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Phylogeny and age of chromidotilapiine cichlids (Teleostei: Cichlidae)

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Abstract Chromidotilapiine cichlid fishes (Teleostei: Cichlidae) of West and Central Africa represent the most species rich ancient African cichlid lineage. In contrast to the mega-diverse haplotilapiine cichlids from the African rift valley and crater lakes, very little is known about their phylogenetic history. Based on mitochondrial and nuclear DNA sequences and a representative taxon sampling, we present a first molecular phylogenetic hypothesis and propose age estimates for their origin and diversification. Our data support the monophyly and an Oligocene/Eocene origin

of chromidotilapiines. Within chromidotilapiines, two large, reciprocally monophyletic clades are present and the enigmatic genus *Teleogramma* could be phylogenetically placed for the first time. The two distantly distributed species *Limbochromis robertsi* and *Chromidotilapia schoutedeni* were identified as sister group to the Congolian species complexes of *Nanochromis* and *Congochromis*. This unexpected phylogenetic link between a region in West Africa and the Congo basin suggests an ancient hydrogeographic corridor spanning almost half of the African continent. The nearly complete taxon sampling, good knowledge on species distribution patterns and well resolved phylogenies allow the presumption that paleogeographic patterns rather than ecological factors shaped the ancient divergence within chromidotilapiines, which predates the origin of the mega-diverse austrotilapiine lineage, comprising the majority of African cichlid species.

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Introduction

The majority of cichlid fish diversity (Teleostei: Cichlidae) is represented by the lacustrine species flocks of the East African Great Lakes Tanganyika, Malawi, and Victoria (Kocher, 2004; Seehausen, 2006). Phylogenetic analyses showed that these species flocks represent a single lineage (haplotilapiines: Schlieven & Stiassny, 2003; Schwarzer et al., 2009; Dunz & Schlieven, 2013), whereas the remaining African cichlid diversity can be grouped into five major lineages (chromidotilapiines, pelmatochromines, Tylochromini, hemichromines, and *Heterochromis*). There is overwhelming evidence that African cichlids represent a monophylum (e.g., Sparks & Smith, 2004; Keck & Hulsey, 2014), but neither the interrelationships of the major lineages (except for the sister group position of *Heterochromis* and the remaining African cichlids), nor the intrarelationships of the most speciose and widespread non-haplotilapiine clade, the chromidotilapiines (sensu Greenwood, 1987, Fig. 1 showing their distribution), have been resolved (Stiassny, 1991; Schlieven & Stiassny, 2003; Dunz & Schlieven, 2013; McMahan et al., 2013; Friedman et al., 2013). The term “chromidotilapiines” was coined by Greenwood (1987) for a putatively monophyletic group of cichlids, which was once classified in Regan’s genus *Pelmatochromis*. Thys van den Audenaerde (1968a) presented the first systematic revision of all species of Regan’s *Pelmatochromis* known at that time. He created a novel subgenus for *Pelvicachromis*, i.e., *Pelmatochromis* (*Pelvicachromis*), and suggested to use *Pelmatochromis*, *Chromidotilapia*, and *Nanochromis* as further valid subgenera. He considered his revision as preliminary and recognized that species of the subgenus *Pelmatochromis* form a polyphyletic assemblage including problematic species that might rather belong to *Tilapia* or *Hemichromis* than to *Pelmatochromis*. Several of these problematic taxa were indeed later reassigned to other genera, e.g., *P. ruweti* to *Tilapia* (Thys van den Audenaerde, 1968b) and *P. exsul* to *Hemichromis* (Trewavas, 1973), the subgenus *Pelmatochromis* (*Pelmatochromis*) was transferred to *Tilapia* as a subgenus *Tilapia* (*Pelmatochromis*) (Thys van den Audenaerde, 1968b), and finally resurrected as a full

genus by Trewavas (1973). With resurrecting *Chromidotilapia* to generic rank by Trewavas (1973), the remainder of Thys’ subgenera, *Pelvicachromis* and *Nanochromis*, implicitly gained full generic rank, too. Greenwood (1987) presented the first and only cladistic phylogenetic review of *Pelmatochromis*-related cichlids that were recognized at that time: *Pelmatochromis*, *Pterochromis*, *Thysochromis*, *Chromidotilapia*, *Nanochromis*, and *Pelvicachromis*. His analysis was based mainly on osteological investigations. The most important result of his study was the recognition of “chromidotilapiines” as a monophyletic lineage comprising the already described genera *Chromidotilapia*, *Nanochromis*, *Pelvicachromis*, *Thysochromis* as well as two newly described ones, *Limbochromis* and *Parananochromis*. Due to the lack of informative morphological characters, intrarelationships of chromidotilapiines remained unresolved, although he suggested that *Thysochromis* is the sister taxon to the other chromidotilapiines, and *Parananochromis* and *Nanochromis* could be sister taxa. After investigation of additional material, he indicated a further subdivision of *Nanochromis* and *Pelvicachromis* into two genera. After Greenwood’s study, numerous descriptions of novel species and genera were published (Lamboj, 1999, 2001, 2002, 2003, 2004a, 2005, 2009, 2012, 2013; Lamboj & Snoeks, 2000; Lamboj & Stiassny, 2003; Lamboj & Schelly, 2006), but no phylogenetic studies have been presented that would account for the drastically increased richness of the most speciose group of West-Central African cichlids. Today, chromidotilapiine species richness comprises 10 genera with 48 described and at least 10 undescribed species (Eschmeyer, 2014, AL & UKS, unpubl. data).

