Age of Cichlids: New Dates for Ancient Lake Fish Radiations

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Timing divergence events allow us to infer the conditions under which biodiversity has evolved and gain important insights into the mechanisms driving evolution. Cichlid fishes are a model system for studying speciation and adaptive radiation, yet, we have lacked reliable timescales for their evolution. Phylogenetic reconstructions are consistent with cichlid origins prior to Gondwanan landmass fragmentation 121–165 MYA, considerably earlier than the first known fossil cichlids (Eocene). We examined the timing of cichlid evolution using a relaxed molecular clock calibrated with geological estimates for the ages of 1) Gondwanan fragmentation and 2) cichlid fossils. Timescales of cichlid evolution derived from fossil-dated phylogenies of other bony fishes most closely matched those suggested by Gondwanan breakup calibrations, suggesting the Eocene origins and marine dispersal implied by the cichlid fossil record may be due to its incompleteness. Using Gondwanan calibrations, we found accumulation of genetic diversity within the radiating lineages of the African Lakes Malawi, Victoria and Barombi Mbo, and Palaeolake Makgadikgadi began around or after the time of lake basin formation. These calibrations also suggest Lake Tanganyika was colonized independently by the major radiating cichlid tribes that then began to accumulate genetic diversity thereafter. These results contrast with the widely accepted theory that diversification into major lineages took place within the Tanganyika basin. Together, this evidence suggests that ancient lake habitats have played a key role in generating and maintaining diversity within radiating lineages and also that lakes may have captured preexisting cichlid diversity from multiple sources from which adaptive radiations have evolved.

Introduction

Cichlid fishes are the only freshwater representatives of the suborder Labroidei and are naturally distributed across Africa, Madagascar, South and Central America, the Middle East, and the Indian subcontinent. Their species richness and diversity of morphology, color, and behavior has made them model organisms for the study of speciation and adaptive evolution (Kocher 2004). However, dates of cichlid diversifications and major geological events associated with present distributions are poorly resolved. Molecular phylogenies have shown that cichlids are monophyletic (Streelman and Karl 1997; Sparks and Smith 2004) and that cichlid faunas of major biogeographical regions split in a chronological order congruent to the breakup of the Gondwanan landmass (Sparks and Smith 2004, 2005). Thus, it has been proposed that cichlids originated during the Late Jurassic or Early Cretaceous and had a Mesozoic Gondwanan distribution that started to fragment as Madagascar–India broke away from South America–Africa 121–165 MYA (Sparks and Smith 2005).

However, the oldest known fossil cichlids are *Mahengechromis* from Eocene Tanzania, dated to ~46 MYA (Murray 2001a), and *Proterocara* from Eocene Argentina (33.9–55.8 MYA) (Malabarba et al. 2006), whereas the earliest fossil labroids have been dated to around 65 MYA (Lundberg 1993). Moreover, few spiny-rayed teleost fish (Acanthomorpha) fossils are known between their first appearances in the Late Cretaceous and the Late Palaeocene/Early Eocene when they became common in the fossil record, exhibiting extensive diversity in morphology (Patterson 1993; Chakrabarty 2004). If the earliest cichlid fossils are approximately coincident with the origin of the family, their present distribution must be explained by intercontinental marine dispersal and not vicariance during the breakup of Gondwana (Vences et al. 2001).

The cichlid fishes of African lakes are particularly well known for their rapid speciation and extensive adaptive radiation. Reliable molecular clock estimates could help estimate the extent to which speciation and adaptive diversification have been dependent on the presence of long-lasting lacustrine habitats, if the onset of radiation occurred at around the time of entering a lacustrine habitat, whether divergence was initiated in surrounding drainages from where lakes were colonized multiple times, or whether recent desiccation events were associated with phases of diversification. Addressing these questions is of fundamental importance for understanding the causes and dynamics of adaptive radiation in this textbook evolutionary system.

Until now, attempts to date African cichlid evolutionary events have been predominantly based on rates of molecular evolution extrapolated from mammals (Meyer et al. 1990), assumptions of monophyletic origins of cichlid radiations within lake catchments of known age (Vences et al. 2001), or assumptions of lineage splitting being synchronized with lake-level changes (Sturmbauer et al. 2001; Verheyen et al. 2003). Although these published date estimates are informative, they do have the potential to be misleading because of inconsistencies in rates of molecular evolution among evolutionary lineages, uncertainties over the timing, extent and biological implications of lake-level changes, and the possibility that lake radiations may have been founded by multiple colonizers. As a consequence, many calibrations may be required. Here, we estimate the timing of divergence among the major cichlid taxa (subfamilies and tribes), employing calibration points first from

Key words: molecular clock, Gondwanan fragmentation, fossil record, adaptive radiation, speciation.

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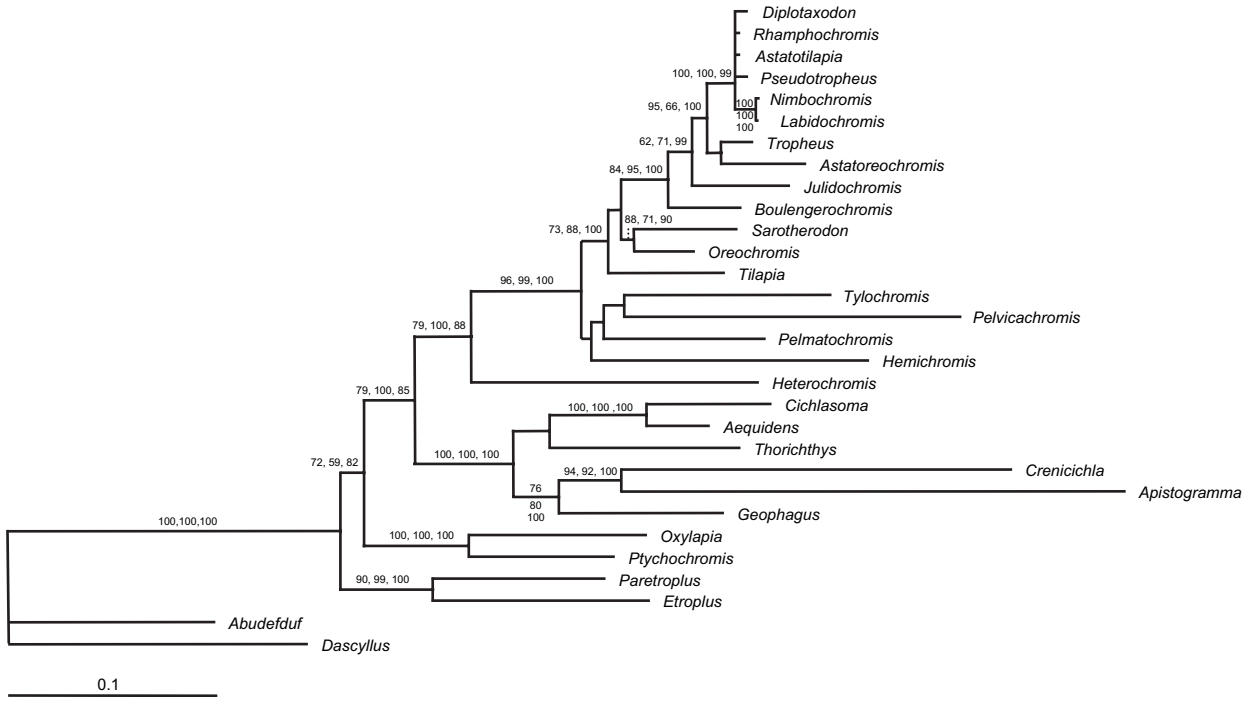


FIG. 1.—ML phylogram of global cichlid divergence. Numbers on branches indicate NJ bootstrap support (1,000 replicates), ML bootstrap support (1,000 replicates), and BI posterior probabilities, respectively. All labeled nodes received branch support >50% for NJ and ML and >70% for BI.

the cichlid fossil record and second from the fragmentation of Gondwana. We then compare these with rates estimated using a range of relatively well-dated non-cichlid fish fossils. Finally, we use the dates derived from the cichlid fossil record and Gondwana fragmentation calibrations to estimate the timing of molecular divergence in radiating African cichlid clades.

Materials and Methods

Dating Global Cichlid Divergence

From published records, the earliest known fossils of extant cichlid taxa were identified, and from published phylogenies, extant sister lineages of fossil taxa were identified. Phylogenetic relationships of these, together with representatives of all major cichlid lineages and 2 outgroups from the related family Pomacentridae, were analyzed using DNA sequences from 2 mitochondrial genes (cytochrome *b* and 16S) and one nuclear gene (TMO-4C4).

