,16,34,((((10,11),9),(7,8)), (5,6),4), ((1,2),3))),(93,94),95).

In the second step we focused on the branching order among the major groups by analyzing a combined data set of the two protein coding genes and the control region selecting 26 representative taxa for all 13 genera. To further evaluate the support of the branching order of the four major clades we applied the four cluster likelihood mapping (Strimmer and von Haeseler 1997) implemented in the computer program PUZZLE using the combined data set.

The third step of analysis focused on the branching order within the *Asprotilapia*- and the *Ophthalmotilapia*-clade. We analyzed the two clades separately using maximum likelihood. All three gene segments were combined, and the likelihood parameters and substitution models calculated, by the program Modeltest (version 3.04. [Posada and Crandall 1998]) for each of the four subdata sets were used for our ML-analyses. We applied heuristic search procedures with 100 replicates with the PAUP* option "heuristic search with random stepwise addition of taxa" and 1,000 replications for bootstrapping. The clades were rooted both by the most ancestral clade (*Grammatotria lemairii*) and by the *Callochromis*-clade (*Callochromis macrops*, *C. stappersii*, and *C. pleurospilus*). The phylogenetic hypothesis derived from the three analytical steps is presented in the form of a composite consensus tree, which was used to trace evolutionary transitions of habitat specialization and breeding mode.

Age Estimates

To elucidate the relative timing of major cladogeneic events in the Ectodini and to test for the influence of historic lake level fluctuations on speciation bursts, we applied the linearized tree method described by Takezaki et al. (1995) using their computer program LINTRE. This analysis was based on 402 bp of the cytochrome *b* and 1,047 bp of the ND2, because it was not possible to use the mitochondrial control region due to beginning saturation of transition mutations. We used a subset of the available data comprising one representative per species and *Cyprichromis leptosoma*, a member of the tribe Cyprichromini, as the outgroup. Since the construction of a linearized tree requires an equal rate of base substitution among all analyzed taxa, we tested for differences in the rate of base substitution using the branch length test implemented in LINTRE. To calculate the linearized tree we applied the substitution model TrN + Γ (Tamura and Nei 1993), which was identified as the most appropriate algorithm for our data set by Modeltest.

For estimating relative ages of major diversification events during the evolution of the Ectodini we calculated average pairwise TrN + Γ distances (arithmetic mean and standard deviation) within and among the four major clades on the basis of 402 bp of the cytochrome *b* and 1,047 bp of the ND2. We are aware that absolute age comparisons using a constant divergence rate for all vertebrates are problematic, because the rate is influenced by several factors such as body size, metabolic rate, and generation time (Martin and Palumbi 1993). For this reason and due to the fact that there is no molecular clock calibration for a combined data set of cyt *b* and ND2 available, we refrained from attempting an estimate of the absolute age.

To make our divergence estimates comparable with those of Sturmbauer and Meyer 1993, we additionally applied the model of base substitution of their choice (Jukes and Cantor 1969) for cytochrome *b*.

Results

Phylogenetic Analyses

Likelihood mapping demonstrated the presence of a strong phylogenetic signal in all data sets (Fig. 2). Varying levels of fully resolved quartets were found for cyt *b* (91.7%), ND2 (95.0%), control region (95.2%), and the combined data set of all three gene segments (92.8%). Pairwise sequence divergence (uncorrected p-distance) within the Ectodini varied from 0.0 to 11.2% in cyt *b*, from 1.4 to 12.6% in ND2, and from 0.0 to 30.5% in the control region. For the combined data set pairwise differences between 1.5 and 20.0% were observed.

Step 1: Identification of Major Lineages. The MP analysis of cyt *b* yielded 725 most parsimonious trees of a length of 850 steps (consistency index (CI) excluding uninformative characters, 0.51; retention index (RI), 0.88; and rescaled consistency index (RC), 0.54; tree not shown). The MP analysis of ND2 resulted in two most parsimonious trees of a length of 3421 steps (CI excluding uninformative characters, 0.46; RI, 0.63; and RC, 0.38; tree not shown). The MP analysis of the control region resulted in 7271 most parsimonious trees of 737 steps (CI excluding uninformative characters, 0.45; RI, 0.86; and RC, 0.41; tree not shown). In the ML analysis the appropriate likelihood parameters for the three gene segments were as follows: for cyt *b* PAUP estimated $g_A = 0.2506$, $g_C = 0.3187$, $g_G = 0.1604$, $g_T = 0.2703$, and the gamma shape parameter $\alpha = 0.2998$. For ND2 the estimated likelihood parameters were $g_A = 0.2671$, $g_C = 0.3555$, $g_G = 0.1072$, $g_T = 0.2702$, and the gamma shape parameter $\alpha = 0.3108$. For the control region we obtained the base frequencies $g_A = 0.3974$, $g_C = 0.1739$, $g_G = 0.1096$, and $g_T = 0.3192$. The proportion of invariable sites (I) was 0.4482, and α estimated at 0.7525. The phylogenetic tree resulting from the ML analysis of the control region using *Triglachromis otostigma*, *Limnochromis auritus* (Limnochromini), and *Cyprichromis leptosoma* (Cyprichromini) as outgroup taxa is shown in Fig. 3. The NJ analysis of the control region was congruent in all major branchings. The bootstrap values of this analysis formed the basis for the defi-

