Ancient Hybridization and Phenotypic Novelty within Lake Malawi's Cichlid Fish Radiation

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Abstract

Does hybridization play a broad innovative role in evolution? Many studies have shown hybrid origins of individual species, particularly in major adaptive radiations, but this may be a consequence, rather than a cause, of the existence of many closely related species. Cases of hybridization in the early stages of major adaptive radiations are comparatively rare. Here, we report phylogenetic evidence for ancient introgression between distinct lineages of the species-rich Lake Malawi haplochromine cichlid fishes. Mitochondrial DNA (mtDNA) sequences indicated surprisingly close relationships between the shallow-water rocky habitat "Mbuna" species and a group of dark-adapted "Deep-Benthic" species specialized for feeding in low-light conditions (dawn/dusk, under overhangs, and deep water). By contrast, analyses of nuclear amplified fragment length polymorphism data demonstrated that these Deep-Benthic cichlids were more closely related to shallow water "Shallow-Benthic" soft-sediment feeders, a group that shares similar head and body morphology. A coalescent-based computer simulation indicated that the mtDNA similarity of rocky habitat Mbuna species and dark-adapted Deep-Benthic species was due to hybridization rather than incomplete lineage sorting. Comparisons of morphology indicated that some Deep-Benthic species possessed novel morphology not present in other Lake Malawi species groups. Thus, these analyses support the hypothesis that ancient hybridization occurred within the Lake Malawi cichlid radiation, that the event occurred before the radiation of a species group adapted to low-light benthic habitats, and that this group went on to dominate the deep-water regions of Lake Malawi. The results of this study contribute to a growing literature consistent with a creative role of hybridization in the evolution of species diversity and adaptive radiations.

Key words: incomplete lineage sorting, introgression, adaptive radiation, AFLP, mitochondrial DNA.

Introduction

It has been proposed that introgressive hybridization may provide novel gene combinations that promote speciation and adaptive radiation by generating new transgressive phenotypes for natural selection to act upon (Seehausen 2004; Mallet 2007; Mavárez and Linares 2008). Phylogenetic evidence for natural hybridization has been found within the African lake cichlid radiations, including Lake Malawi (Smith et al. 2003), Lake Victoria (Seehausen et al. 2008), and Lake Tanganyika (Nevado et al. 2009) but also in other major adaptive radiations, including Hawaiian *Laupala* crickets (Shaw 2002) and Darwin's finches (Grant et al. 2005). Evidence that hybridization can create new transgressive phenotypes has also been found in numerous evolutionary lineages including fish (Stelkens et al. 2009), mammals (Larsen et al. 2010), and plants (Johansen-Morris and Latta 2006), and there is evidence that greater periods of isolation lead to greater transgressive differences following hybridization (Stelkens et al. 2009). Moreover, it has been shown that hybrids can occupy novel habitats distinct from those colonized by parental species (Rieseberg et al. 2003), which may thus promote adaptive radiation.

One of the problems with assigning a role for hybridization in large-scale adaptive radiation is that cases of natural hybridization reported to date tend to involve relatively recently diverged species pairs, resulting in the evolution of a single taxon per hybridization event (see Mavárez and Linares 2008). Indeed, the frequency of hybridization observed in rapidly diversifying clades may be the consequence rather than the cause of their rapid radiation (Wiens et al. 2006). Thus, the critical test of the role of hybridization in generation of adaptive radiation is whether there is evidence that introgression frequently occurs at the base of major radiations (Seehausen 2004). To date, the strongest evidence of this comes from Hawaiian silverswords, plants that are believed to have radiated following hybridization resulting in allopolyploidy (Barrier et al. 1999). As gene duplication is also proposed to enhance adaptive evolution (Lynch and Conery 2000), the two potential causal factors are confounded. Thus, if hybridization per se plays a creative role in the generation of adaptive diversity and species richness, introgression must be demonstrated to occur at the base of major nonpolyploid adaptive radiations.

Lake Malawi contains an adaptive radiation with an estimated 835 haplochromine cichlid fish species (Konings 2007), although at present only 387 have been formally described (http://malawicichlids.com/). The species in the

radiation have diverged in habitat use (depth, substrate), ecological traits (diet, trophic morphology, and body size), and signaling traits (visual signals, visual sensitivity, and perhaps olfactory and acoustic signals). Phylogenetic evidence, mainly using mitochondrial DNA (mtDNA), suggests that this species flock evolved from a single common ancestor in the region of 2.3–4.6 Ma (Genner, Seehausen, et al. 2007). The radiation is almost entirely endemic, with only two described species, *Astatotilapia calliptera* and *A. swynnertoni*, known to occur outside of the Lake Malawi catchment (Joyce et al. 2011). Much of the diversity is found within the largely shallow-water rock-associated "Mbuna" group that comprises an estimated 327 species (Konings 2007) in 13 genera (Oliver and Arnegard 2010). The offshore open waters tend to be dominated by the predatory species within *Diplotaxodon–Pallidochromis* (estimated 19 species; Konings 2007) and *Rhamphochromis* (estimated 15 species; Genner, Nichols, Carvalho, Robinson, Shaw, Smith, et al. 2007; Konings 2007). The remaining estimated 474 species are placed within 38 typically "Benthic" genera, and in this group, individual species tend to be associated with either sand, mud, rock, or macrophyte-dominated habitat, and possess strong differences in their depth distributions (Konings 2007).