Sequences not already available on GenBank were generated for this study. DNA was isolated from ethanol-preserved fin tissue using the CTAB–chloroform method. Primers used were TMO-4C4F and TMO-4C4R (Streelman et al. 2002), 16SAR and 16SBR (Palumbi et al. 1991), and L14724 and H15149 (Kocher et al. 1989). All polymerase chain reactions (PCRs) were performed in 25 μl reactions including 1 μl genomic DNA, 2.5 μl 10× PCR buffer, 2.5 μl dNTPs (1 mM), 1 μl each primer (10 mM stock), 1 μl $MgCl_2$ (25 mM stock), 0.5 units Taq, and 14.9 μl double-distilled water. For all primers, PCR conditions were as follows: 1 min at 95 °C; then 34 cycles of 95 °C for 30 s, 43 °C for 30 s, and 72 °C for 1 min followed by 72 °C for 5 min. Cleaned PCR products were directly sequenced using a Beckman CEQ sequencer and Quickstart cycle sequencing kits (Beckman Coulter, Fullerton, CA) according to manufacturer's protocol. New sequences have GenBank accession numbers EF470866–EF470904 (Supporting Information S1, Supplementary Material online).

Sequences were aligned using ClustalW in Dambe (Xia and Xie 2001). Phylogenetic trees were reconstructed using Neighbor-Joining (NJ) in PAUP*4.0b10 (Swofford 2002), maximum likelihood (ML) in PhyML (Guindon and Gascuel 2003), and Bayesian inference (BI) using MrBayes 3.04 (Huelsenbeck and Ronquist 2001). Prior to analyses, MrModeltest 1.1b (http://www.abc.se/~nylander) was used to determine the best-fitting model of sequence evolution for the concatenated data, and the GTR + Γ + *I* model was selected. NJ was undertaken using ML distances with parameters inferred from MrModeltest. Branch support for ML and NJ was calculated as bootstrap percentages of 1,000 replicates. BI analyses were performed with the GTR + Γ + *I* model using 4 Markov chains and 5,000,000 generations, a burn-in of 10% was discarded.

Branches not supported by >70% in BI and >50% in ML and NJ were collapsed resulting in a conservative dating topology (fig. 1). First, we employed 3 Gondwanan fragmentation events as calibration points. Following Vences et al. (2001), we used the separation of East and West Gondwana 121–165 MYA, the separation of Africa from South America 86–101 MYA, and the separation of Madagascar and India 63–88 MYA. Second, we employed 8 cichlid fossil calibrations (table 1 and fig. 2). Calculations followed a Bayesian "relaxed-clock" approach using PAML (Yang 1997), Multidivtime (Thorne

Table 1
Calibrations Used for Bayesian Dating of the Global Cichlid Phylogeny Using 8 Representatives of Extant Taxa in the Fossil Record

Calibration Node	Taxon	Interpretation	Period	Earliest (MYA)	Latest (MYA)	Location	Reference
C	*Aequidens saltensis*	Earliest Cichlasomatini	Miocene	23.03	5.33	Salta, Argentina	Bardack (1961), Casciotta and Arratia (1993), Murray (2001b)
D	cf. *Crenicichla*	Earliest *Crenicichla*	Miocene	23.03	5.33	Salta, Argentina	Casciotta and Arratia (1993), Murray (2001b)
E	*Protocara argentina*	Earliest "GCC" superclade[a]	Eocene	55.80	33.90	Lumbrera Formation, Salta, Argentina	Malabarba et al. (2006)
F	cf. Geophagini	Earliest Geophaginae	Miocene	23.03	5.33	Salta, Argentina	Casciotta and Arratia (1993), Murray (2001b)
G	*Oreochromis lorenzoi*	Earliest *Oreochromis*	Late Miocene	6.00	6.00	Central Italy	Carnevale et al. (2003)
N	cf. *Tylochromis*	Earliest *Tylochromis*	Late Eocene–Early Oligocene	35.90	33.10	Jebel Qatrani Formation, Fayum, Egypt	Murray (2004)
O	cf. *Heterochromis*	Earliest *Heterochromis*	Oligocene	33.90	23.03	Ad Darb Formation, Saudi Arabia	Lippitsch and Micklich (1998), Murray (2001b)
R	*Mahengechromis* sp.	Earliest Cichlidae[b]	Middle Eocene	46.30	45.70	Mahenge, Singida, Tanzania	Murray (2000, 2001a, 2001b)

NOTE.—Dates were based on reported literature, where only epochs were reported International Commission of Stratigraphy dates were used (http://www.stratigraphy.org).

[a] We use the term Geophaginae-Cichlasomatinae-Chaetobranchine superclade for brevity, see Farias et al. (2000) for a comprehensive phylogenetic reconstruction of Neotropical cichlids.

[b] Murray (2001a) placed *Mahengechromis* most conservatively as sister to Etroplines, but the genus could be sister to Hemichromines (Murray 2001b). Although the position is unresolved they represent the oldest well-dated cichlids so were used to calibrate the basal node.

and Kishino 2002), and the procedures outlined by Rutschmann (2004). Briefly, we used the F84 + Γ model with parameters estimated using BaseML in PAML, Paml2-modelinf to generate the input files for Estbranches program, and Multidivtime to generate estimated dates of lineage divergence using all genes combined. The F84 + Γ distances for each gene showed strong linear correlations with distances from the best-fitting models selected by MrModeltest (Supporting Information S3, Supplementary Material online), indicating the F84 + Γ model was appropriate. The earliest and latest dates of all calibration nodes were treated as hard constraints. In all, 5,000 samples of the chain were taken with 10 cycles between each sampling following a 10,000-cycle burn-in. Expected time between tip and root was 1 (=100 MYA) (standard deviation [SD] = 1), and rate at the root was 0.5 (SD = 0.5). Largest possible time between tip and root was 3 (=300 MYA). Other parameters were as default.

Comparing Rates of Molecular Evolution of Cichlids and Other Bony Fishes

Dating of cichlid divergence events using first Gondwanan fragmentation and second cichlid fossils produced 2 distinct scenarios of cichlid evolution (see Results and Discussion). Thus, we aimed to determine which set of evolutionary rates most closely matched those suggested by 6 independently dated phylogenies of bony fishes. One of these phylogenies was a topology with fossil calibrations (Benton and Donoghue 2007), whereas the remaining 5 were fossil-dated phylogenies based on mitochondrial genomic data (Inoue et al. 2005; Yamanoue et al. 2006; Hurley et al. 2007) or nuclear sequence data (Steinke et al. 2006; Hurley et al. 2007). We incorporated our global cichlid topology (fig. 1) into the published topologies, and aligned cytochrome *b* and 16S mitochondrial DNA sequences from all taxa included in each phylogeny. We then dated cichlid divergence events using the published divergence times of non-cichlids as calibration points and the Bayesian "relaxed-clock" method described above. The priority for the largest possible time between tip and root was set to 5 (=500 MYA) for the data sets of Inoue et al. (2005), Yamanoue et al. (2006), and Hurley et al. (2007). For the Benton and Donoghue (2007) fossil dates, we treated both earliest and latest dates of each calibration node as hard constraints, and for the remainder of the dated phylogenies, we employed published 95% confidence or credibility intervals for each calibration node as hard constraints. Two sets of dates were available for each of the phylogenies of Inoue et al. (2005) and Yamanoue et al. (2006), so here we used each set separately. Finally, we compared rates of evolution between 1) cichlid and non-cichlid teleosts represented in the 6 previously dated phylogenies and 2) cichlids and other labroid families, by compiling published TMO-4C4, cytochrome *b*, and 16S sequences from GenBank and using RRTree (Robinson-Rechavi and Huchon 2000).