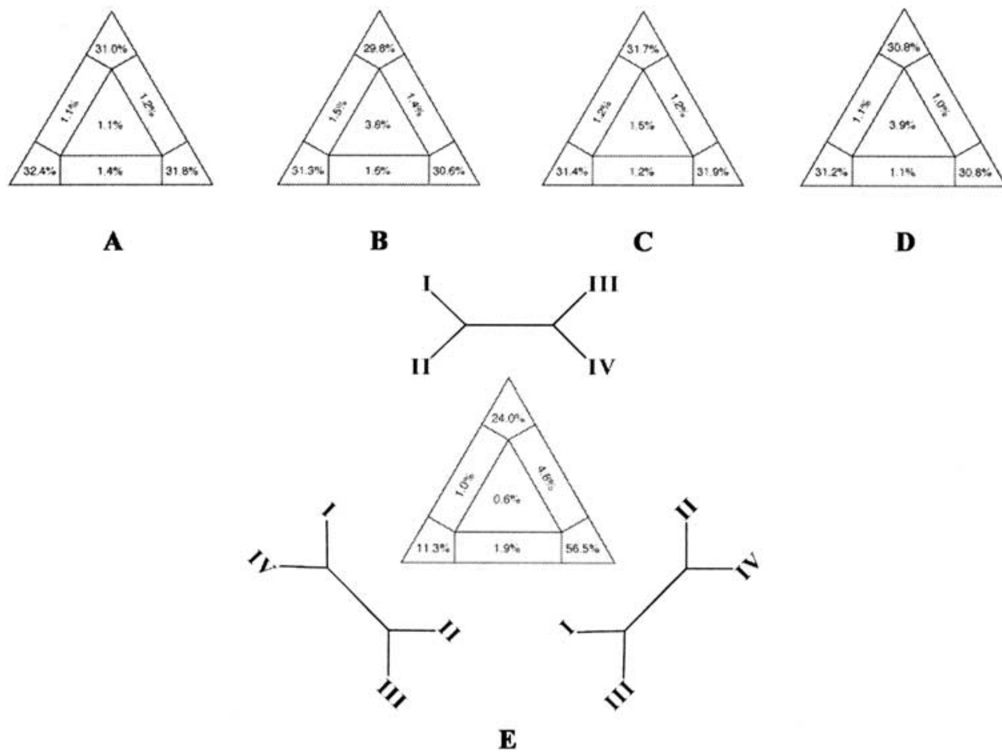


Fig. 2. Results of the likelihood mapping analysis (Strimmer and von Haeseler 1997) of (**A**) the control region (90 taxa plus outgroup), (**B**) the cytochrome *b* (63 taxa plus outgroup), (**C**) the ND2 (28 taxa plus outgroup), and (**D**) the combined data set (26 taxa plus outgroup) presented as barycentric triangles. Values at the corners indicate the percentage of fully resolved quartet topologies, numbers in the rectangular sections give the percentages of partially resolved topologies, and the value at the center of the triangle represents the percentage of unresolved trees. **E** 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 major clades of the Ectodini. The corners of the triangle are labeled with the corresponding tree topology. I, *Grammatotria*-clade; II, *Callochromis*-clade; III, *Xenotilapia*-clade; IV, *Ophthalmotilapia*-clade.

nition of the constrained taxon groups. Four major clades were identified, which we named according to Sturmbauer and Meyer (1993) the *Grammatotria*-, the *Callochromis*-, the *Asprotilapia*-, and the *Ophthalmotilapia*-clade (Fig. 3). The names of the clades are according to Sturmbauer and Meyer (1993), except for their *Asprotilapia*-clade, which we decided to name *Xenotilapia*-clade, because this genus is by far the most species-rich of the clade. The *Grammatotria*-clade (I in Fig. 3) represented the most ancestral branch, consisting of the monotypic genus *Grammatotria lemairii*. Of the three remaining clades, the *Callochromis*-clade (II in Fig. 3), containing the four species of the genus *Callochromis*, branched as the most ancestral split. Within this genus, *C. macrops* and *C. melanostigma* form sister taxa, so as *C. stappersii* and *C. pleurospilus*. The *Xenotilapia*-clade (III in Fig. 3) includes the genera *Xenotilapia*, *Microdontochromis*, *Asprotilapia*, and *Enantiopus*. Their branching order conflicted with respect to the phylogenetic algorithm used and was supported by relatively low quartet puzzling and bootstrap values, indicating a radiation immediately after the origin of this clade. Moreover, the genera *Xenotilapia* and *Microdontochromis* could not be resolved as a monophyletic assemblage. *Xenotilapia caudafasciata* and *X. longispinis* represented the most ancestral split and were grouped as sister taxa. The next ancestral branches were occupied by *Microdontochromis tenuidentata* and *X. spiloptera* forming a paraphyletic assemblage. The next ancestral clade comprised three subclades: the first was comprised of *X. ochrogenys*, the second had *X. papilio* "sunflower," *X. bathyphila*, and *X. boulengeri*. The third subclade contained *X. sima*, *Asprotilapia leptura*, *Microdontochromis rotundiventralis*, *Enantiopus melanogenys* and *X. flavipinnis*. Within the *Ophthalmotilapia*-clade (IV in Fig. 3) two distinct subclades were consistently found: the first consisted of *Ectodus descampsii* and *Ectodus* cf. *descampsii* "north," representing the most ancestral branch, followed by a branch containing *Lestradea stappersii*, *L. perspicax*, *Cunningtonia longiventralis*, and *Aulonocranus dewindti*. The second subclade comprised the genera *Cardiopharynx*, *Cyathopharynx*, and *Ophthalmotilapia*. *Cyathopharynx furcifer* was recovered as the sister group to the sister species pair *O. ventralis* and *O. heterodonta*, whereas *Cardiopharynx schoutedeni* formed the sister group of *O. boops* and *O. nasuta*. Thus, our data do not support the monophyly of the genus *Ophthalmotilapia*.

Cyathopharynx furcifer (65)
Cyathopharynx furcifer (68)
Cyathopharynx furcifer (69)
Cyathopharynx furcifer (63)
Cyathopharynx furcifer (67)
Cyathopharynx furcifer (62)
Cyathopharynx furcifer (66)
Cyathopharynx furcifer (61)
Cyathopharynx furcifer (70)
Cyathopharynx furcifer (64)
Ophthalmotilapia ventralis (57)
Ophthalmotilapia ventralis (58)
Ophthalmotilapia ventralis (56)
Ophthalmotilapia ventralis (53)
Ophthalmotilapia ventralis (54)
Ophthalmotilapia ventralis (55)
Ophthalmotilapia heterodonta (59)
Ophthalmotilapia heterodonta (60)
Ophthalmotilapia nasuta (50)
Ophthalmotilapia nasuta (52)
Ophthalmotilapia nasuta (51)
Ophthalmotilapia boops (48)
Ophthalmotilapia boops (49)
Cardiopharynx schoutedeni (45)
Cardiopharynx schoutedeni (46)
Cardiopharynx schoutedeni (47)
Cardiopharynx schoutedeni (44)
Aulonocranus dewindti (90)
Aulonocranus dewindti (91)
Aulonocranus dewindti (92)
Aulonocranus dewindti (88)
Aulonocranus dewindti (86)
Aulonocranus dewindti (87)
Aulonocranus dewindti (85)
Aulonocranus dewindti (89)
Cunningtonia longiventralis (83)
Cunningtonia longiventralis (84)
Lestradea perspicax (82)
Lestradea stappersii (79)
Lestradea stappersii (81)
Lestradea stappersii (80)
Ectodus descampsii (73)
Ectodus descampsii (75)
Ectodus descampsii (74)
Ectodus descampsii (72)
Ectodus descampsii (71)
Ectodus cf. descampsii "north" (76)
Ectodus cf. descampsii "north" (77)
Ectodus cf. descampsii "north" (78)
Xenotilapia flavipinnis (29)
Xenotilapia flavipinnis (30)
Xenotilapia flavipinnis (31)
Xenotilapia flavipinnis (32)
Xenotilapia flavipinnis (33)
Enantiopus melanogenys (36)
Enantiopus melanogenys (37)
Enantiopus melanogenys (38)
Enantiopus melanogenys (39)
Microdontochromis rotundiventralis (43)
Asprotilapia leptura (34)
Xenotilapia sima (27)
Xenotilapia sima (28)
Xenotilapia bathyphila (23)
Xenotilapia boulengeri (21)
Xenotilapia bathyphila (24)
Xenotilapia cf. bathyphila (22)
Xenotilapia papilio "sunflower" (25)
Xenotilapia papilio "sunflower" (26)
Xenotilapia ochrogenys (16)
Xenotilapia spiloptera (17)
Xenotilapia spiloptera (18)
Xenotilapia spiloptera (19)
Microdontochromis tenuidentata (41)
Microdontochromis tenuidentata (42)
Xenotilapia spiloptera (20)
Xenotilapia caudafasciata (12)
Xenotilapia caudafasciata (13)
Xenotilapia longispinis (15)
Callochromis macrops (11)
Callochromis macrops (10)
Callochromis macrops (9)
Callochromis melanostigma (7)
Callochromis melanostigma (8)
Callochromis stappersii (5)
Callochromis stappersii (6)
Callochromis pleurospilus (4)
Grammatotria lemairii (1)
Grammatotria lemairii (2)
Grammatotria lemairii (3)
Limnochromis auritus (93)
Triglachromis otostigma (94)
Cyprichromis leptosoma (96)