Attempts to reconstruct the phylogenetic relationships of these cichlids have been confounded by their relatively recent evolutionary history (Albertson et al. 1999). This has resulted in a lack of genetic diversity at many nuclear markers investigated to date (Won et al. 2006), and problems of retained ancestral polymorphisms within more rapidly evolving genomic regions, such as the mtDNA control region (Moran and Kornfield 1993; Genner, Nichols, Carvalho, Robinson, Shaw, Smith, et al. 2007; Genner, Nichols, Carvalho, Robinson, Shaw, and Turner 2007; Mims et al. 2010). Moreover, there is evidence of hybridization between relatively closely related species, violating the assumptions of standard dichotomously branching cladistic models (Smith et al. 2003; Mims et al. 2010). Despite this, there is evidence of lineage sorting among some groups, for example, the *Diplotaxodon–Pallidochromis* and *Rhamphochromis* clades have been resolved as reciprocally monophyletic using mtDNA (Shaw et al. 2000; Turner et al. 2004). Similarly, most Mbuna belong to a single mtDNA haplogroup (Joyce et al. 2011), whereas most shallow-water species of Benthic cichlids belong to a distinct "Shallow-Benthic" haplogroup. Surprisingly, however, representatives of a large group of deep-water Deep-Benthic species are placed firmly in the Mbuna-dominated mtDNA haplogroup (Moran and Kornfield 1993), despite strong ecological and morphological similarities to species with Shallow-Benthic mtDNA. It was originally proposed that Deep-Benthic cichlids are placed within the Mbuna mitochondrial haplogroup as a consequence of incomplete lineage sorting (Moran and Kornfield 1993). However, more recently, there has been speculation that the Deep-Benthic taxa may indeed be more closely related to the Shallow-Benthic cichlids but have inherited Mbuna mtDNA through introgression prior their radiation into the diversity observed today (Joyce et al. 2011).

If the Deep-Benthic cichlids acquired their mtDNA following unidirectional introgression into the Shallow-Benthic from Mbuna, then this would provide novel evidence of hybridization at the base of a species-rich evolutionary radiation. Here, we report an investigation of the plausibility of this scenario by reconstructing wide-ranging phylogenies of the Lake Malawi haplochromines using mtDNA and the largely nuclear DNA amplified fragment length polymorphisms (AFLPs). Specifically, we hypothesized that if Mbuna are most closely related to Deep-Benthic using AFLP markers, this would support convergent evolution of cichlids with a Benthic phenotype. An alternative scenario where Shallow-Benthic and Deep-Benthic cichlids are more closely related to one another in nuclear markers would instead support mtDNA introgression into the Benthic lineage from the Mbuna or a retention an ancestral mtDNA polymorphism in Deep-Benthic cichlids through incomplete lineage sorting. To statistically distinguish between these possibilities, we used an approach that simulated mtDNA evolution under conditions where introgression between the Mbuna and Benthic cichlids was absent. Should the pattern of mtDNA haplogroup sharing between Deep-Benthic and Mbuna cichlids be replicable by chance, this would support incomplete lineage sorting. If instead the pattern could not be readily replicated, this would support ancient hybridization between Benthic cichlids and Mbuna. Finally, on the basis of evidence from this study, we classified Lake Malawi genera into major multispecies groups and compared species richness and ecophenotypic diversity among them. This provided an indication of the extent of phenotypic novelty now present within the Deep-Benthic cichlids.

Materials and Methods

Phylogenetic Analyses—Sample Selection

Samples included 61 Lake Malawi species and the outgroups *Astatotilapia tweddlei*, *Pseudocrenilabrus philander*, and *Julidochromis regani* (fig. 1; supplementary table S1, Supplementary Material online). Using mtDNA and AFLP data, *A. tweddlei* has recently been identified as a sister species to *A. bloyeti* and together these represent the sister group of the radiating Lake Malawi haplochromine flock (Joyce et al. 2011). *Pseudocrenilabrus philander* is a more distantly related haplochromine cichlid, whereas *J. regani* is a lamprologine cichlid from Lake Tanganyika. Fin tissue was stored in absolute ethanol, and DNA was extracted using the Wizard DNA extraction kit (Promega, Madison, WI).