Dating African Cichlid Radiations

We compiled mitochondrial DNA sequences selecting widely sampled genes with broad within-radiation taxonomic coverage and including as many taxa in the global topology as possible (Supporting Information S5, Supplementary Material online). Three genes, $NADH_2$, cytochrome *b*, and the control region (D-loop), were concatenated for the Lake Barombi Mbo and Tanganyika radiations, whereas control region alone was employed for the Lake Malawi, serranochromine, and Lake Victoria Region haplochromine radiations (Supporting Information S4, Supplementary Material online). Polymorphisms at

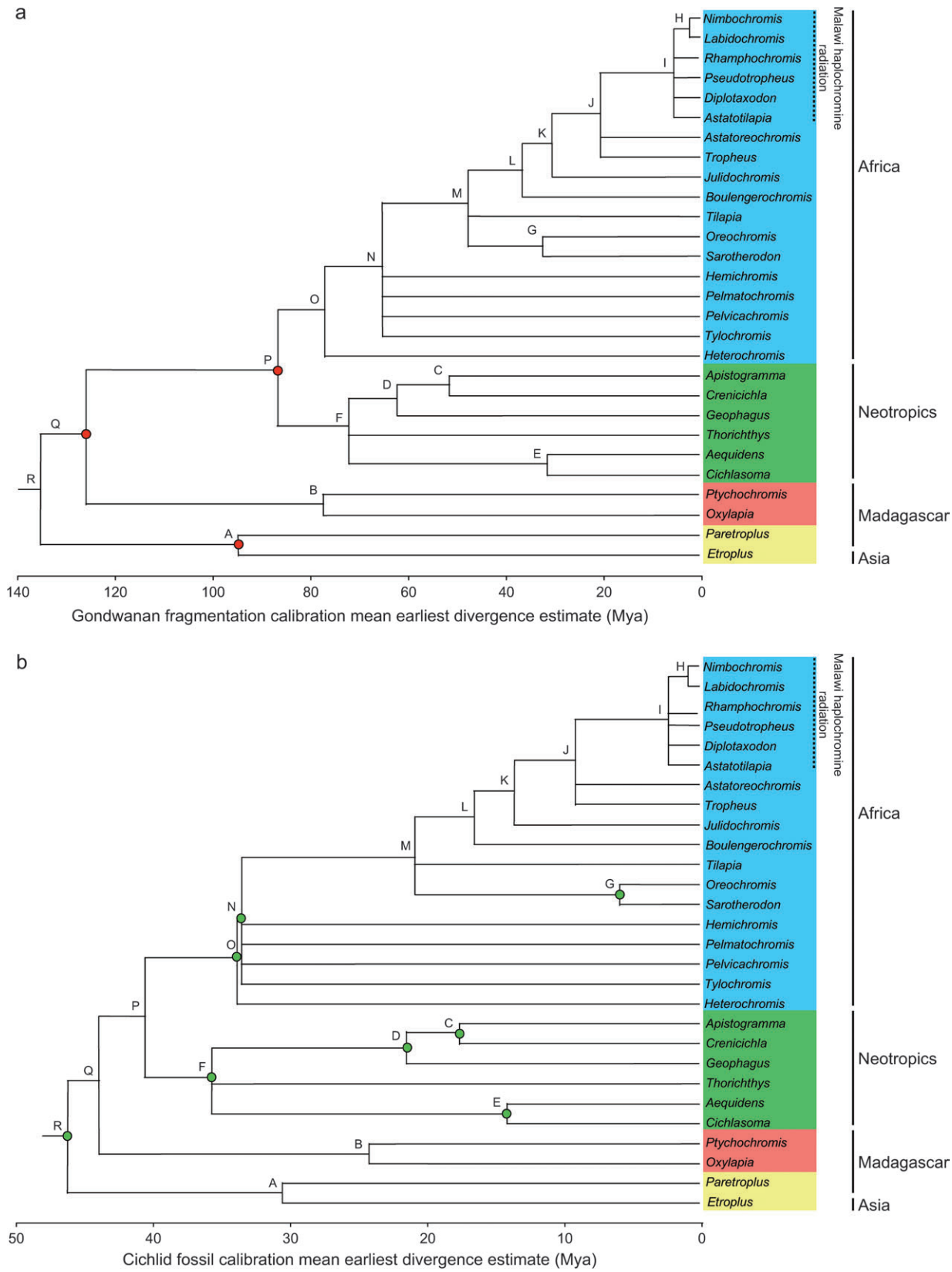


FIG. 2.—Phylogeny of global cichlid lineages with mean node dates based on calibrations from (*a*) Gondwanan fragmentation (red circles) and (*b*) the cichlid fossil record (green circles). Colors indicate subfamily classifications; blue, Pseudocrenilabrinae; green, Cichlinae; red, Ptychochrominae; yellow, Etroplinae. All labeled nodes received branch support >70% for BI and >50% for NJ and ML.

individual nuclear genes tend to either be limited both within and between haplochromine radiations (Sültmann et al. 1995; Mayer et al. 1998; Booton et al. 1999; Won et al. 2006) or lack lineage sorting (Nagl et al. 1998; Terai et al. 2002). Hence, the more rapidly evolving and faster coalescing mitochondrial genes may provide the best available means of dating haplochromine divergence events. We aligned these "African sequence sets" in MUSCLE (Edgar 2004). Phylogenies were constructed using NJ methods in PAUP* using uncorrected *P* distances. Trees were rooted using the most ancient cichlid lineages identified from the global topology (fig. 1). Resultant topologies were highly congruent with published phylogenies.

We then applied a novel dating strategy to each African sequence set independently. First, we calculated mean *P* distances between pairs of taxa in the African sequence set that were also present in the dated global phylogeny. We then identified the nodes that each pair of taxa converged upon in the dated global phylogeny, enabling us to calculate mean *P* distance for these nodes using the African sequence set genes. Time dependency of rates of molecular evolution can influence calculations of divergence times, particularly of more recent divergences (Ho et al. 2005; Ho and Larson 2006). Thus, for the mitochondrial DNA control region, we also employed a series of 10 post-Pliocene calibration points from biogeographic events estimated to have occurred between 5,000 and 50,000 years ago (table 3). We next plotted genetic distances against estimated ages using dates estimated first from cichlid fossils and second from Gondwanan fragmentation (fig. 5). The power curves fitted to these relationships (all $r^2 > 0.95$) enabled us to extrapolate dates from our global phylogeny to all pairwise divergences in the African sets. We then identified taxa converging at nodes in African sequence set topologies and calculated node dates. Species relationships and basal nodes proved difficult to identify within the haplochromine radiations, as a result of extensive incomplete mitochondrial DNA interspecific lineage sorting. In these cases, we examined mean differences among the unique haplotypes within clades (table 6).

Mapping Lake Formation on African Cichlid Phylogeny

We reconstructed a phylogram of African cichlids using a 1,047 bp alignment of 438 mitochondrial $NADH_2$ mitochondrial DNA sequences using PhyML. Branches not supported by >50% bootstrap support were collapsed. Node dates were then placed using combined information from first the global phylogeny and then the ages of the radiations calculated using the African sequence sets. Where dates for nodes were not available, these were estimated using the same power curve extrapolation procedure used for dating the main African sequence sets. For clarity, well-supported monophyletic clades were collapsed at basal nodes. Periods of lake formation were taken from the literature.

Results and Discussion

Global Diversification

Our recovered topology of the deeper level evolutionary relationships of cichlids using 2 mitochondrial genes (16S and cytochrome *b*) and 1 nuclear gene (TMO-4C4) (fig. 1) was congruent with those of prior analyses (Sparks and Smith 2004), except for the relationships among *Hemichromis*, *Tylochromis*, *Pelvicachomis*, and *Pelmatochromis*. Using calibrations based on the fragmentation of Gondwana, we calculated cichlid origins at 133.2 MYA (95% credibility interval: 122.5–151.8 MYA). This was well over twice the age estimate obtained from cichlid fossil calibrations, which indicated African Eocene origins 46.0 MYA (95% credibility interval: 45.7–46.3 MYA) (fig. 2 and table 2).

Because the Gondwanan and cichlid fossil calibrations predict such radically different scenarios for the diversification of cichlids, we have also analyzed 6 independently calibrated data sets. All suggested cichlids originated during the Early Cretaceous and thus showed a much closer agreement with dates calculated using the Gondwanan-calibrated cichlid clock than with those estimated from cichlid fossils (table 2 and fig. 3). In light of this, we conclude there is good reason to reassess both the timescale and nature of the evolutionary divergence process in cichlids. Moreover, relative rate tests yielded no evidence of different rates of sequence evolution in cichlids compared with the teleosts included in the 6 published dated phylogenies or to other labroid families (Supporting Information S2, Supplementary Material online). These results, together with evidence that the Gondwanan landmass fragmented in the same chronological order as cichlid phylogenetic reconstructions, support Early Cretaceous cichlid origins. These results also strongly suggest that reliance on the known cichlid fossil record leads to substantial underestimation of divergence times.