IV
III
II
I
OUTGROUP

Bootstrap values: 84, 73, 71, 93, 100, 66, 92, 100, 99, 100, 73, 90, 100, 55, 73, 100, 68, 96, 100, 95, 90, 84, 61, 98, 100, 100, 100, 99, 100, 95, 100, 85, 100, 100

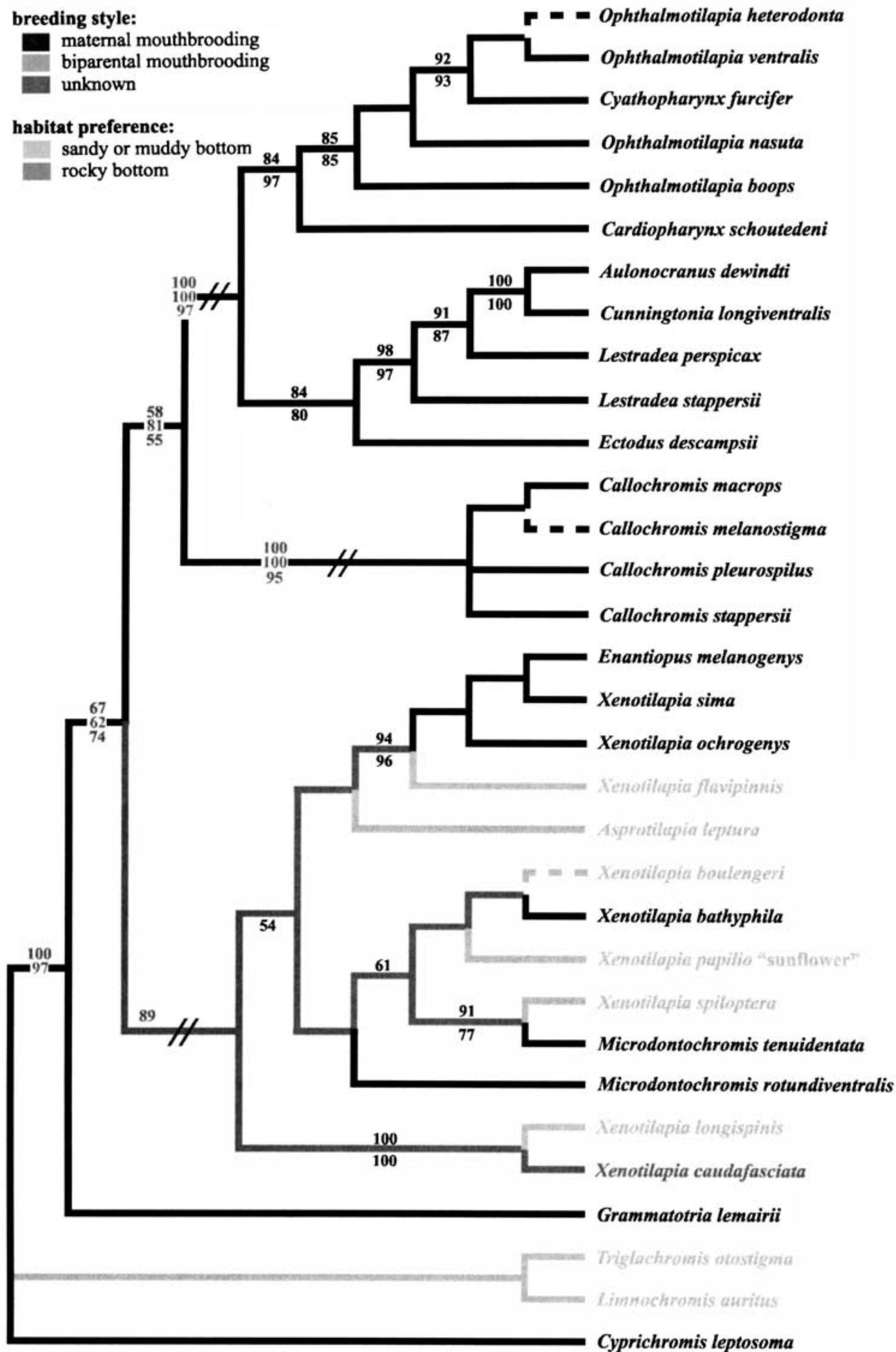
— 0.01 substitutions/site

The corresponding NJ and MP analyses yielded similar and widely congruent topologies (not shown). Only slight differences were found, mainly concerning the branching order within the *Xenotilapia*-clade. Also, *Ectodus* was placed as a third lineage within the *Ophthalmotilapia*-clade (MP) or as ancestral split in relation to the remaining taxa (NJ) of the *Ophthalmotilapia*-clade. However, these branchings were supported by very low bootstrap values. The phylogenetic trees obtained by analyses of ND2 and cyt *b* showed similar results (topologies not shown). Most importantly, the placement of *Grammatotria lemairii* changed with regard to the tree-building algorithm used in the analyses of ND2: in ML it was placed as the most ancestral branch, in NJ it formed the most ancestral split at the base of the *Xenotilapia*-clade, and in MP it was placed within the *Xenotilapia*-clade. Again, the bootstrap support was very weak in each case. In all analyses of ND2 and cyt *b* the topology of the *Xenotilapia*-clade was poorly resolved, and the taxa often changed their relative position. Also, *Cardiopharynx schoutedeni* was ancestral to a subclade containing all *Ophthalmotilapia*-species and *Cyathopharynx furcifer*. All analyses of the cyt *b* data set consistently placed *Grammatotria lemairii* as the most ancestral lineage, followed by the *Xenotilapia*-, the *Callochromis*-, and the *Ophthalmotilapia*-clade. In summary, four clades were consistently found, with the exception of the MP analysis of the ND2. The relative positions of the four clades changed depending on the gene segment(s) analyzed and the algorithm.