Phylogenetic Analysis—mtDNA

The mtDNA control region was amplified following Joyce et al. (2005) and sequenced using standard techniques. Sequences were checked, and a 488 bp alignment was generated with ClustalW in DAMBE (Xia and Xie 2001). MrModeltest 2.1 (http://www.ebc.uu.se/systzoo/staff/nylander.html) executed in PAUP* (Swofford 1999) was used to identify the most appropriate model of sequence evolution, and the general time reversible (GTR)+Γ model

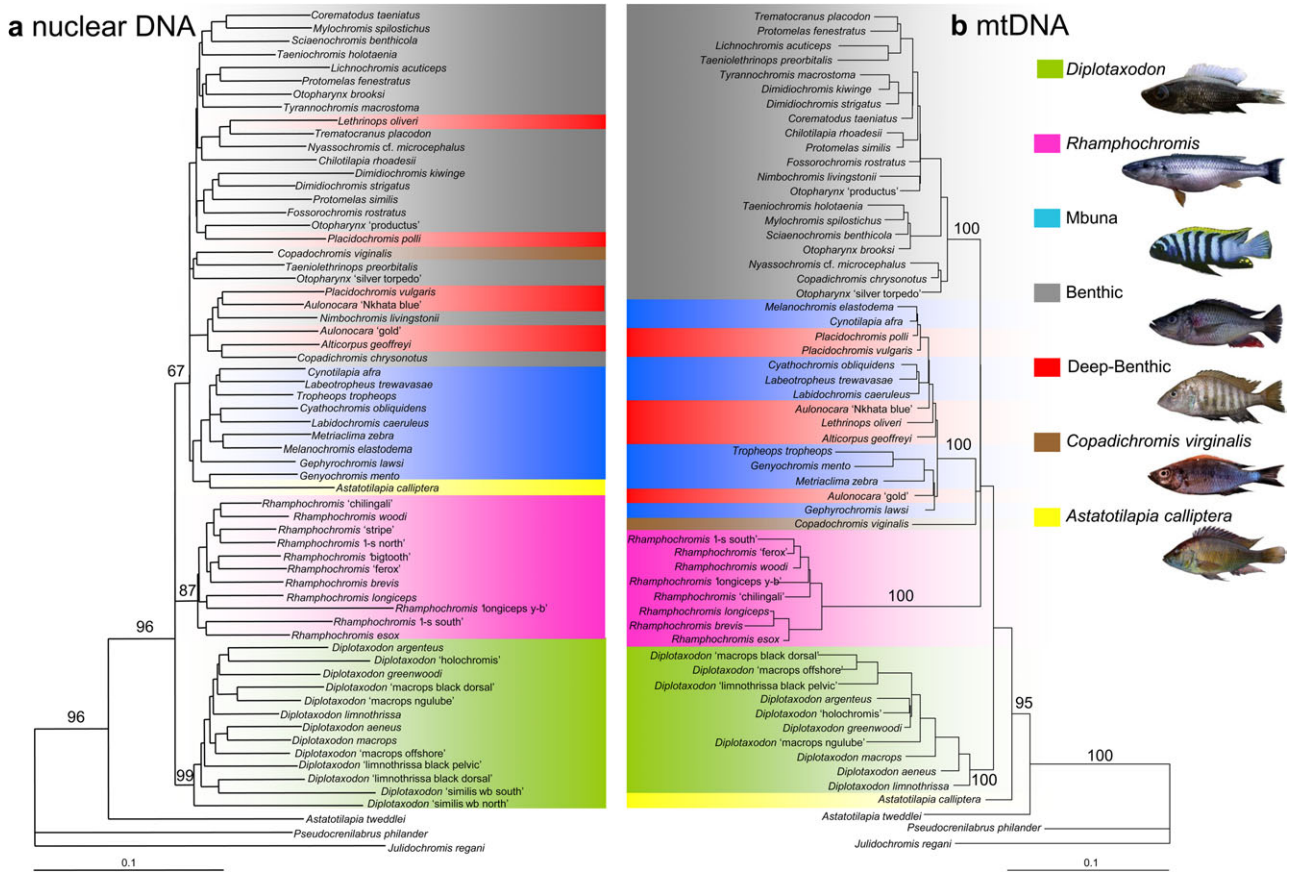

FIG. 1. Phylogenetic reconstructions based on (*a*) nuclear AFLP fragments using a neighbor joining algorithm and mean character differences and (*b*) mtDNA control region sequences using a neighbor joining algorithm and GTR+Γ distances. Percentage Bayesian posterior probabilities indicate support on key nodes.

was selected. MrBayes (Huelsenbeck and Ronquist 2001) was used for phylogenetic reconstruction, and chains were run for 2,000,000 generations. Posterior probability branch support was estimated from the final 50% of trees generated. A neighbor joining tree constructed in PAUP* based on GTR+Γ distances was used to illustrate phylogenetic relationships.