Species of the genus *Teleogramma*, endemic to rapids of the lower Congo (three species) and the Kasai drainage (one species), a large southern affluent of the Congo, were never considered to belong to one of the six major African cichlid lineages (Stiassny, 1991; Takahashi & Nakaya, 2002). In fact, *Teleogramma gracile*, the type species of the genus, was originally placed into the marine family Labridae (Boulenger, 1899). Myers (1939) tentatively placed it into the Cichlidae by referring to the presence of only a single pair of nostrils in *Teleogramma*. Takahashi & Nakaya (2002) corroborated the placement of *Teleogramma* within the Cichlidae on the basis of numerous osteological, myological, and additional soft anatomical characters. Lippitsch (1995) suggested that *Teleogramma* forms a sister group to the

Distribution of chromidotilapiines

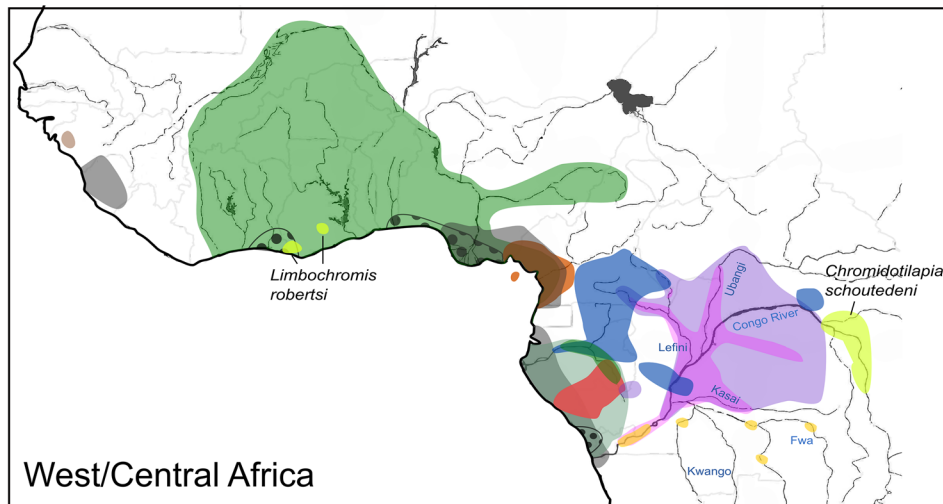
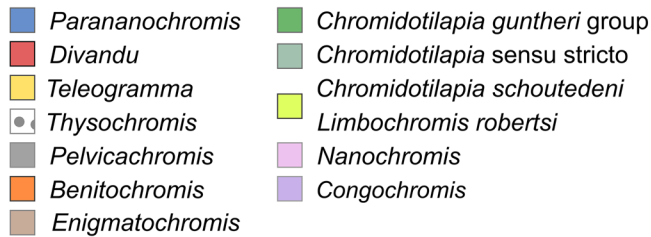


Fig. 1 Distribution of chromidotilapiine lineages. The distribution center of chromidotilapiines in West/Central Africa is shown enlarged. The dark blue area indicates the proposed distribution of *Parananochromis* as based on recently collected

specimens in the Congo area (Vreven, Snoeks & Stiassny, pers. comm). Distribution ranges of *Nanochromis* and *Congochromis* species largely overlap, but no *Congochromis* species are known from the lower Congo

hemichromines based on scale characteristics. Stiassny (1997) proposed that the genus either belongs to lamprologines (Lamprologini following Poll, 1986) or forms a sister group to them, based on the sharing of (1) a variable number of large canines on the anterior part of both premaxilla and dentary, (2) four or more anal fin spines, and (3) an abrupt scale size change above the upper lateral line, and (4) a naked cheek. Takahashi & Nakaya (2002) revised this and provided evidence that the genus belongs to the Pseudocrenilabrinae sensu Kullander, 1998, i.e., all African cichlids except *Heterochromis*. *Teleogramma* shares with this clade two apomorphic characters, first the loss of the posterior supraneural, and second the presence of the opercular spot. Both Stiassny (1997) and Takahashi & Nakaya (2002) concluded that refined morphological and/or molecular-based phylogenies are required for resolving the position of *Teleogramma* within the African cichlids. Preliminary molecular phylogenetic results had

previously suggested a close relationship of *Teleogramma* with chromidotilapiines (Schliewen, 2000).

This study, based on multiple nuclear and mitochondrial genes and an extensive taxon sampling, was conceived to: (i) resolve internal phylogenetic relationships within chromidotilapiines, (ii) identify their phylogenetic position within African cichlids and provide age estimates for their emergence, and (iii) resolve the phylogenetic placement of the enigmatic genus *Teleogramma* within the Pseudocrenilabrinae.

Materials and methods

Samples and sequences

Tissue samples (fin clips) of 78 taxa of all currently described chromidotilapiine (except for *Enigmatochromis*) and pelmatochromine genera, *Teleogramma* and

11 African cichlids representing all remaining major pseudocrenilabrine lineages sensu Sparks & Smith (2004) (*Heterochromis*, Tylochromini, hemichromines, and haplotilapiines) were obtained from field collections, donations, and aquarium trade specimens (Table S1). *Heterochromis multidentis* served as out-group with respect to the rest of the African cichlid radiations (Stiassny, 1990; Lippitsch, 1995; Salzburger et al., 2002). Total genomic DNA was isolated from fin clips or muscle tissue using the Qiagen Tissue Extraction Kit (DNeasy Blood & Tissue Kit) following the manufacturer's protocol. The mitochondrial gene NADH dehydrogenase 2 (ND2) and 16S rRNA, three nuclear protein-coding genes (ENCI, Ptr, Sh3px3) and the first intron of the ribosomal protein-coding gene S7 were amplified and sequenced as described in Schwarzer et al. (2009). Alignment of the sequences was conducted using BioEdit using the ClustalW algorithm (Hall, 2011) and MUSCLE v.3.6. (Edgar, 2004). Coding genes were translated into amino acid sequences to check for stop-codons or frame shifts and the single alignments were checked separately for saturation at each codon position. Base frequencies were homogeneous for all markers (χ^2 tests, df 246, all $P > 0.9$). A control for ambiguous alignment positions was conducted using ALISCORE v.0.2 under default settings (Misof & Misof, 2009) and identified positions (randomized alignment sections) were masked.