Step 2: Branching Order Among Major Lineages. This analysis combined the three gene segments (1,814 bp). The MP analysis resulted in two most parsimonious trees with a length of 4,616 steps (CI excluding uninformative characters, 0.46; RI, 0.63; RC, 0.37). The likelihood parameters for the combined data set of ND2, cyt *b* and control region were: $g_A = 0.2838$, $g_C = 0.3132$, $g_G = 0.1198$, $g_T = 0.2831$, $I = 0.3582$, and $\alpha = 0.5889$. All three phylogenetic algorithms (MP, NJ, ML) resulted in fully congruent branching order among the four clades. The MP phylogeny is shown in Fig. 4. *Grammatotria* was consistently placed as the most ancestral lineage, supported by very high bootstrap or quartet puzzling values. The next ancestral split was formed by the *Xenotilapia*-clade. Its monophyly was strongly supported by high bootstrap and quartet puzzling values. Alternative branching orders were found within the *Xenotilapia*-clade only, indicated by weak bootstrap and quartet puzzling values. As in the first step of analysis the monophyly of the genera *Xenotilapia* and *Microdontochromis* could not be supported. *Microdontochromis tenuidentata* seems to be closely related to *Xenotilapia spiloptera*, whereas *M. rotundiventralis* represents an independent lineage within the radiation of the *Xenotilapia*-clade. *Enantiopus melanogenys* clustered together with *Xenotilapia flavipinnis*, *X. sima*, and *X. ochrogenys*, whereas *Asprotilapia leptura* seems to represent a relatively separate lineage within this subclade. The third major split in the radiation of the Ectodini was the split between the *Callochromis*-clade and the *Ophthalmotilapia*-clade. In the *Ophthalmotilapia*-clade the same subclades were identified as in step 1 of our analysis. In the first subclade the most ancestral split was represented by the genus *Ectodus*. The next ancestral branches were occupied by *Lestradea stappersii*, *Lestradea perspicax*, *Cunningtonia longiventralis*, and *Aulonocranus dewindti*. The genus *Lestradea* formed a paraphyletic assemblage, regardless of the phylogenetic algorithm used. In the second subclade *Cardiopharynx schoutedeni* represented the most ancestral branch. *Ophthalmotilapia ventralis* appeared more closely related to *Cyathopharynx furcifer* than to the other species of the genus *Ophthalmotilapia*, except in the NJ analysis, where *O. nasuta* was the closest relative to *C. furcifer*. As in the first step of our analysis the monophyly of the genus *Ophthalmotilapia* was not supported.

Step 3: Evolution Within Each Lineage. Here, the *Xenotilapia*-clade and the *Ophthalmotilapia*-clade were analyzed separately. These analyses resulted in a widely congruent branching order in the *Ophthalmotilapia*-clade and supported the subdivision into two subclades, with all nodes supported by high bootstrap values ($\geq$80). The separate analysis of the *Xenotilapia*-clade did not yield a more consistent branching order. Again, the species pair *Xenotilapia longispinis* and *X. caudafasciata* could be clearly identified, as well as the species pair *Microdontochromis tenuidentata* and *X. spiloptera*. Also, a monophyletic assemblage consisting of *Xenotilapia ochrogenys*, *X. sima*, *X. flavipinnis*, and *Enantiopus melanogenys* could be identified, supported by a bootstrap value of 94 (outgroup *Grammatotria*), and 96 (outgroup *Callochromis*). The analysis using *Callochromis* as an outgroup yielded two monophyletic

Fig. 3. Constrained Maximum likelihood tree using the substitution model TrN + I + Γ (Tamura and Nei 1993), comprising 90 taxa (30 species) of the Tanganyikan cichlid tribe Ectodini plus three outgroup taxa obtained from analysis of the most variable part of the mitochondrial control region. The tree topology was constrained on the basis of the results of a neighbor joining analysis including all taxa. Nodes supported by NJ bootsrap values >80 were constrained. Bootstrap values obtained in neighbor joining are shown above the branches. Roman numerals indicate the four distinct clades of the Ectodini: I, *Grammatotria*-clade; II, *Callochromis*-clade; III, *Xenotilapia*-clade; IV, *Ophthalmotilapia*-clade. Numbers following the species name correspond to the sample list (Table 1).



lineages within the *Xenotilapia*-clade. One was comprised of the species pair *Xenotilapia longispinis* and *X. caudafasciata* (bootstrap 100), the other contained the remaining species of that clade. The monophyly of this assemblage was supported by a bootstrap value of 54.

Four Cluster Likelihood Mapping

This analysis attempted to further investigate the branching order among the four major clades of the Ectodini. The Four Cluster Likelihood Mapping analysis (Strimmer and von Haeseler 1997) of the

Fig. 4. Composite consensus tree summarizing steps 2 and 3 of our phylogenetic analysis. The tree combines the resulting topologies of two most parsimonious trees, the neighbor-joining tree, and the ML tree of 29 species of the tribe Ectodini based upon analysis of three gene segments (365 bp of the control region, 402 bp of cytochrome *b*, 1,047 bp of ND2), for step 2, and the ML tree for the *Xenotilapia*- and *Ophthalmotilapia*-clade. Bootstrap and quartet puzzling values derived from the overall combined data set (step 2) are shaded in gray. Bootstrap values obtained from neighbor joining are shown above the branches, while numbers in the middle represent parsimony bootstrap values. Quartet puzzling values are shown below the branches. Black shaded numbers refer to bootstrap values obtained from separate maximum likelihood analysis of the *Xenotilapia*- and *Ophthalmotilapia*-clade (step 3). Values above the branches are derived from the analysis using *Grammatotria lemairii* as outgroup. Values below the branches resulted from analysis using the *Callochromis*-clade as outgroup. Only bootstrap and quartet puzzling values >50 are shown. Taxa placed on stippled branches were tentatively placed in the phylogenetic tree based upon analysis of the control region only (*Ophthalmotilapia heterodonta*) or based upon the analysis of a combined data set of cytochrome *b* and the control region (*Callochromis boulengeri* and *Xenotilapia boulengeri*). Roman numerals symbolize the four major clades: I, *Grammatotria*-clade; II, *Callochromis*-clade; III, *Xenotilapia*-clade; IV, *Ophthalmotilapia*-clade. Branch colors symbolize the breeding behavior, according to Kuwamura (1997) and Konings (pers. comm.). Gray bars on the right refer to habitat preference, according to Brichard (1989) and Konings (1998).

combined data set (ND2, cyt *b*, control region) (see Fig. 2e) favored the topology in which the *Callochromis*-clade was the sister group to the *Ophthalmotilapia*-clade. The most highly supported branching order of the four major clades was *Grammatotria* as the most ancestral branch, followed by the *Xenotilapia*-, the *Callochromis*-, and the *Ophthalmotilapia*-clade. Although this topology was supported by only 56.5% of all quartets, this value was much higher than for the other possible topologies (11.3% and 24.0%).