Divergence dates derived from the fragmentation of Gondwana imply a gap in the cichlid fossil record as wide as 90 Myr. Nevertheless, in principle, the fossil record is not inconsistent with Cretaceous cichlid origins. The time disparity may be explained by the incomplete nature of the fossil record. Fossils can only provide hard minimum divergence dates (Heads 2005; Benton and Donoghue 2007), and the cichlid fossils we used as calibrations may represent late first occurrences of the taxon within the palaeontological record. In support of this hypothesis is the substantial decay in numbers of cichlid fossil discoveries with increasing age suggesting a low probability of finding pre-Oligocene cichlid fossils (fig. 4). Importantly, the reliability of cichlid fossil dates also depends upon accurate identification. We employed only fossils attributed to extant lineages as calibrations. It is possible that extinct fossil genera, such as *Palaeofulu* from the Early Miocene of Kenya and *Macfadyena* from the Oligocene of Somalia (Van Couvering 1982), actually belong to extant lineages that may have diverged earlier than would be supposed on the basis of more reliably assigned fossils. The use of non-cichlid bony fishes in dating divergence events has the potential to increase accuracy by providing more well-identified and dated fossils for use as calibration points, plausibly explaining why cichlid divergence dates calibrated using other bony fish fossils show a closer match to those suggested by the Gondwanan vicariance calibrations. Divergence dates estimated from cichlid fossils would be supported by convincing evidence for post-Oligocene marine dispersal, but none has been found (Chakrabarty 2004, 2006; Sparks and Smith

Table 2
Bayesian Estimated Cichlid Divergence Times (MYA) Using TMO-4C4, 16S, and Cytochrome *b* Genes

Node	Gondwana Fragmentation	Cichlid Fossil	Independent Calibration Set A (Benton and Donoghue 2007)	Independent Calibration Set B (Steinke et al. 2006)	Independent Calibration Set Ci (Inoue et al. 2005, Set 1)	Independent Calibration Set Cii (Inoue et al. 2005, Set 2)	Independent Calibration Set Di (Yamanoue et al. 2006, Set 1)	Independent Calibration Set Dii (Yamanoue et al. 2006, Set 2)	Independent Calibration Set E (Hurley et al. 2007, mtDNA)[a]	Independent Calibration Set F (Hurley et al. 2007, Nuclear)[a]	CytB Divergence (Mean *P* Distance)	16S Divergence (Mean *P* Distance)
A	92.9 (86.3, 100.5)	30.3 (19.6, 41.6)	81.5 (55.8, 107.2)	67.3 (38.8, 97.1)	87.6 (54.3, 124.0)	105.1 (64.0, 148.7)	122.2 (77.0, 168.9)	108.6 (68.4, 150.6)	97.3 (63.0, 135.7)	95.0 (57.1, 127.0)	0.1657	0.0693
B	75.6 (48.1, 103.6)	24.0 (13.7, 34.4)	85.2 (59.9, 111.3)	67.1 (37.6, 97.0)	73.8 (42.6, 109.7)	88.2 (51.0, 131.2)	97.8 (54.6, 146.0)	87.2 (47.3, 130.1)	83.7 (50.2, 120.7)	68.9 (31.2, 108.4)	0.1514	0.0794
C	50.2 (26.4, 72.2)	17.3 (8.8, 22.3)	48.4 (29.0, 71.3)	47.9 (13.7, 79.8)	68.7 (34.3, 104.0)	82.8 (40.9, 125.0)	81.7 (34.9, 130.1)	73.4 (28.9, 112.4)	80.1 (48.6, 115.0)	54.4 (14.0, 92.2)	0.2096	0.1524
D	61.0 (40.2, 80.0)	21.3 (16.8, 23.0)	59.1 (38.9, 82.7)	65.9 (39.0, 94.8)	83.3 (53.6, 113.5)	100.1 (63.2, 138.2)	100.7 (61.3, 144.0)	89.9 (52.9, 127.7)	90.9 (59.7, 125.9)	75.0 (41.7, 105.6)	0.1997	0.1239
E	30.3 (14.5, 48.2)	14.2 (7.6, 21.1)	20.9 (6.9, 40.5)	13.4 (1.5, 31.9)	43.3 (21.6, 69.3)	52.9 (25.9, 86.8)	55.4 (24.6, 93.0)	48.6 (20.0, 81.2)	44.9 (21.3, 74.6)	27.0 (4.4, 55.3)	0.1135	0.0159
F	70.6 (52.8, 85.0)	35.5 (33.9, 39.3)	68.1 (47.2, 92.4)	75.0 (47.3, 102.7)	93.1 (66.0, 122.1)	112.1 (76.1, 150.1)	119.0 (83.7, 157.6)	106.1 (73.5, 139.9)	101.8 (71.2, 135.1)	87.0 (57.0, 115.6)	0.1878	0.1067
G	30.9 (16.4, 47.5)	6.0 (6.0, 6.0)	49.8 (28.6, 73.4)	34.3 (15.9, 58.0)	36.0 (18.8, 58.9)	45.0 (23.5, 73.9)	40.8 (20.7, 69.8)	35.9 (18.1, 59.9)	53.4 (29.0, 82.1)	37.7 (18.1, 62.3)	0.1000	0.0214
H	1.6 (0.1, 5.0)	0.7 (0.02, 2.2)	1.8 (0.1, 6.4)	2.3 (0.1, 8.0)	1.0 (0.04, 3.3)	1.2 (0.05, 4.2)	1.1 (0.05, 3.3)	0.91 (0.04, 3.0)	1.5 (0.04, 5.4)	1.8 (0.07, 5.9)	0.0054	0.0000
I	5.0 (1.4, 10.1)	2.2 (0.7, 4.3)	6.6 (1.8, 15.8)	7.5 (1.8, 17.8)	2.7 (0.6, 6.6)	3.6 (0.9, 8.9)	2.7 (0.8, 6.4)	2.4 (0.7, 6.0)	4.7 (1.3, 11.3)	4.3 (1.0, 10.9)	0.0186	0.0069
J	19.5 (10.2, 31.0)	8.9 (5.2, 13.4)	30.8 (15.0, 52.2)	24.5 (9.7, 44.5)	14.2 (6.3, 27.6)	18.4 (7.6, 36.5)	15.3 (6.9, 28.9)	13.6 (6.2, 27.331)	23.4 (10.4, 43.1)	19.2 (7.3, 36.6)	0.0649	0.0263
K	29.5 (17.7, 43.2)	13.5 (8.7, 18.8)	42.1 (22.5, 66.2)	33.6 (16.2, 55.7)	26.6 (13.6, 45.5)	33.6 (16.7, 58.8)	27.3 (13.6, 48.3)	24.3 (11.7, 44.6)	39.6 (20.1, 65.8)	30.6 (14.7, 51.9)	0.0977	0.0305
L	35.6 (22.3, 50.6)	16.3 (11.2, 21.9)	51.5 (31.0, 75.0)	42.2 (22.5, 64.7)	32.9 (18.2, 54.2)	41.6 (22.3, 70.3)	36.7 (19.4, 62.2)	32.7 (17.5, 56.3)	48.2 (26.3, 76.5)	39.0 (20.8, 62.3)	0.0983	0.0341
M	46.4 (31.9, 61.7)	20.6 (15.9, 25.6)	66.2 (44.6, 89.3)	52.8 (31.5, 76.5)	45.8 (27.5, 70.0)	57.1 (33.8, 89.2)	53.0 (31.4, 83.6)	46.9 (27.5, 74.5)	66.6 (41.6, 96.3)	50.6 (29.6, 76.4)	0.1235	0.0361
N	63.7 (46.6, 79.6)	33.4 (33.1, 33.8)	80.6 (57.2, 104.4)	67.5 (42.4, 93.4)	82.7 (57.1, 110.4)	100.0 (66.9, 136.4)	102.5 (68.9, 141.2)	91.1 (62.2, 124.3)	97.8 (68.3, 130.8)	83.9 (56.3, 110.4)	0.1713	0.0817
O	75.7 (60.5, 86.1)	33.6 (33.2, 33.9)	88.3 (64.5, 112.7)	78.3 (51.7, 102.1)	101.3 (73.4, 128.5)	121.2 (87.0, 155.7)	129.2 (90.6, 167.2)	115.6 (81.6, 149.6)	111.0 (79.7, 145.1)	97.8 (71.7, 121.0)	0.2038	0.0831
P	85.1 (77.8, 87.9)	40.5 (35.7, 44.9)	98.4 (75.4, 121.9)	91.0 (66.4, 111.4)	110.7 (82.9, 136.1)	132.5 (97.5, 166.1)	144.8 (110.5, 179.2)	129.6 (98.2, 160.0)	124.3 (92.6, 156.7)	106.4 (81.3, 127.2)	0.1945	0.1162
Q	124.7 (121.1, 134.6)	43.7 (39.5, 46.0)	106.9 (83.8, 129.1)	101.3 (79.0, 117.8)	117.4 (89.3, 142.0)	140.2 (105.4, 172.7)	154.9 (120.9, 189.1)	138.6 (105.8, 169.0)	130.7 (98.8, 162.6)	114.1 (88.9, 132.2)	0.1841	0.1044
R	133.2 (122.5, 151.8)	46.0 (45.7, 46.3)	110.4 (87.3, 132.42)	107.7 (86.3, 121.4)	123.7 (95.4, 147.9)	147.9 (112.8, 178.7)	167.8 (132.7, 200.1)	149.8 (117.0, 178.8)	136.9 (104.6, 168.0)	121.7 (98.8, 136.5)	0.1877	0.1012

NOTE.—Ninety-five percent credibility intervals are presented in parentheses. Nodes correspond with those in figure 1.