Phylogenetic reconstruction

Bayesian inference (BI) and maximum likelihood (ML) approaches were used for phylogenetic inferences. The concatenated dataset was partitioned according to coding vs. non-coding and mitochondrial vs. nuclear genes yielding four partitions, i.e., two partitions for mitochondrial genes (rRNA and first and second codon position of ND2) and two for nuclear genes (exons and introns). The third codon position of ND2 was excluded from phylogenetic analyses, as previous tests (e.g., plotting the number of transversions against the number of transitions) showed saturation between chromidotilapiines and basal taxa (data not shown). For each partition, model parameters were estimated separately. For BI, best fitting models of sequence evolution were estimated using the Bayes factor test (Nylander et al., 2004). Bayesian analyses were performed using MrBayes v. 3.1.2 (Huelsenbeck & Ronquist, 2001) with four parallel runs each

with 10^6 generations starting with random trees and sampling of trees every 1,000 generations. To ensure convergence, the first 10^5 generations of each run were treated as burn-in and excluded. The remaining trees from all Bayesian analyses were used to build a 50% majority rule consensus tree. The program RAXML v. 7.0.3 (Stamatakis, 2006) was used for ML analyses. Branch support was evaluated for the best scoring ML tree using non-parametric bootstrapping (BS) consisting of 1,000 pseudo replicates (using RAXML) and Bayesian posterior probabilities (BPP). Mitochondrial and nuclear genes were analyzed separately using the ML approach described above to be able to detect cytonuclear discordances. The leaf stability index (Thorley & Wilkinson, 1999) was calculated using Phyutility v. 2.2 (Smith & Dunn, 2008) based on 1,000 ML bootstrap trees.

Dating and diversification rates

Divergence times for chromidotilapiines were estimated using a Bayesian approach with a relaxed clock implemented in BEAST v. 1.5.3 (Drummond & Rambaut, 2007). The BI tree obtained from the previously described phylogenetic analysis was used as starting tree. The Yule model was selected as tree prior and an uncorrelated log-normal model was used to estimate rate variation along branches. The same priors used in Schwarzer et al. (2009) were applied: an exponential prior (zero offset 5.98 mya, O_1) at the root of all oreochromines (following Carnevale et al., 2003) and a uniform prior (53–84 mya, A_1) at the root of all African cichlids except *Heterochromis* (Azuma et al., 2008). The first calibration point (O_1) was based on the fossil of *Oreochromis lorenzoi*† (Azuma et al., 2008) from the Baid Formation of the Early Miocene (5.98 mya, Krijgsman et al., 1999). This fossil is, in our point of view, one of the few reliable cichlid fossils suitable for calibration, as the type specimens are in a well-preserved state and its phylogenetic placement within the African cichlid phylogeny is less ambiguous than for other cichlid fossils, as the oreochromines are a clearly monophyletic group (Schwarzer et al., 2009). The second calibration point (A_1) was placed at the split between *Tylochromis* and the remaining African cichlids (except *Heterochromis multidentis*) and corresponds to the 95% credible interval (53–84 mya) of age estimates for this node based on non-cichlid teleostean fossils from a study of Azuma

et al. (2008). For a more detailed discussion on the choice of these priors, see Schwarzer et al. (2009). Two recently published age estimates for the emergence of African cichlids (McMahan et al., 2013; Friedman et al., 2013) were set as alternative priors at position A_1 in two additional runs. All analyses were run for 10^7 generations. Convergence and stationarity of runs and an effective sample size (ESS) of all priors above 200 for each run was ensured using Tracer v. 1.4 (Rambaut & Drummond, 2007).

Results

Due to alignment ambiguities within the S7 intron (16 positions) and saturation in the third codon position of the mitochondrial ND2 locus, 347 positions were excluded from further analyses. The final alignment had 3776 bp. The Bayes factor test identified the HKY model as best fitting model for all partitions except for nuclear exons (ENC1, Ptr, and Sh3px3) and 16S, which were assigned to GTR + Γ . The final dataset had 739 variable sites and empirical base frequencies of $A = 26$, $C = 26$, $G = 23$, $T = 25$. The nuclear (nc-DNA) and mitochondrial (mt-DNA) datasets alone had 359 and 381 variable sites and base frequencies of $A = 25$, $C = 24$, $G = 25$, $T = 26$ and $A = 26$, $C = 31$, $G = 18$, $T = 25$, respectively. Nuclear genes gave a better resolution in the more basal splits, whereas mitochondrial genes provided increased resolution in terminal groups. The leaf stability index representing the stability of single taxa within the concatenated dataset revealed a comparatively high support for all included chromidotilapiines (between 0.93 and 0.95). Highest values were present for all *Paranochromis* and *Divandu albimarginatus* and lowest values for the phylogenetic positioning of *Teleogramma*, *Chromidotilapia schoutedeni* and *C. guntheri*, *Thysochromis ansorgii*, *Nanochromis minor* and all included *Pelvicachromis* species.

Phylogenetic results

Tree reconstructions based on the concatenated dataset yielded good resolution for all major clades and recent relationships within chromidotilapiines (Fig. 2). ML and BI analyses revealed congruent topologies for all major clades. Both approaches corroborated the

monophyly of the chromidotilapiines including *Teleogramma*, whereas a sister group relationship of this group with the haplotilapiines within the African cichlids gained moderate BS and BPP values (70/0.97, Fig. 2). Within the chromidotilapiines two reciprocally monophyletic clades were present. One was composed of *Divandu albimarginatus* and *Paranochromis* and three Lower Guinea members of the genus *Chromidotilapia* (99/1.0), the second of *Teleogramma*, *Thysochromis*, *Pelvicachromis*, three mainly western African *Chromidotilapia* as well as *Benitochromis*, *Limbochromis robertsi*, *Chromidotilapia schoutedeni* and the two mainly Congolian genera *Nanochromis* and *Congochromis* (93/1.0, Fig. 2). Within the first clade the following topology was highly supported: *Divandu* and the Lower Guinea *Chromidotilapia* appeared as sister group to a monophyletic clade composed of *Paranochromis* (99/1.0). In the second clade, *Teleogramma* was a sister group (93/1.0) to a strongly supported monophyletic clade of remaining taxa (100/1.0). Sister group relationships among genera within this clade were very weakly supported (all BS/BPP <50/<0.93, Fig. 2), except for a clade composed of the Congolian taxa *Chromidotilapia schoutedeni*, *Congochromis*, *Nanochromis* (100/1.0, Fig. 2), and the West-Central African *Limbochromis robertsi*, *Benitochromis* and three *Chromidotilapia* species. The Congolian genera *Nanochromis* and *Congochromis* species formed monophyletic sister groups (80/1.00, monophyly of subclades each: 100/1.0) with the northeastern Congolian endemic *C. schoutedeni* and the Ghanaian endemic *Limbochromis robertsi* as unresolved sister taxa (100/1.0, Fig. 2).