Estimates of Divergence Time Within and Among Major Lineages

The test for constancy of the rate of base substitution among the four major lineages of the Ectodini using the combined data set of a 402 bp segment of the cytochrome *b* and a 1,047 bp segment of the ND2 showed that two taxa (*Xenotilapia caudafasciata*, *X. spilopterus*) fell out of the 99% confidence interval surrounding the average root to tip distance (Table 2). The linearized tree (Fig. 5) suggests two major cladogeneic events. The first represents the primary radiation of the Ectodini, i.e., the split into the *Callochromis*-, *Xenotilapia*-, and *Ophthalmotilapia*-clade, for which we obtained an average TrN + Γ; distance of 13.3% (±1.5%). Interestingly, the *Grammatotria*-clade branched earlier (TrN + Γ distance, 16.4 ± 2.0%). A second cladogeneic event happened within the *Xenotilapia*- and *Ophthalmotilapia*-clade at a divergence level of 8.0–8.8%. In the *Xenotilapia*-clade this event concerns the branching of *Microdontochromis rotundiventralis* (TrN + Γ distance, 8.8 ± 0.8%), the further subdivision into two distinct sublineages (TrN + Γ distance, 8.5 ± 0.9%), as well as the branching of *Asprotilapia leptura* (TrN + Γ distance, 8.2 ± 0.9%). In the *Ophthalmotilapia*-clade the cladogeneic event represents the formation of two subclades (TrN + Γ distance, 8.0 ± 0.7%). As observed for the primary radiation, one lineage of the *Xenotilapia*-clade *Xenotilapia caudafasciata*—branched slightly earlier (TrN + Γ distance, 10.1 ± 0.7%). The branching of the three extant species in the *Callochromis*-clade is younger (TrN + Γ distances, 5.6–5.3%) and happened almost contemporaneously with the speciation of *Xenotilapia papilio* "sunflower" and *X. bathyphila* (TrN + Γ distance, 6.2%) and of *X. flavipinnis*, *X. ochrogenys*, and *X. sima* (TrN + Γ distance, 6.1 ± 0.3%). The members of the *Ophthalmotilapia*-clade are likely to be the youngest species of the Ectodini. All speciation events within the two subclades showed TrN + Γ distances ≤4.8%, except for *Ectodus descampsii*, which branched soon after the formation of the two subclades (TrN + Γ distance 7.1% ± 0.8%).

The use of Jukes–Cantor distances for our much more comprehensive data set resulted in an average JC69-distance between the *Grammatotria*-clade and the three remaining clades of 10.79 ± 0.93%, similar to the maximum value of 10.1% reported by Sturmbauer and Meyer (1993).

Discussion

Evolutionary and Taxonomic Implications

The Ectodini are a member tribe of the H-lineage, containing the majority of the mouthbrooding cichlid lineages of Lake Tanganyika, as well as several hundreds of haplochromine cichlids forming the species flocks of Lakes Malawi and Victoria (Nishida 1991, 1997; Kocher et al. 1995; Salzburger et al. 2002a). Previous morphological (Liem 1981; Greenwood 1983; Poll 1986) and molecular investigations (Sturmbauer and Meyer 1993) suggested the monophyly of the Ectodini. Sturmbauer and Meyer (1993) suggested the Cyprichromini to be the sister lineage of the Ectodini, whereas a ND2 based investigation of Kocher et al. (1995) identified the Perissodini as sister group of the Ectodini. The most recent work on Lake Tanganyika cichlids (Salzburger et al. 2002a) could not unequivocally identify a sister group

Table 2. Branch length test for a combination of cytochrome *b*, ND2, and control region of 26 species of the Ectodini

No.[a]	Species	δ	SE	*Z*
3	*Grammatotria lemairii*	0.0017	0.0079	0.21
4	*Callochromis pleurospilus*	0.0131	0.0056	2.33
5	*Callochromis stappersii*	0.0134	0.0057	2.36
9	*Callochromis macrops*	0.0151	0.0055	2.75
71/72[c]	*Ectodus descampsii*	0.0097	0.0048	2.02
79	*Lestradea stappersii*	0.0020	0.0045	0.44
82	*Lestradea perspicax*	0.0025	0.0044	0.56
89	*Aulonocranus dewindti*	0.0122	0.0050	2.45
84	*Cunningtonia longiventralis*	0.0057	0.0047	1.22
45	*Cardiopharynx schoutedeni*	0.0029	0.0040	0.72
50	*Ophthalmotilapia nasuta*	0.0058	0.0045	1.29
48	*Ophthalmotilapia boops*	0.0016	0.0043	0.38
55	*Ophthalmotilapia ventralis*	0.0022	0.0039	0.56
66	*Cyathopharynx furcifer*	0.0025	0.0042	0.59
19	*Xenotilapia spiloptera*[b]	0.0114	0.0045	2.54
41	*Microdontochromis tenuidentata*	0.0100	0.0044	2.30
15	*Xenotilapia longispinis*	0.0086	0.0046	1.88
12	*Xenotilapia caudafasciata*[b]	0.0131	0.0043	3.07
31	*Xenotilapia flavipinnis*	0.0071	0.0042	1.68
16	*Xenotilapia ochrogenys*	0.0019	0.0050	0.39
34	*Asprotilapia leptura*	0.0090	0.0041	2.17
25/26[c]	*Xenotilapia papilio* "sunflower"	0.0084	0.0041	2.04
23	*Xenotilapia bathyphila*	0.0070	0.0045	1.56
36	*Enantiopus melanogenys*	0.0009	0.0047	0.19
43	*Microdontochromis rotundiventralis*	0.0077	0.0048	1.59
28	*Xenotilapia sima*	0.0047	0.0044	1.05

Note: Only one representative per species was selected. Substitution model TrN + Γ; average root-to-tip distance = 0.0541.
[a] Numbers correspond to the sample list (Table 1).
[b] Taxon shows a significant deviation at the 1% level.
[c] Sequences of two specimens were combined (cyt *b*/ ND2).

(tentative sister group: *Orthochromis malagarazensis*, formerly called *Schwetzochromis melagarazensis*) and suggested that the Ectodini evolved in parallel to the tribes of the H-lineage during the primary lacustrine radiation. Takahashi et al. (2001) suggested the possibility of ancient incomplete lineage sorting on the basis of SINEs among the ancestors of the tribes related to the Ectodini. If this was the case, identification of the sister lineage must be quite difficult since different loci will show different genealogies. A lepidology-based investigation (Lippitsch 1998) identified a monophyletic assemblage consisting of the Limnochromini and the Ectodini. Distinguishing the two tribes was not possible on the basis of scale characters, due to a lack of autapomorphies in each of the tribes. Notably, Salzburger et al. (2002a) suggested that the tribe Limnochromini is not monophyletic and therefore in need of taxonomic revision.