[a] We used dates for the halecostome topologies, including *Brachydegma*.

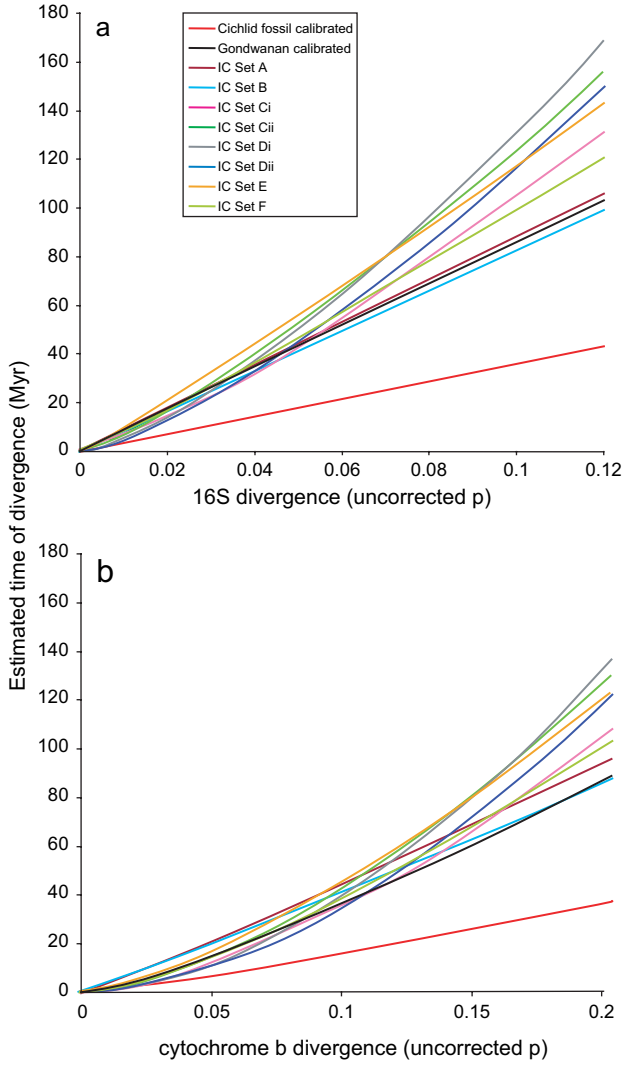


FIG. 3.—Evolutionary rates of mitochondrial genes (*a*) 16S and (*b*) cytochrome *b* in cichlids suggested by calibrations based on cichlid fossils, the fragmentation of Gondwana, and a series of independent calibrations (IC sets, table 2). Curves fitted to data shown in table 2. Only nodes including more than one sequence pair comparison were included.

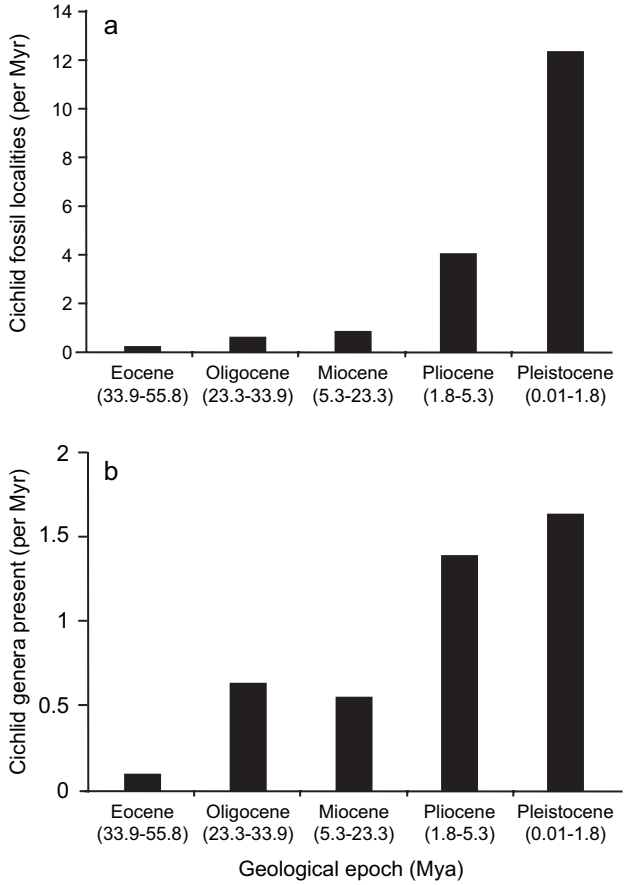


FIG. 4.—Frequency of fossil cichlid discoveries as a function of time within geological epochs; data compiled using sources cited in table 1. Note that many cichlid fossils are only tentatively classified into genera.

2005), despite saline-tolerant, brackish water taxa being known from a number of cichlid lineages, including the Etroplinae, Geophagini, Hemichromini, and Tilapiini. The presence of Etropline cichlids on both Sri Lanka and India provides no evidence of marine dispersal because terrestrial connections were present as recently as 10,000 years ago (Bossuyt et al. 2004).

Accumulation of Genetic Diversity within African Radiations

Lake Tanganyika contains the highest diversity of ancient lacustrine cichlid lineages. The basin is believed to have started to form around 20 MYA, initially as an extensive swampland (Tiercelin and Mondeguer 1991), but attaining deep-lake conditions some 6–12 MYA (Cohen et al. 1993). Calibrations based on cichlid fossils yielded dates of basal nodes (table 4, fig. 6) consistent with suggestions that the cichlids began radiating early during formation of deep-lake conditions into a few surviving taxa now represented as distinct tribes (Salzburger et al. 2002, 2005). By contrast, dates derived from Gondwanan fragmentation indicate that ancestors of every major tribe entered the lake independently and that molecular diversity within some tribes began to accumulate around the time of colonization, or indeed in 2 cases (Trematocarini and Ectodini) possibly before colonization (table 4 and fig. 6). The Gondwanan estimates also suggest that the Haplochromini may have been split into several lineages already prior to the formation of deep-water conditions in Lake Tanganyika and that at least 2 haplochromine lineages colonized the basin independently prior to initial rifting. The first lineage, *Pseudocrenilabrus*, has one species in the catchment. The second lineage contains Tropheini (a group endemic to the lake basin that has radiated into a large number of species/geographic races), *Astatoreochromis* (2 species in the lake) and *Astatotilapia* (2 species in the lake). The latter genus includes *Astatotilapia burtoni*, from a relatively deep East African haplochromine mtDNA lineage (fig. 2), and *Astatotilapia stappersi*, a member of the Lake Victoria Region superflock mtDNA lineage (fig. 2)(Salzburger et al. 2005).

The tilapiine radiation of the crater lake Barombi Mbo comprises 11 known species in 4 genera. Gondwanan

Table 3
Additional Biogeographic Calibrations Used for Assessment of Post-Pliocene Rates of Evolution of the Mitochondrial DNA Control Region

Geological Event	Inferred Biogeographic Consequence	Event Date (Earliest Estimate) Years Ago	*n* Sequence Comparisons Employed[b]	Mean Control Region *P* Distance[c]	SD	Reference
Formation of Lake Apoyo	Separation Lake Apoyo–Lake Nicaragua *Amphilophus*	23,000	30	0.0023	0.0009	Barluenga et al. (2006)
Formation of Lake Lutoto	Isolation of Lake Lutoto from Western Rift haplochromines	50,000	9	0.0062	0.0009	Sato et al. (2003)
Formation of Lake Nshere	Isolation of Lake Nshere from Western Rift haplochromines	50,000	10	0.0024	0.0016	Sato et al. (2003)
Isolation of Lake Nabugabo from Lake Victoria	Isolation of Lake Nabugabo superflock haplochromines	5,000	8	0.0008	0.0007	Stager et al. (2005)
Isolation of Lake Nabugabo from Lake Victoria	Isolation of Lake Nabugabo *Astatoreochromis alluaudi*	5,000	2	0.0000	0.0000	Stager et al. (2005)
Isolation of Lake Kanyaboli from Lake Victoria[a]	Isolation of Lake Kanyaboli superflock haplochromines	5,000	10	0.0019	0.0020	
Isolation of Lake Kanyaboli from Lake Victoria[a]	Isolation of Lake Kanyaboli *Astatoreochromis alluaudi*	5,000	6	0.0010	0.0015	
Isolation of Lake Malimbe from Lake Victoria[a]	Isolation of Lake Malimbe superflock haplochromines	5,000	6	0.0025	0.0024	
Isolation of Lake Kivu from Western Rift lakes	Isolation of Lake Kivu superflock haplochromines	25,000	28	0.0033	0.0019	Snoeks 1994
Formation of southern outflow of Lake Kivu	Colonization of Lake Tanganyika catchment by Kivu haplochromines[d]	14,000	3	0.0047	0.0042	Snoeks 1994

NOTE.—There was a significant association between mean control region *P* distance and earliest age estimate of the event ($r^2 = 0.40$; $F_{1,8} = 5.40$; $P < 0.05$).