Cytonuclear discordance

Separate analyses of mtDNA and nuclear genes revealed differences concerning the position of *Teleogramma*, *Thysochromis*, one *Chromidotilapia* clade (*C. guntheri*, *C. linkei*, *C. regani*) *Limbochromis robertsi*, *Congochromis sabinae* “Lui Kotale” and *Nanochromis minor* (Fig. 3). In the mtDNA dataset, *Teleogramma* appeared together with *Thysochromis ansorgii* in an unresolved clade as sister group to all remaining chromidotilapiines, whereas in the nuclear dataset *Teleogramma* formed the sister group to the chromidotilapiine clade 2. Here, *T. ansorgii* clustered

with *Pelvicachromis* and the three West-Central African *Chromidotilapia* (Fig. 3). The monophyly of a clade comprising *Chromidotilapia schoutedeni*, *Limbochromis robertsi*, *Nanochromis*, and *Congochromis* was supported in both datasets with high statistical support (nuclear DNA: BS = 100 and mtDNA: BS = 99). Within the clade, discordant signal was present concerning internal sister group relationships: *C. schoutedeni* appeared as sister group to *Congochromis* and *Nanochromis* species in the mtDNA dataset (BS = 74), but *Limbochromis robertsi* was their closest relative based on the nuclear dataset (BS = 82, Fig. 3). Intra-generic topological differences were also present within *Nanochromis* and *Congochromis* (Fig. 3).

Node age estimates

The median age of the split between haplotilapiines and chromidotilapiines dates to estimated 30.1/36.9/40.3 mya [depending on the prior placed at the root of the African cichlids; 38.2–51.6 mya (Friedman et al., 2013)/48–72 mya (McMahan et al., 2013)/53–84 mya (Azuma et al., 2008)] with a total range between 23.9 and 50.5 mya (minimum and maximum of 95% credibility intervals (HPC) of all three dating approaches, node CHRO1, Table 1). The median age for the most recent common ancestor (clade CHRO2 in Fig. 4 and Table 1) of chromidotilapiines was estimated at 25.7/31.3/34.2 (20.3–43.6) mya. The two subsequent, well supported, splits (CHRO3, CHRO4) were estimated to have emerged at 24.1/29.4/32.5 (18.8–40.6) mya and 22.7/27.6/30.1 (17.7–38.4) mya. Soon after, several phylogenetically not well resolved lineages (*Thysochromis*, two *Pelvicachromis*-lineages, *Benitochromis*, *Chromidotilapia guntheri*-lineage, and a large clade comprising *Limbochromis*, *Chromidotilapia schoutedeni*, *Nanochromis* and *Congochromis*) split off. Two major internal splits are younger, i.e., the diversification of the Congo Basin clade with *Limbochromis* (LIMB) and without *Limbochromis* (CONG1), were estimated at 15.4/18.4/20.1 (11.4–26.4) mya and 14.3/17.1/18.6 (10.8–24.4) mya, respectively. The age of the most recent common ancestor (MRCA) of *Nanochromis* and *Congochromis* (node CONG2) was estimated at 12.6/15.1/16.5 (9.3–21.9) mya and the age for *Nanochromis* and *Congochromis* at 5.2/6.5/7.1 (3.5–10.3) mya and 10.7/12.6/13.9 (7.9–18.5) mya (nodes NANO and COCH, Table 1, Fig. 4), respectively. The divergence

Fig. 2 Consensus BI tree of the concatenated dataset based on six genes. Consensus tree (50% majority rule) of chromidotilapiines including representatives of the remaining major African cichlid tribes (pelmatochromines, Tylochromini, haplotilapiines, and hemichromines) as well as *Heterochromis multidens* as outgroup. The dataset comprises mitochondrial and nuclear sequences of six independent markers. *Green numbers* at nodes refer to bootstrap-values (BS, 1,000 replicates) of the ML run and *black numbers* to Bayesian posterior probabilities (BPP). *Red circles* represent a 100% BS support and 1.00 BPP and *black circles* 1.00 BPP and lower BS values. The leaf stability index exceeded 0.93 for all specimens

of the Lower Guinea endemic clade (node PARA) into a coastal *Divandu/Chromidotilapia* sensu stricto lineage and the inland *Parananochromis* lineages was estimated to be much older [19.5/23.8/26.2 (13.4–35) mya]. Within this clade, the deep split within *Parananochromis* was dated to 14.8/18.4/20.3 (9.88–27.8) mya and the split between *Divandu* and *Chromidotilapia* sensu stricto was estimated to have emerged at 13.7/17.3/18.7 (9.88–27.8) mya.

Discussion

The chromidotilapiines comprise the largest non-haplotilapiine group of African cichlids. Most species are riverine, except the species from the central Congo basin lakes Mai Ndombe and Tumba, with distribution centers in West Africa, Lower Guinea and the Congo basin (Fig. 1). This study including members of all genera except one (*Enigmatochromis*), corroborated the monophyletic origin of the chromidotilapiines, placed *Teleogramma* firmly within the chromidotilapiine radiation, and provided first evidence for the paraphyly of the genera *Chromidotilapia* and *Pelvicachromis*.