Our analysis identified *Grammatotria* as the most ancestral branch of the Ectodini. This finding is in agreement with the previous published molecular phylogeny of Sturmbauer and Meyer (1993) but contrary to the results obtained by a previous study based upon comparative osteology and myology (Liem 1981) and a very recent investigation using different internal and external morphological characters (Takahashi 2003a). Liem (1981) regarded *Grammatotria lemairii*, which was resolved as the most ancestral branch in the molecular approaches, as the morphologically most derived species of the Ectodini. His suggestion was based upon characters considered specialized when compared with the morphology of *Astatotilapia*, which he treated as generalized and hence ancestral (for a discussion see Sturmbauer and Meyer 1993). Recent molecular studies showed *Xenatilapia* to be a member of the H-lineage and a sister group to the endemic Tanganyikan tribe Tropheini (Nishida 1991, 1997; Salzburger et al. 2002a).

Our phylogenetic analysis of three mitochondrial gene segments confirms the subdivision of the Ectodini into four clades, the *Grammatotria*-clade, the *Xenotilapia*-clade, the *Callochromis*-clade, and the *Ophthalmotilapia*-clade. Due to the much more comprehensive species sample in our present work, especially the large number of *Xenotilapia* species, we are now able to derive a much more fine-scale phylogenetic hypothesis. The most striking new insights concern the *Xenotilapia*-clade, which comprises about 50% of the diversity of the Ectodini. This clade underwent a major radiation immediately after its origin as one of the four distinct clades. The relative instability of the branching order of the *Callochromis*-, *Xenotilapia*-, and *Ophthalmotilapia*-clade, depending on the algorithm used, the low bootstrap values for these branches, and most importantly the inferences drawn from the linearized tree, suggest that the separation of the three clades occurred nearly simultaneously. The observed instability of branching order with respect to the algorithm used suggests that phylogenetic analysis is on its limit of resolution at this section of the phylogenetic tree. This may be due to the short time span of the diversification event resulting in few diagnostic synapomorphies, and sometimes also resulting in ancient incomplete lineage sorting, as recently suggested by Kazuhiko Takahashi et al. (2001) for the radiation of the MVhL clade (H-lineage + Lamprologini). Tetsumi Takahashi (2003a) was able to support the monophyly of the *Xenotilapia*-clade, but in his study the *Ophthalmotilapia*-clade did not form a monophyletic cluster. Furthermore, the branching order obtained by T. Takahashi (2003a) among the different clades is in agreement with the present and previous molecular phylogenies (Sturmbauer and Meyer 1993) when the tree is rooted with *Grammatotria lemairii*. The differences concerning the branching order of the four major clades in the study might be due to the choice of outgroup and the assignment of ancestral states.

Within the *Callochromis*-clade *C. macrops* and *C. melanostigma* clearly form a species pair. Interestingly, we were not able to unambiguously identify *C. pleurospilus* and *C. stappersii* as sister taxa,

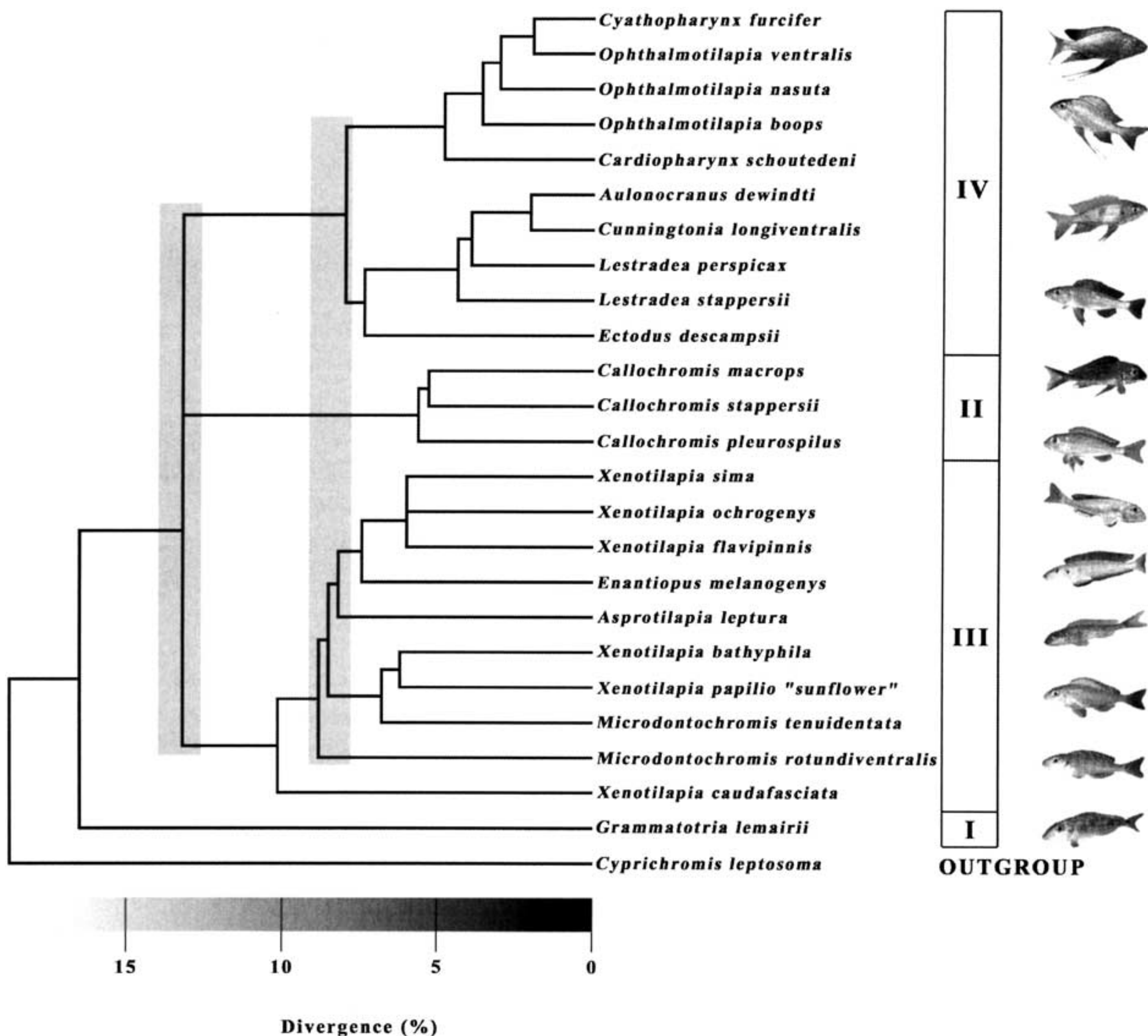


Fig. 5. Linearized tree based on a combination of a 402 bp segment of cytochrome *b* and a 1,047 bp segment of ND2. The linearized tree was compiled with the computer program LINTRE (Takezaki et al. 1995) after performing a branch length test (Takezaki et al. 1995) to test for differences in base substitution rates, using the substitution model TrN + Γ (Tamura and Nei 1993). The scale below the phylogenetic tree corresponds to the observed mean sequence divergence using the substitution model TrN + Γ as appropriate algorithm. Roman numerals refer to the four major clades: I, *Grammatotria*-clade; II, *Callochromis*-clade; III, *Xenotilapia*-clade; IV, *Ophthalmotilapia*-clade. Gray bars refer to major cladogeneic events.