[a] Both Lake Kanyaboli and Lake Malimbe are satellite lakes of Lake Victoria, separated by papyrus swamp. They are likely to have become isolated from Lake Victoria during lake-level falls revealed by Lake Nabugabo sediment cores (Stager et al. 2005).

[b] Based on the sampling location with the smallest number of unique haplotypes.

[c] *P* distance between closest haplotypes using each unique haplotype in smallest sample once.

[d] Lake Victoria Region superflock haplochromines present in the Ruzuzi river and Malagarasi river.

fragmentation and cichlid fossil calibrations indicate this radiation started to accumulate molecular diversity 0.47 (±0.19 SD) and 0.19 (±0.09 SD) MYA, respectively. The lake is estimated to be approximately 1 Myr old (Cornen et al. 1992), and so both sets of dates are consistent with the hypothesis of within-catchment diversification (Schliewen and Klee 2004) (table 4 and fig. 6).

The southern Africa serranochromines, the haplochromines of Lake Malawi, and the haplochromines of the Lake Victoria region were dated using sequences from the rapidly evolving mitochondrial control region, including calibration from post-Pliocene divergence events (table 3). Like previous studies (Ho et al. 2005; Ho and Larson 2006), we found that rates of molecular change showed a sharp decline with increasing divergence time until a baseline substitution rate was reached after a divergence time of approximately 1,000,000 years (fig. 5). This pattern has been attributed to purifying selection and/or saturation at mutational "hot spots" along rapidly evolving sequences (Ho et al. 2005; Ho and Larson 2006).

The onset of accumulation of molecular diversity within the Lake Malawi haplochromine radiation, comprising 450–600 species in around 50 genera, was dated to 4.63 (±2.14 SD) and 2.44 (±1.01 SD) MYA using Gondwanan fragmentation and cichlid fossil calibrations, respectively. Both estimates support the hypothesis that the flock radiated within the timescale of the basin history. Rifting that formed Lake Malawi is estimated to have begun 8.6 MYA with deep-water conditions attained by 4.5 MYA (Ebinger et al. 1993; Delvaux 1995). The earliest known fossil freshwater assemblages in the catchment are from the Chiwondo beds laid down in lacustrine conditions ~2.3 MYA (Van Damme and Pickford 2003), but taxonomic identities of cichlids within the deposits are unclear (Murray 2001b).

Of greater controversy is the age of the Lake Victoria region cichlid fishes. Over 500 closely related haplochromine cichlid species comprise the East African "superflock" inhabiting Lakes Victoria, Kyoga, Rukwa, Kivu, Albert, George, Edward, and surrounding water bodies. Diversity between unique haplotypes within the widespread superflock was dated on average to 0.273 (±0.216 SD) and 0.189 (±0.133 SD) MYA using the Gondwanan fragmentation and cichlid fossil calibrations, respectively. Within the region occupied by the superflock, Lake Victoria forms a phylogeographic zone largely distinct in mitochondrial DNA from the neighboring western rift lakes Albert, George, Edward, and Kivu (Verheyen et al. 2003). We dated divergence within the Lake Victoria catchment to 0.120 (±0.110 SD) and 0.089 (±0.074 SD) MYA using the Gondwanan fragmentation and cichlid fossil calibrations, respectively. Both estimates are within the age of the Lake Victoria catchment formation, approximately 400,000 years ago (Johnson et al. 2000).

The extant members of the serranochromine radiation comprise at least 24 species broadly distributed across southern Africa. It has been proposed that much of their species richness and morphological diversity arose in Palaeolake Makgadikgadi (Joyce et al. 2005), a vast lake that formed between 315,000 and 460,000 years ago and dried up within the last few thousand years, leaving the

Table 4
Estimated Dates of Onset of Diversification of Major Lacustrine Clades Using Gondwanan- and Fossil-Derived Calibrations of Mitochondrial DNA Sequence Divergence

Radiation	Clade (mtDNA)	Gondwanan Mean Date (MYA ± SD)	Fossil Mean Date (MYA ± SD)	Estimated Number of Species in Clade
Tanganyika Psuedocrenilabrinae	*Bathybates*	16.791 ± 1.247	7.646 ± 0.581	8
	Benthochromis	0.154	0.064	2
	Cyprichromini	10.215	4.600	10
	Ectodini	23.530 ± 4.144	10.800 ± 1.944	33–41
	Eretmodini	6.812 ± 0.626	3.040 ± 0.285	4
	Tropheini	6.758 ± 1.068	3.016 ± 0.486	19–36
	Lampologini	16.012 ± 2.444	7.286 ± 1.137	79–83
	Limnochromini	10.828 ± 2.000	4.884 ± 0.922	13
	Perissodini	9.240 ± 2.007	4.153 ± 0.921	8
	Trematocarini	27.450	12.637	10
	Haplochromini[a]	22.72 ± 3.498	10.460 ± 1.626	~1,500
Barombi Mbo Tilapiini	Basal	0.473 ± 0.198	0.199 ± 0.085	11
	Konia	0.179 ± 0.014	0.074 ± 0.059	2
	Sarotherodon	0.108 ± 0.050	0.044 ± 0.021	4
	Stomatepia	0.085 ± 0.024	0.034 ± 0.010	3
Malawi Haplochromini	Basal	4.632 ± 2.140	2.435 ± 1.008	~450–600
	Diplotaxodon–Pallidochromis	0.637 ± 0.426[b,c]	0.403 ± 0.252[b,c]	12–23
	Rhamphochromis	0.995 ± 0.654[b,c]	0.606 ± 0.365[b,c]	12–15
	"Mbuna" dominated	0.486 ± 0.476[b,c]	0.313 ± 0.274[b,c]	66–225
	"Malawi Hap" dominated	1.447 ± 0.684[b,c]	0.855 ± 0.369[b,c]	~350
Southern Africa serranochromines	Basal	7.472 ± 2.087	3.194 ± 0.795	24–33
	Within radiating clade "I"	0.425 ± 0.362[b,c]	0.279 ± 0.216[b,c]	15[e]
	Within radiating clade "III"	0.630 ± 0.430[b,c]	0.401 ± 0.249[b,c]	6[e]
East Africa Haplochromini	Lake Victoria Region "superflock"	0.273 ± 0.216[c,d]	0.189 ± 0.133[c,d]	500–1,000
	Lake Victoria catchment only	0.120 ± 0.110[c,d]	0.089 ± 0.074[c,d]	500–800

NOTE.—Post-Pliocene biogeography-derived calibrations were additionally used for the haplochromine radiations. The term "basal" represents the divergence of major mitochondrial DNA component clades.

[a] The hypothesis that Haplochromini originated in Lake Tanganyika (Salzburger et al. 2005) remains speculative. An equally plausible and arguably more conservative interpretation of published evidence is that the Tanganyika Basin has been independently colonized from riverine systems by at least 4 lineages of Haplochromini (Tropheini, *Pseudocrenilabrus*, *Astatoreochromis*, and *Astatotilapia*), with radiation only occurring in Tropheini.

[b] Mean interspecific mitochondrial DNA difference only. Intraspecific differences were excluded to avoid underestimating divergence times.

[c] There is evidence of extensive incomplete lineage sorting within these clades. Thus, mitochondrial DNA divergence is not a measure of the age of the adaptive radiation or species flock perse because speciation rates are faster than lineage sorting and ancestral polymorphisms are likely to have been retained within the flocks. It represents only the age of mitochondrial DNA diversity, thus enabling comparisons with ages of lake basins and determination of whether high preexisting genetic diversity was present in colonizing taxa. Species radiations are likely to be younger than haplotype radiations.