Ancient origin and early allopatric divergence of major chromidotilapiine lineages

Phylogenetic analyses of the concatenated dataset revealed congruent, well resolved splits supporting an ancient (Oligocene/Eocene, i.e., 20.3–43.6 mya, Table 1, Fig. 4) monophyletic origin of chromidotilapiines with largely, but not completely resolved internal relationships (Fig. 2). Based on the ML tree, two reciprocally monophyletic and comparatively old (Oligocene age) clades are present within chromidotilapiines, which started to radiate mostly allopatrically into

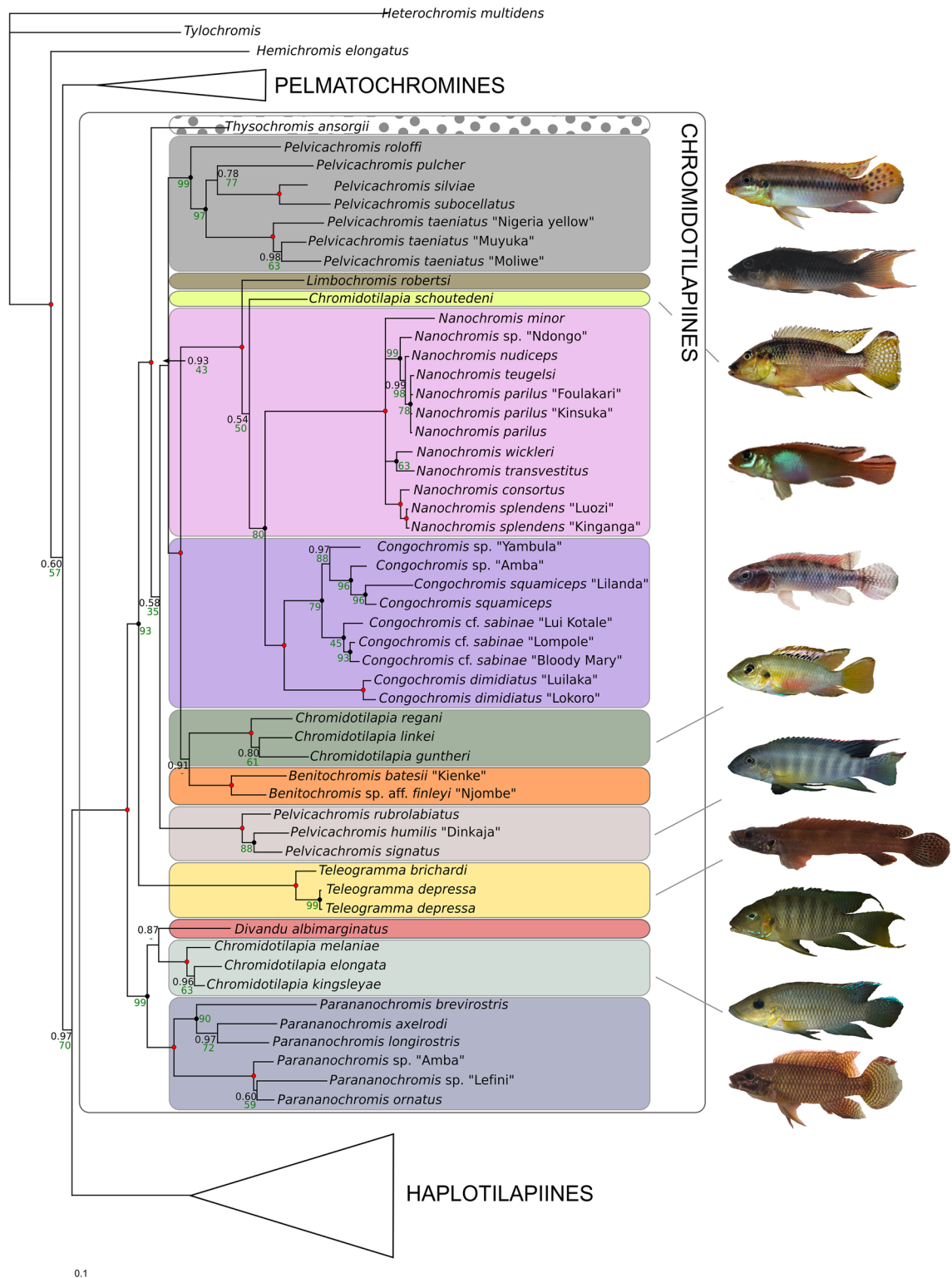




Fig. 3 ML trees generated based on ncDNA-genes (*left*) and mtDNA genes (*right*). Maximum likelihood phylogeny calculated separately based on mitochondrial (1177 bp) or nuclear genes (2602 bp). Numbers at nodes refer to bootstrap-values (BS, 1,000 replicates) and *gray dots* to a BS value of 100. Species names shaded in *gray* indicate differences between the trees

the extant species diversity during the late Oligocene/early Miocene. Members of the first ancient clade (PARA, Fig. 4) are restricted to adjacent regions comprising the Lower Guinea coastal forest and savannah block (*Divandu* and *Chromidotilapia* sensu stricto) and the interior Lower Guinea forest block and disjunct areas of the northern part of the Congo basin (*Parananochromis*); members of the second ancient clade (CHRO3, Fig. 4) overlap in distribution only along a narrow coastal lowland belt in Lower Guinea and are otherwise widely distributed in West and Central Africa, from the Upper Guinea ecoregion to the central and

southern Congo basin (Fig. 1). Included in this large second clade are also two ancient lineages, which had diverged already in the Oligocene/early Miocene (nodes CHRO3 and CHRO4): one endemic to the rapids of the southern Congo affluent drainage of the Kwango and Kasai and to the adjacent lower Congo rapids (*Teleogramma*; CHRO3), and one endemic to the ancient lowland swamps distributed along a narrow coastal region along the western coast of Africa (*Thysochromis*). The revealed largely allopatric pattern strongly suggests that ancient geographic patterns rather than ecology have shaped the ancient divergence within chromidotilapiines. This is most evident for the two species rich, but largely allopatric, clades PARA and CHRO3, both comprising ecologically divergent taxa whose ecology is replicated in both clades, e.g., sand-dwellers (e.g., *Parananochromis longirostris* vs. *Nanochromis* spp.), leaf litter inhabitants (*Parananochromis brevirostris* vs. *Congochromis*) and inhabitants of

Table 1 Age estimates and 95% confidence intervals resulting from different molecular clock approaches