although these two species were synonymized by Takahashi and Nakaya (1998). According to our mitochondrial phylogeny, the taxonomic assignments of species and genera in the *Xenotilapia*-clade are in need of revision. The genera *Xenotilapia*, *Enantiopus*, *Asprotilapia*, and *Microdontochromis* were consistently resolved as para- or polyphyletic. This finding is in agreement with the results of Takahashi (2003a) although the branching order of the species within the *Xenotilapia*-clade differs slightly. The most striking differences concern the phylogenetic position of *Xenotilapia caudafasciata*, the genus *Microdontochromis* and the species pair *X. bathyphila* and *X. boulengeri*. Due to our findings, *Microdontochromis tenuidentata* seems to be a close relative of *X. spiloptera*, whereas *M. rotundiventralis*, which exhibits morphological characteristics intermediate between *Xenotilapia* and *Microdontochromis* (Takahashi et al. 1997), represents an independent lineage within this clade. *Xenotilapia caudafasciata* and *X. longispinis* were consistently grouped as sister species, whereas Takahashi (2003a) placed *X. caudafasciata* as ancestral to a cluster consisting of *Microdontochromis tenuidentata*, *M. rotundiventralis*, and *Asprotilapia leptura*. Moreover, *Xenotilapia sima* and *X. boulengeri*, which were often confused with each other or

even synonymized in the past (Axelrod et al. 1988; Hermann 1990; Konings 1998), never resulted as sister taxa. This finding is in agreement with the study of Takahashi and Nakaya (1997). Furthermore, *X. bathyphila* and *X. boulengeri* consistently formed a closely related assemblage, but never resulted in a clade containing *X. flavipinnis*, *X. ochrogenys*, *X. sima*, and *Enantiopus melanogenys*. The *Ophthalmotilapia*-clade contained two subclades, the first including the genera *Ectodus* as most ancestral branch, followed by *Lestradea*, *Cunningtonia*, and *Aulonocranus*. The second subclade consisted of the genera *Cardiopharynx*, *Cyathopharynx*, and *Ophthalmotilapia*. Interestingly, the genus *Lestradea* appeared paraphyletic in our analysis, as well as the genus *Ophthalmotilapia*. *Ophthalmotilapia ventralis* and *O. heterodonta* formed a cluster together with *Cyathopharynx furcifer*, whereas *O. nasuta* and *O. boops* formed a sister species pair. Due to our results, we suggest the need of taxonomic revision of these taxa.

Evolution of Breeding Behavior

Among the species forming the Lake Tanganyika cichlid flock, many different types of mouthbrooding are displayed. Recent works have shown that mouthbrooding is likely to have evolved several times independently in various African cichlid lineages (Barlow 1991; Goodwin et al. 1998; Klett and Meyer 2002). The same is true for Lake Tanganyika cichlids, since various seeding lineages, such as the Tylochromini, one of the two species of the Tilapiini, the Bathybatini, Trematocarini and Eretmodini perform mouthbrooding (Sturmbauer and Meyer 1993; Goodwin et al. 1998; Salzburger et al. 2002a). Other Tanganyikan lineages evolved to mouthbrooders during the radiation itself. These were shown to constitute the "H-lineage," comprising the Limnochromini, Perissodini, Cyprichromini, Ectodini, Haplochromini, Tropheini, and *Cyphotilapia* (see Nishida 1991 and Salzburger et al. 2002a), which was suggested to have evolved from a nonmouthbrooding *Lamprologus*-like ancestor (Salzburger et al. 2002a). The H-lineage comprises tribes with biparental and maternal mouthbrooding, so that it was suggested that specific modes of mouthbrooding evolved within each lineage during their diversification. The mode of biparental mouthbrooding which is displayed in the tribes Limnochromini and Perissodini is considered ancestral and as an intermediate stage between substrate-breeding and mouthbrooding (Yanagisawa 1985; Salzburger et al. 2002a). They attach their small eggs to a solid substrate and both parents take up the fry in their buccal cavity and continue to guard the fry. The Ectodini are the only tribe of the H-lineage in which both maternal and biparental mouthbrooding is found. We reconstructed the evolution of parental care behavior in the Ectodini by mapping these traits on our phylogenetic hypothesis (Fig. 4). The extant representative of the most ancestral branch of the Ectodini, *Grammatotria lemairii*, exhibits maternal mouthbrooding, as well as all species of the genus *Callochromis* and all members of the *Ophthalmotilapia*-clade. All species displaying biparental mouthbrooding are part of the *Xenotilapia*-clade. They do not attach their eggs to solid subtrate but immediately incubate them orally. These differences suggest that the *Xenotilapia*- type of biparental mouthbrooding has evolved as a specific adaptation from maternal mouthbrooding and is not comparable to the mode displayed in the Limnochromini and Perissodini. All biparentally mouthbrooding species of the Ectodini are monogamous, and all maternal mouthbrooderes are polygynous. The maternal mouthbrooders either display male territory-visiting polygamy or nonterritorial polygamy, as found in *Grammatotria lemairii* (Kuwamura 1997). Reasons for such transitions in the sex representing the caregiver are believed to be mainly influenced by ecological factors such as predation pressure, food abundance (Townshend and Wootton 1985), and the operational sex ratio (Balshine-Earn 1996). According to these hypotheses, biparental care would be selectively favored by high predation risk for the fry, whereas maternal mouthbrooding would be supported by a high probability of remating opportunities for the male due to a female-biased sex ratio (Klett and Meyer 2002).