[d] Most samples were not identified to species; includes only differences between unique haplotypes; many haplotypes shared between species.

[e] Clades I and III of the southern African serranochromines refer to those of Joyce et al. (2005). Some species belong to both haplotype clades.

present-day Makgadikgadi salt pans (Moore and Larkin 2001). Mean interspecific mitochondrial divergence within the 2 radiating clades was dated, irrespective of calibration used, to the middle Pleistocene (Gondwana fragmentation means: Clade "I" 0.425 ± 0.36 MYA, Clade "III" 0.630 ± 0.43 MYA, cichlid fossil means Clade I 0.279 ± 0.22 MYA, Clade III 0.401 ± 0.25 MYA), approximately the period when the now-extinct lake was one of Africa's largest water bodies.

Timescale of Speciation and Adaptive Radiation within Lake Tanganyika

Since the pioneering molecular phylogenetic studies of African cichlids, numerous studies have presented estimates of divergence times. Although most studies have only tentatively calculated these dates, a consensus timescale of cladogenesis has emerged (see Kocher 2004; Salzburger and Meyer 2004). The development of this paradigm began with extrapolation of an estimated rate of mitochondrial DNA (cytochrome *b*) evolution from mammals (Meyer et al. 1990). Using this rate, haplochromine radiations of the Lake Victoria region and Lake Malawi were estimated at 200,000 years and 700,000 years, respectively, whereas the divergence of the Haplochromini and Lamprologini was dated to ~4.5 MYA (Meyer et al. 1990). Subsequently, divergence dates have been calculated employing assumptions that the basal radiations of the Lake Tanganyika endemic cichlid tribes occurred between 6 and 12 MYA based on evidence that during this period deep lacustrine conditions began to form in Lake Tanganyika (Sturmbauer et al. 1994; Duftner et al. 2005; Koblmüller et al. 2005; Won et al. 2005, 2006). Additionally, the basal radiation of the Lake Malawi haplochromine flock has commonly been used for calibration with the assumptions that it occurred between 0.7 and 4 MYA (Sturmbauer and Meyer 1993; Nagl et al. 1998, 2000, 2001; Salzburger et al. 2005). Calibrations have also been based on assumptions of divergence events being synchronized with Lake Malawi surface-level changes. Sturmbauer et al. (2001) calculated sequence evolution rates assuming that the typically rock-associated "mbuna" and the often sand-associated "utaka" (=Malawi Hap dominated; table 4) mitochondrial DNA lineages of Lake Malawi haplochromines diverged during a rise in water level 0.59–1 MYA following a desiccation

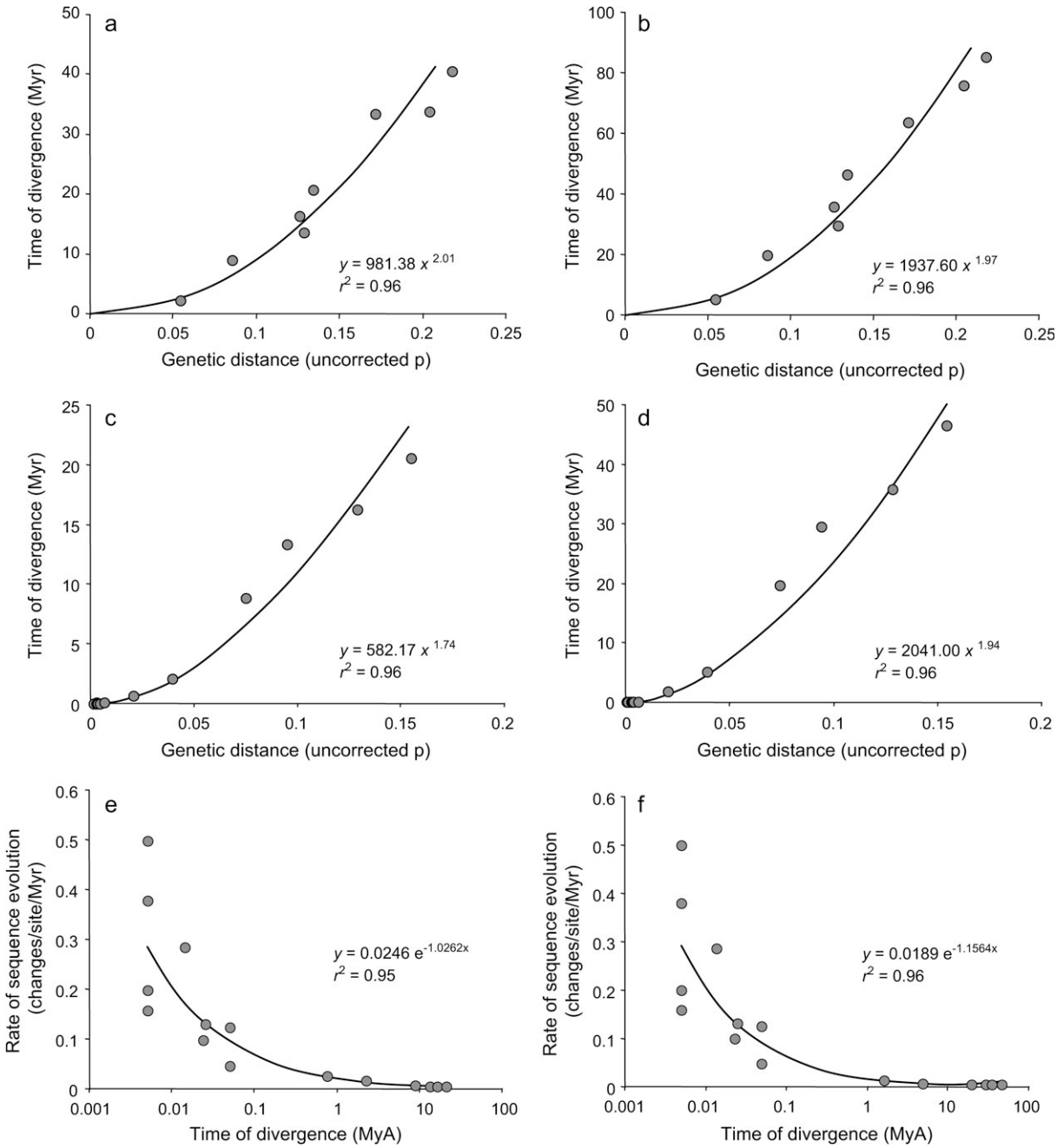
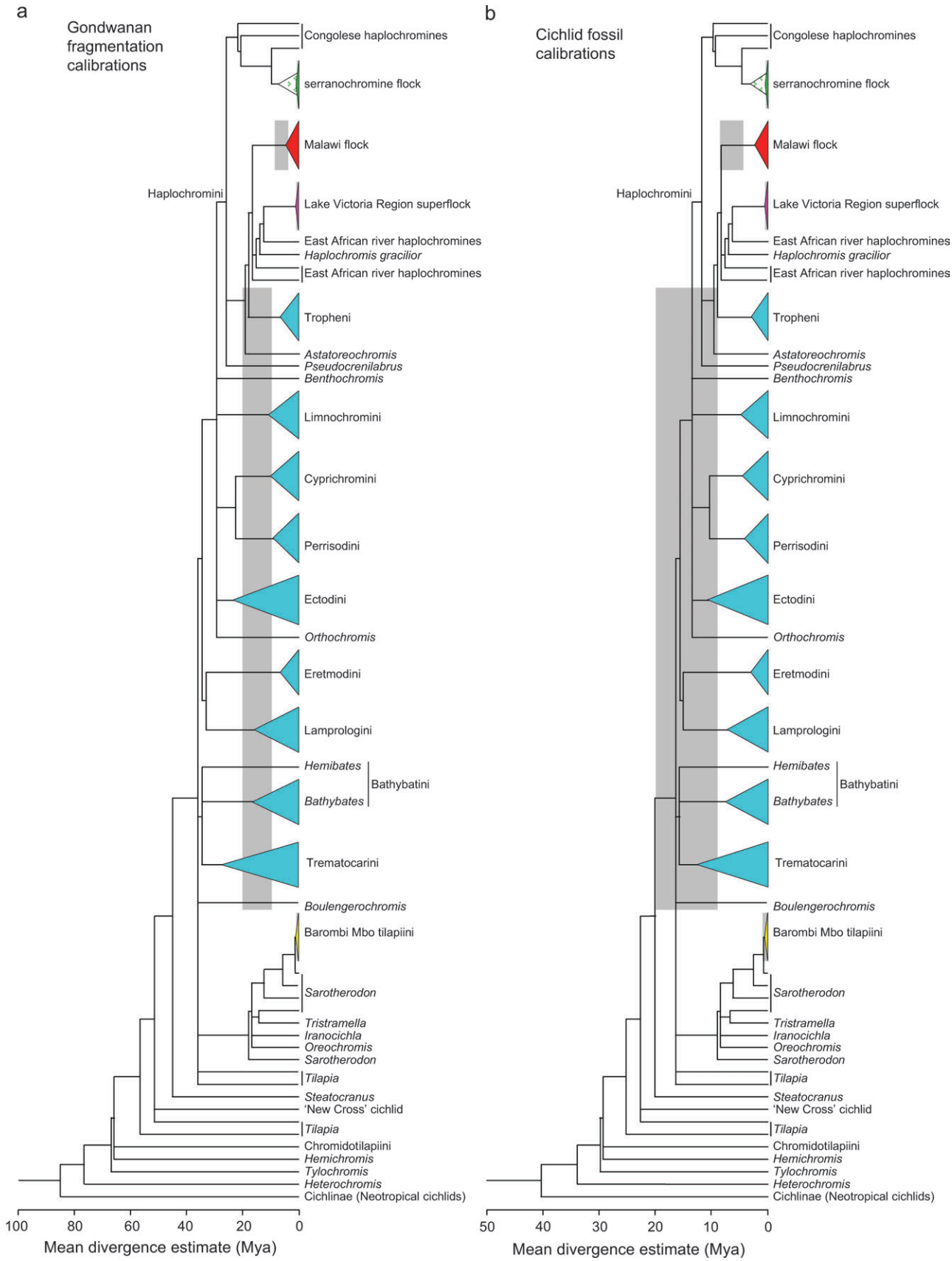