Node	Date estimates in Myr (95% credibility intervals)						
	This study ₁ (53–84 mya)		This study ₂ (48–72 mya)		This study ₃ (38.2–51.6 mya)		Genner et al. (2007)
A ₁	56.4	(53, 68.2)	51.2	(48, 61.9)	41.3	(38.2, 48.8)	(46.6, 79.6)
O ₁	9.1	(6.2, 12.4)	8.4	(6, 11.4)	7.4	(5.9, 9.9)	
PELM	19	(9.8, 31.4)	17.9	(10.2, 28.9)	14.7	(8.1, 22.1)	
PARA1	26.2	(17.9, 35.0)	23.8	(16, 32)	19.5	(13.4, 26.2)	
PARA2	20.3	(13.4, 27.8)	18.4	(9.9, 25.6)	14.8	(7.9, 21.1)	
PARA3	18.7	(10.4, 27.8)	17.3	(12.3, 25.9)	13.8	(9.9, 21.1)	
CHRO1	40.3	(31.2, 50.5)	36.9	(28.8, 46.2)	30.1	(23.9, 36.8)	
CHRO2	34.2	(25.9, 43.6)	31.3	(24.3, 39.7)	25.7	(20.3, 31.9)	
CHRO3	32.5	(24.1, 40.6)	29.4	(22.6, 37.2)	24.1	(18.8, 29.8)	
CHRO4	30.1	(22.7, 38.4)	27.6	(20.8, 34.8)	22.7	(17.7, 28.4)	
CHRO5	28.6	(21.4, 36.7)	26.3	(20.2, 33.5)	21.7	(16.7, 27.0)	
PELV	22.8	(16.2, 29.5)	20.8	(14.6, 27.3)	17.4	(12.8, 22.4)	
HUMI	9.4	(5, 14.8)	8.5	(4.5, 13.7)	7.4	(3.9, 12.1)	
LIMB	20.1	(14.8, 26.4)	18.4	(14.1, 24.2)	15.4	(11.4, 19.5)	
CONG1	18.6	(13.5, 24.4)	17.1	(12.5, 22.1)	14.3	(10.8, 18.3)	
CONG2	16.5	(12.0, 21.9)	15.1	(11.2, 19.7)	12.6	(9.3, 16.2)	
COCH	13.9	(9.8, 18.5)	12.6	(9.2, 16.9)	10.7	(7.9, 14.1)	
NANO	7.1	(4.7, 10.3)	6.5	(4.3, 9.3)	5.2	(3.5, 7.4)	

Single dating points (mean height, shown in bold) and 95% confidence intervals are given for molecular clock estimates based on three different calibration time intervals for node prior A₁. Priors A₁ and O₁ were taken from Schwarzer et al. (2009). Alternative age estimates were calculated based on different time intervals (set as uniform priors at position A₁) from recent publications (McMahan et al. (2013): 48–72 mya and Friedman et al. (2013): 38.2–51.6 mya). Resulting age estimates were compared with published studies when possible (Genner et al., 2007). Age estimates for node A₁ from Genner et al. (2007) correspond to their dataset calculated with Gondwana priors. Names of the nodes correspond to those marked in Fig. 4