To test the robustness of our hypothesis for the evolution of the breeding behavior in the Ectodini we traced alternative pathways of character evolution based on the dataset including all the three genes by means of the computer program MacClade (Maddison and Maddison 1992). The resulting tree had a length of 1,706 evolutionary steps. According to this evolutionary hypothesis, biparental mouthbrooding either evolved once in the common ancestor of the clade to be reverted to the ancestral state at least three times, or evolved at least five times independently from a maternally mouthbrooding ancestor (Fig. 4). On the basis of the tree topology it is difficult to decide whether biparental or maternal mouthbrooding is the ancestral state of the *Xenotilapia*-clade because the species-pair forming its most ancestral branch is likely to display both types of mouthbrooding: *Xenotilapia longispinis* is a biparental mouthbrooder (Kuwamura 1997), whereas the breeding style of *Xenotilapia caudafasciata* is not known to date. However, due to the presence of a clear sexual dimorphism in this species it seems more likely to be a maternal mouthbrooder (Konings pers. comm.). If so, it would seem more parsimonious to infer a single transition to biparental mouthbrooding in the ancestor of the *Xenotilapia*-clade and several

reversals to maternal mouthbrooding. *Xenotilapia bathyphila*, *Microdontochromis tenuidentata* and *M. rotundiventralis* belong to a different lineage than the other maternal mouthbrooders, *X. ochrogenys*, *X. sima* and *Enantiopus melanogenys* (Fig. 4). In contrast to previous studies on the breeding behavior of *Microdontochromis tenuidentata* and *M. rotundiventralis* (Kuwamura 1986; Takahashi et al. 1997), recent observations in the wild and in the aquarium indicate that these species are maternal mouthbrooders (Konings, pers. comm.). The score of our phylogenetic hypothesis was better than the topology assuming two monophyletic groups, either displaying biparental or maternal mouthbrooding—at least 1,746 evolutionary steps needed to be inferred. The alternative topology in which a single reversal to maternal mouthbrooding within a clade of biparental mouthbrooders was reinforced, resulted in tree lengths of at least 1,719 evolutionary steps. Taken together, these observations indicate that the transition from maternal to biparental mouthbrooding is much more flexible in the *Xenotilapia*-clade than previously thought and likely to depend predominantly on ecological factors such as predation pressure, food abundance and mate competition.

Colonization of Different Habitat Types

Among the Tanganyikan lineages large ecological and morphological differences are found. These may indicate that major habitat types were rapidly occupied during the early stages of the Tanganyika radiation and that niche differentiation proceeded in step with the origin of the major lineages (Salzburger et al. 2002a). Further speciation events within each of the lineages resulted in a more fine-scale subdivision of these "fundamental niches" but rarely involved switching to other types of habitat. The Ectodini seem to represent an exception to this general observation, such as the major substrate breeding tribe Lamprologini, and some representatives of the Tropheini, whereby several underwent habitat shifts. Based on our results, we postulate that the ancestors of the Ectodini were benthic dwellers, utilizing sandy or muddy substrates. *Grammatotria lemairii*, representing the most ancestral split, is a roamer over sandy substrate. Additionally, all species of the genus *Callochromis*, most species of the *Xenotilapia*-clade, and about half of the members of the *Ophthalmotilapia*-clade live on sandy substrate (Fig. 4). Several species colonized rocky substrates. Interestingly, a habitat switch happened in the *Xenotilapia*-clade as well as in the *Ophathalmotilapia*-clade. In the *Xenotilapia*-clade, *Asprotilapia leptura* and *Xenotilapia papilio* "sunflower" live in rocky habitats, and within the *Ophthalmotilapia*-clade the four species of the genus *Ophthalmotilapia*, *Cyathopharynx furcifer*, and *Cunningtonia longiventralis* switched to rocky bottom. Interestingly, *A. leptura* and *X. papilio* "sunflower" do not form a monophyletic group, and neither do *Ophthalmotilapia*, *C. furcifer*, and *C. longiventralis*. Within the subassemblage of the genera *Ophthalmotilapia*, *Cyathopharynx*, and *Cardiopharynx*, *Cardiopharynx schoutedeni* is the only species living on sandy bottom, whereas in the cluster of *Ectodus*, *Lestradea*, *Cunningtonia*, and *Aulonocranus*, all species except for *Cunningtonia longiventralis* prefer sandy bottom. A switch to the rocky habitat happened relatively recently and independently in the *Xenotilapia*- and *Ophthalmotilapia*-clade, possibly after these types of habitat expanded due to the rise of the lake level.

Age Estimate for Major Cladogeneic events

The rate of nucleotide substitution is almost never the same for all taxa. However, the extent of rate heterogeneity is usually moderate, when sequences of relatively closely related taxa are compared. Thus, it is possible to obtain rough estimates of divergence time between species from molecular sequence data. Nevertheless, it is possible that some taxa show a significantly higher or slower rate of base substitution. These taxa have to be excluded from further analysis on the basis of branch length tests. For the remaining sequences it is possible to construct a linearized tree for a given topology, based on a NJ tree using the appropriate substitution model under the assumption of rate constancy (Takezaki et al. 1995). If the rate of base substitution is known from other sources this tree can be used to estimate the divergence time for any sequence pair.

Molecular clocks of bony fishes have been studied using a variety of taxa, genes, and assumptions (see Martin and Palumbi 1993; Orti et al. 1994; Murphy et al. 1996; Penzo et al. 1998; Zardoya and Doadrio 1999; Baric et al. 2003). Due to the absence of a reliable fossil record, the calibration of a molecular clock for Tanganyikan cichlid fish still remains difficult. Thus, datings of cladogeneic events can only be estimated by calculating average genetic distances, compared with the assumed age of geological events during lake formation. This was recently attempted by obtaining an independent divergence estimate for the most variable section of the control region, which was derived from two ancestral lineages of the Lake Malawi species flock (Sturmbauer et al. 2001). Due to the much older age of the Ectodini in relation to the Lake Malawi species flock (Sturmbauer and Meyer 1993), it is not possible to use this calibration, because the mitochondrial control region is already affected by saturation of transition mutations. We therefore reconstructed a chronicle of the diversification of the Ectodini using a linearized tree analysis

(Fig. 5) based on 402 bp of cytochrome *b* and 1,047 bp of ND2. Our tentative dating rests on the 402 bp section of cytochrome *b* only, since no calibration is available for ND2. The earlier investigation by Sturmbauer and Meyer (1993) based on the same gene and rate estimated that the age of the Ectodini is about 3.7 MY. This analysis used JC69-distances (Jukes and Cantor 1969). Using the same correction algorithm for our data set we obtain roughly the same age (3.42 ± 0.29 MYA). However, our analyses suggest that the TrN+Γ nucleotide substitution model may be more appropriate to our data, but a molecular clock calibration for a combined data set of the two protein-coding genes cytochrome *b* and ND2 is not available at present. Salzburger et al. (2002a) showed in a linearized tree analysis that the radiation of the Ectodini into its major lineages cannot be separated from the primary lacustrine radiation of the H-lineage. Their radiation is thus likely to have proceeded slightly after the fusion of the three proto-lakes into a single lake with deepwater conditions, dated 5 to 6 MYA (Tiercelin and Mondeguer 1991). Within the Ectodini two cladogeneic events become evident from our analysis. The split into the *Xenotilapia*-, *Callochromis*-, and *Ophthalmotilapia*-clade can be tentatively dated to 4.1 to 4.9 MYA, according to the geological age of the onset of deepwater conditions in Lake Tanganyika. Accordingly, the second diversification event may have occurred 2.5 to 3 MYA and concerned the diversification within the *Xenotilapia*- and *Ophthalmotilapia*-clade. These tentative age estimates can be improved as soon as more precise information about the rate of nucleotide substitutions is available for the protein-coding genes of cichlid fish.

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