FIG. 5.—Rates of evolution of the concatenated cytochrome *b*, $NADH_2$, and control region using (*a*) cichlid fossil and (*b*) Gondwana fragmentation calibrations. These were used for determining ages of Barombi Mbo and Lake Tanganyika radiations. Rates of evolution of the control region using (*c*) cichlid fossil and (*d*) Gondwana fragmentation calibrations. These were used for determining ages of Lake Malawi, Lake Victoria Region, and southern serranochromine haplochromine radiations. A series of additional calibration points based on post-Pliocene biogeographic events were also included in the control region calibrations to account for time dependence the rates of molecular sequence evolution shown on logarithmic scales in (*e*) for cichlid fossil calibrations and (*f*) Gondwana fragmentation calibrations. Note that continued decline in rate of change following 1 MYA is also due to saturation.

→

FIG. 6.—Divergence times of African cichlids using (*a*) Gondwanan fragmentation (*b*) cichlid fossil calibrations. Each set of calibrations was combined with biogeography-derived dates for the Lake Malawi, Lake Victoria Region, and serranochromine radiations. The topology was generated using $NADH_2$ sequences, all branches had ML bootstrap support >50%. Triangles indicate the start of radiation where blue = Lake Tanganyika; red = Lake Malawi; yellow = Barombi Mbo; purple = Lake Victoria; and green = serranochromine (dotted triangle = serranochromine basal divergence and solid triangle = radiating clades). Gray boxes indicate periods of lake formation.


a
Gondwanan fragmentation calibrations
Congolese haplochromines
serranochromine flock
Malawi flock
Haplochromini
Lake Victoria Region superflock
East African river haplochromines
Haplochromis gracilior
East African river haplochromines
Tropheni
Astatoreochromis
Pseudocrenilabrus
Benthochromis
Limnochromini
Cyprichromini
Perrisodini
Ectodini
Orthochromis
Eretmodini
Lamprologini
Hemibates
Bathybatini
Bathybates
Trematocarini
Boulengerochromis
Barombi Mbo tilapiini
Sarotherodon
Tristramella
Iranocichla
Oreochromis
Sarotherodon
Tilapia
Steatocranus
'New Cross' cichlid
Tilapia
Chromidotilapiini
Hemichromis
Tylochromis
Heterochromis
Cichlinae (Neotropical cichlids)
100 80 60 40 20 0
Mean divergence estimate (Mya)
b
Cichlid fossil calibrations
Congolese haplochromines
serranochromine flock
Malawi flock
Haplochromini
Lake Victoria Region superflock
East African river haplochromines
Haplochromis gracilior
East African river haplochromines
Tropheni
Astatoreochromis
Pseudocrenilabrus
Benthochromis
Limnochromini
Cyprichromini
Perrisodini
Ectodini
Orthochromis
Eretmodini
Lamprologini
Hemibates
Bathybatini
Bathybates
Trematocarini
Boulengerochromis
Barombi Mbo tilapiini
Sarotherodon
Tristramella
Iranocichla
Oreochromis
Sarotherodon
Tilapia
Steatocranus
'New Cross' cichlid
Tilapia
Chromidotilapiini
Hemichromis
Tylochromis
Heterochromis
Cichlinae (Neotropical cichlids)
50 40 30 20 10 0
Mean divergence estimate (Mya)

event identified by Delvaux (1995). Rates based on this calibration have subsequently been employed several times for both African and Neotropical cichlids (Baric et al. 2003; Sturmbauer et al. 2003; Verheyen et al. 2003; Barluenga and Meyer 2004; Brandstätter et al. 2005; Barluenga et al. 2006).

Notably, no molecular phylogenetic studies have presented dates that strongly conflict with the established timescale of African cichlid evolution presented by Kocher (2004) and Salzburger and Meyer (2004), and this evolutionary scenario is generally supported by the timescale derived from the fossil cichlid calibrations (fig. 6). Briefly, this scenario would suggest that a small number of colonizing species entered a proto-lake Tanganyika when swamp and shallow lake conditions were present between approximately 12 and 20 MYA. Upon entering this habitat, some of these species began to radiate forming the present-day Lake Tanganyika cichlid tribes (Salzburger et al. 2002). Next, during the formation of deep-water conditions approximately 6–12 MYA, these lineages diversified further and began to accumulate species richness and morphological diversity (Salzburger et al. 2002).

By contrast, the timescale of African cichlid evolution suggested by the Gondwanan calibrations is very different. It proposes Lake Tanganyika as a reservoir of more than 10 riverine lineages that subsequently underwent adaptation for deep-water lacustrine conditions and radiated but became extinct in river systems over a timescale of approximately 20 Myr. One possible mechanism driving such extinctions could be climatically driven cycles of desiccation and flooding of the shallow rivers and streams in the Lake Tanganyika catchment. There is substantial evidence that East Africa has been through dramatic climatic changes, for example, from substantial lake-level changes recorded in the sediments of the East African great lakes (Johnson et al. 2002) and in the East African molluscan fossil record (for discussion, see Genner et al. 2007). If the Gondwanan calibrations are reliable, then the molecular lineage diversity present in Lake Tanganyika must have formed elsewhere. It is alternatively possible that this took place in one or more palaeolakes that formerly existed in this region. Lake formation and extinction appear to be common phenomena within sub-Saharan Africa (Cahen 1954; Stewart 2001; Van Damme and Pickford 2003), and several large palaeolakes are known from the upper Congo basin (Cahen 1954). If so, then the emerging Lake Tanganyika may have captured existing molecular diversity from previous lacustrine radiations, which in turn seeded new radiations (Seehausen 2006).

Conclusions

This study has provided 2 dichotomous timescales for the evolution of cichlid fishes and discussed the evidence surrounding both. In doing so, we hope to have contributed to the debate concerning timescales of cichlid evolution. Of particular interest are our results using the combined Gondwanan/post-Pliocene biogeographical calibrations that enabled us to reconcile global cichlid distributions and the comparatively young ages of endemic species flocks within lake basins without invoking scenarios of multiple intercontinental marine dispersal events. Further study is needed to investigate the implications of this calibration set for phylogeography and the mechanisms driving speciation and adaptive radiation. Nevertheless, with respect to African cichlid evolution, it is notable that both cichlid fossil and Gondwanan fragmentation calibrations indicate a strong association between onset of molecular radiation and the formation of lacustrine water bodies, in turn suggesting that colonization of lakes is associated with accelerated speciation and adaptive radiation. This may possibly be linked to the diversity of habitats that lakes provide as larger lakes have the largest cichlid flocks (Seehausen 2006). As such, it is clear that ancient lakes have played a key role in generating and maintaining diversity within radiating lineages, and the role of ecological opportunity in generation and maintenance of biodiversity should not be underestimated.

Supplementary Material

Supplementary materials are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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