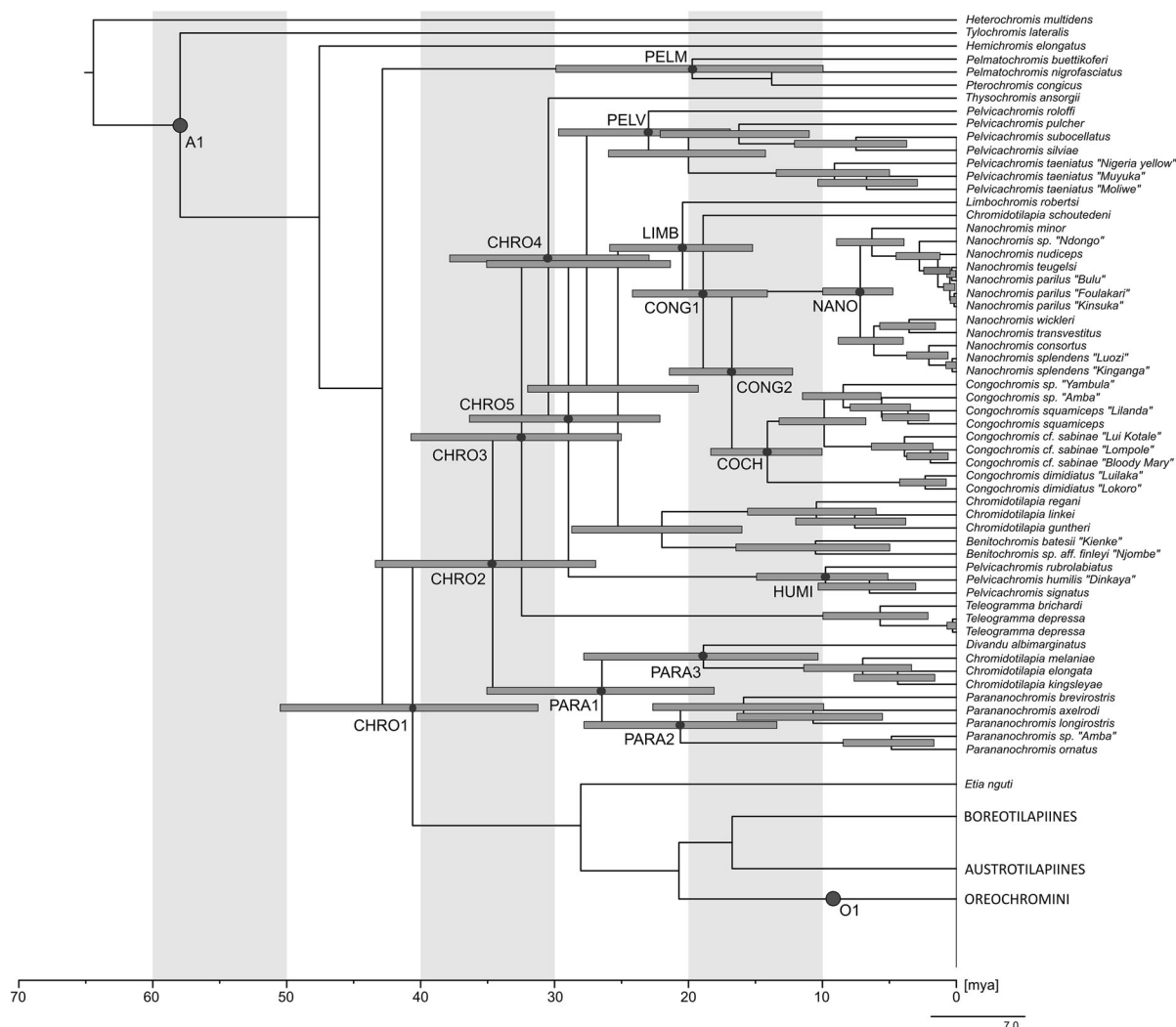


Fig. 4 Chronogram showing age estimates for chromidotilapiines. The chronogram was calculated based on the BI consensus tree. Divergence times were estimated using a partitioned Bayesian analysis implemented in BEAST. Time constraints were used following Schwarzer et al. (2009): A₁ 53–84 mya (uniform prior), published age estimate based on non-cichlid fossils (Azuma et al., 2008) and O₁ 5.98 mya (minimum age),

the age estimate for *Oreochromis lorenzoi* † (Carnevale et al., 2003). The chronogram shows 95% credibility intervals (HPC, gray bars). For labeled 95% credibility intervals marked with black dots age estimates (95% HPC and mean heights) are shown in Table 1. The position of calibration points (O₁ and A₁) in the tree is indicated with dark-gray circles

larger clear rainforest streams with a mixture of rock and sand (*Divandu* and *Chromidotilapia* sensu stricto vs. *Chromidotilapia schoutedeni* and *Chromidotilapia guntheri*-lineage, Lamboj, 2004b). Interestingly, the ancient chromidotilapiine divergence predates the origin and divergence of the mega-diverse austrotilapiine lineage that comprises the majority of all African cichlid species (Schwarzer et al., 2009).

Ancient divergence across the western margin of the northern Congo Basin

Nanochromis and *Congochromis* form, based on our data, a monophylum of two small species assemblages, both with a distribution center in the central Congo basin (Fig. 1). The distribution of the two genera is largely sympatric, but they rarely occur

syntopically, i.e., *Congochromis* lives in small rainforest streams or in the leaf litter of rainforest rivers and lakes, whereas *Nanochromis* lives in large rivers and lake habitats, including the lower Congo rapids, composed of a mixture of sand and rocks (Schliewen & Stiassny, 2006; Stiassny & Schliewen, 2007; Schliewen, 2009). On the basis of the concatenated dataset and the nuclear dataset, the sister group to the *Congochromis*/*Nanochromis* clade is *Chromidotilapia schoutedeni*, distributed in a non-overlapping area in the eastern tributaries of the middle Congo River/Lualaba River (upper central Congo, Fig. 1). In the mtDNA tree, however, *Limbochromis robertsi*, endemic to the upper tributaries of rivers Pra, Ankasa, and Ankobra basins in Ghana (Fig. 1), appears as sister group to *Nanochromis* and *Congochromis*. Both conflicting phylogenetic alternatives are well supported (Fig. 3), indicating ancient reticulate signal or ancient incomplete lineage sorting (Seehausen, 2004). This, however, does not question the presence of a monophylum composed of species distributed in an area spanning ~2,000 km distance from the Congo basin to southwestern Ghana (Fig. 1). Equally surprising is the recent discovery of several, yet undescribed, *Parananochromis* species from the northern Congo basin, which co-occur in some locations with *Congochromis* (*P. sp.* “Amba,” *P. sp.* “Lefini” and *P. sp.* “Lower Itimbiri,” unpubl. own data and pers. comm. E. Vreven and M. Stiassny). Previously, *Parananochromis* was considered to be restricted to the inland portion of northeastern Lower Guinea (Lamboj & Stiassny, 2003; Stiassny et al., 2007). Node age estimates for the basal divergences across the extant western margin of the Congo basin, i.e., the two major *Parananochromis* splits from the Congo basin and Lower Guinea, respectively, as well as the divergence estimate for the Ghanaian *Limbochromis* and the Congolian taxa, indicate that lineages diverged at approximately the same period in the Oligocene/Miocene (nodes PARA1 and LIMB, Fig. 4, Table 1). This period roughly coincides with paleogeographic inferences based on coastal sediment cores, indicating that ancient (Cretaceous) proto-Congo once drained west through the Ogooué valley into the Atlantic, but ceased doing so at approximately the transition of the Eocene to Oligocene (Leturmy et al., 2003). Despite the fact that both the paleogeographic inferences and our molecular clock dates are only rough approximations, this coincidence hints at an ancient biogeographic

connection between the northern Congo basin area, the northern part of Lower Guinea and the Ghanaen-Eburneo region.

Only few other African freshwater fish taxa with a similar distribution pattern have been analyzed with a comparatively good taxon sampling from the northern and western margin of the Congo basin and western Africa: *Synodontis*-catfishes (Siluriformes: Mochokidae) and *Epiplatys*-killifishes (Cyprinodontiformes: Nothobranchiidae).

According to a recent time-tree analysis of Day et al. (2013), *Synodontis* diverged into two major clades roughly at the same time as the chromidotilapiines. Within one of these two major clades, *Synodontis* species from the Central Congo basin form a subclade, which is sister group to a second subclade mainly composed of West African and Lower Guinea (including ghanaen-eburnean taxa). The split of these two subclades is dated to around 26–31 mya (Fig. 2 in Day et al., 2009), i.e., it is older than the Congo basin chromidotilapiine clade including *Limbochromis* from Ghana (15–26 mya), but roughly as old as the *Parananochromis* divergence across Lower Guinea and the northern Congo basin (13.4–35 mya). Unfortunately, support for some relationships forming the base of this second subclade receive little statistical support (clade A1 of Day et al., 2009), hereby rendering refined phylogeographic inferences impossible.

In a study on *Epiplatys*-killifishes, Collier et al. (2009) revealed an ancient West African/Congo basin connection, i.e., *Epiplatys duboisi* from the northern part of the Central Congo basin deeply nested in a predominantly West African/nilo-sudanic clade (Collier et al., 2009). Based on the large distribution of two predominantly nilo-sudanic savannah species, *E. bifasciatus* and *E. spilargyreus*, the latter with a disjunct population in the Pool Malebo area (Congo Basin), the authors infer the existence of an ancient savannah pathway from West Africa into the northern part of the Congo basin. However, as no molecular clock analysis was performed in this study and as it lacks critical taxa from the Ghanaen-eburnean region and the Niger Delta (e.g., *Epiplatys olbrechtsi*, *E. etzeli*, and *E. longiventralis*) no detailed paleogeographical inferences could be drawn.

In summary, while there is a general support for ancient West African/Congo basin hydrological pathways, additional studies are necessary to resolve their exact timing and location.

Cytonuclear discordances

Cytonuclear discordance implies at least two alternative phylogenetic hypotheses either derived from the mitochondrial or the nuclear dataset with contrasting phylogenetic signal. Reasons for this may be incomplete ancient lineage sorting, base compositional biases in different lineages or ancient hybridization/introgression events (Seehausen, 2004). Our dataset does not allow differentiating between these hypotheses due to a comparatively low number of analyzed loci. However, given the evidence for ancient hybridization in cichlid fishes (Schliewen & Klee, 2004; Koblmüller et al., 2007, 2010; Joyce et al., 2011; Schwarzer et al., 2012a, b; Genner & Turner, 2012), we would like to point out that inconsistent phylogenetic patterns identified here cannot be ascribed a priori to ancient incomplete lineage sorting. One potential candidate to be of hybrid origin, based on our data, is *Divandu albimarginatus*, showing an intermediate present day distribution with respect to its putative hybrid partner lineages *Parananochromis* and *Chromidotilapia* sensu stricto (Fig. 1), and conflicting mtDNA and ncDNA hypotheses, each with high statistical support (Fig. 3).

Taxonomic implications

The phylogenetic analysis presented here yielded several results which are in conflict with the current genus-level taxonomy of the genera *Pelvicachromis* Thys van den Audenaerde, 1968 and *Chromidotilapia* Boulenger, 1898, as well as with the current composition of genera within chromidotilapiine cichlids.

Together with *P. taeniatus*, *P. subocellatus*, *P. silviae* and *P. roloffii*, the type species of *Pelvicachromis pulcher* Boulenger, 1901, forms a monophyletic clade in our analysis (see Fig. 2) which is the sister group to a large clade comprising the genera *Nanochromis*, *Congochromis*, *Limbochromis*, *Chromidotilapia* (in part), and *Benitochromis*. The three other species, i.e., *P. signatus*, *P. humilis*, and *P. rubrolabiatus*, are placed outside of this clade and hereby render *Pelvicachromis* paraphyletic. These three species are restricted to a narrow distribution area in the Upper Guinea region and share one morphological and one coloration character distinguishing them from the aforementioned *Pelvicachromis*: (1) two contiguous posterior infraorbital bones vs. lacrymal and three

tubular infraorbital bones with a small gap between tubular infraorbital 2 and 3, and (2) seven or eight bars visible on body in most behavioral situations (Lamboj, 2004a). These three species belong to an undescribed genus, for which no valid genus name is taxonomically available. Since a comprehensive phylogenetic assessment of chromidotilapiine morphological characters is lacking, and since one genus possibly closely related to *Pelvicachromis*, *Enigmatochromis* Lamboj, 2009, could not be included in our molecular analysis, we refrain from explicit taxonomic action, but suggest instead to refer to the group including the type species *P. pulcher* as “*Pelvicachromis* sensu stricto.”

The type species of *Chromidotilapia* is *C. kingsleyae* Boulenger, 1898. It forms together with additional species from Lower Guinea, *C. melaniae* and *C. elongata*, a monophylum which is the sister taxon to *Divandu* Lamboj & Snoeks, 2000. Two additional *Chromidotilapia* clades are identified as being more closely related to other genera: *Chromidotilapia schoutedeni* from the Upper Congo is placed in a clade with the Congolian genera *Congochromis* and *Nanochromis* and the Ghanaian genus *Limbochromis*; the predominantly western African *C. guntheri* is placed with two species from northern Lower Guinea (*C. linkei* and *C. regani*) as the sister group to *Benitochromis*. As several *Chromidotilapia* species are missing in the molecular analysis, of which one (*C. cavalliensis*) was previously placed in a different genus, taxonomic action would be premature. Pending a formal generic revision including a comprehensive phylogenetic assessment of morphological characters, we suggest in the meantime to refer to the group including the type species as “*Chromidotilapia* sensu stricto.” This group is restricted to coastal drainages in Gabon and the Republic of Congo, and includes apart from *C. kingsleyae*, *C. melaniae*, *C. elongata* possibly two other species from the Republic of Congo and Gabon, *C. nana* and *C. mrac* (Lamboj, 2002, 2003). These two other aforementioned species are probably erroneously placed within *Chromidotilapia* and most likely belong to two undescribed genera.

The single phylogenetic assessment of chromidotilapiine morphological characters by Greenwood (1987) had concentrated on type species of the genera described at that time. Therefore, and because additional chromidotilapiine species and genera were described later, an up-to-date phylogenetic analysis of morphological characters together with a sufficient

taxon sampling and with the inclusion of *Teleogramma* is overdue. Our analysis provides a basis for such a study, which should include also a reassessment of characters currently used for generic distinction and the morphological diagnosis of chromidotilapiines.

Conclusions

The chromidotilapiines are the most diverse group of West/Central African cichlids. With this study, we largely resolve internal relationships and place for the first time the Congolian genera *Nanochromis* and the rheophilic *Teleogramma* in a larger phylogenetic framework. Age estimates of the widely distributed *Parananochromis* species support a proposed ancient connection of northern Congo tributaries with the Ogooué. With the present dataset, we elucidate phylogenetic relationships in an old African cichlid tribe (late Eocene/early Oligocene), which significantly contributes to the understanding of the evolution of African cichlids and the emergence of their spectacular diversity.

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