Phylogenetic Analysis—AFLP

We employed nuclear AFLPs to reconstruct the evolutionary history of the Lake Malawi flock. These have previously been proven useful in reconstructing the evolutionary history of cichlid clades in Lake Malawi (Albertson et al. 1999; Allender et al. 2003) and other African cichlid radiations (Schliewen and Klee 2004; Koblmüller et al. 2007). The restriction, ligation, and preamplification steps followed established protocol (Vos et al. 1995). The following end-amplification primers were used (E-AAG M-CCA; E-AAG M-CCT; E-ACA M-CCT; E-AAG M-CTG; E-AAG M-CGT; E-ACA M-CGT; E-AAG M-CAT; E-ACA M-CAT). After end-amplification, samples were loaded on a Beckman CEQ sequencer. AFLP markers were scored by eye using Beckman CEQ fragment analyzer software. Five individuals were repeated from the restriction–ligation stage onward. Repeatability of scoring loci was on average 94.8% across the five individuals, range 92.0–96.5%. The final data set retained only one of each pair of repeated individuals and in total contained 64 individuals and 1,006 loci, of which 81 loci were constant, 246 variable but parsimony uninformative, and 679 parsimony informative. MrBayes was used for phylogenetic reconstruction, with 2,000,000 generations, coding datatype = restriction, and otherwise retaining default parameters. The posterior probability branch support values were estimated from a majority rule tree of the final 50% of trees generated. A neighbor joining tree constructed in PAUP* based on mean character distances was used to illustrate phylogenetic relationships.

MrBayes was used to investigate support for phylogenetic hypotheses from AFLP data, using an approach that compares the harmonic mean log-likelihood of trees generated under constrained (hypothesized) and unconstrained (observed) topologies. The method differs from traditional approaches because it does not lead to rejection of the null hypothesis in favor of an alternative hypothesis but instead evaluates support for a given hypothesis based on available evidence. Specifically, Markov chain Monte Carlo analyses were run in MrBayes for 2,000,000 generations using both constrained and unconstrained topologies, and the final 50% of trees were retained. Data were analyzed coding datatype = restriction, otherwise default parameters were retained. Bayes factors were calculated as twice the difference in harmonic means of log-likelihoods

between null and hypothesized topologies, those >10 were considered unsupportive of hypothesized topologies, whereas those <10 were considered supportive of hypothesized topologies, following Marek and Bond (2006).

Resolving Evolutionary Relationships of Multispecies Groups

A phylogenetic hypothesis for five major multispecies groups identified within the Lake Malawi haplochromine radiation (*Diplotaxodon*, *Rhamphochromis*, Mbuna, Deep-Benthic, and Shallow-Benthic; see Appendix for details) was reconstructed using the AFLP data from sampled individuals and a UPGMA tree based on Nei's (1978) genetic distances in TFPGA (Miller 1997), with percentage bootstrap percentage support estimated from 1,000 replicates. This provided a best estimate of evolutionary relationships of the multispecies groups, but we recognize that true evolutionary relationships may be more complex. AFLP genetic distances between species groups were quantified using F_{ST} in AFLP-SURV (Vekemans 2002), and the significance of differences was tested using 1,000 permutations.

Distinguishing Incomplete Lineage Sorting from Hybridization Using mtDNA Sequences

To test between incomplete lineage sorting (retention of ancestral polymorphism) or hybridization, we used the general approach developed by Joly et al. (2009) and employed in Joyce et al. (2011). Briefly, mtDNA evolution can be simulated for topologies that assume either no hybridization (=incomplete lineage sorting) or hybridization. If observed genetic distances between taxa are different from those expected from simulations under a given topology, then we can reject that hypothesis. Specifically, here, we expected observed genetic distances between potentially hybridizing lineages to be lower than expected under a no-hybridization scenario.

Initially, we assumed a no-hybridization scenario where Deep-Benthic and Shallow-Benthic multispecies groups were not reciprocally monophyletic (fig. 2*b*). We then aligned 150 published unique control region sequences from the five species groups (total 836 bp), including 25 *Rhamphochromis*, 25 *Diplotaxodon*, 35 Mbuna, 30 Deep-Benthic, and 35 Shallow-Benthic (supplementary table S2, Supplementary Material online). We then used MCMCCoal (Rannala and Yang 2003) with the Jukes-Cantor model to estimate all divergence times (τ) and effective population sizes (θ) for each species group, along with 95% credibility intervals (supplementary table S3, Supplementary Material online). Each run of MCMCCoal included 100,000 samples, saving the data every 20 samples, and first 10% of samples were disregarded as burn-in. These divergence times and effective population sizes were used to simulate 1,000 coalescent trees in MCCoal (Rannala and Yang 2003). A sequence alignment was modeled for each coalescent tree using SeqGen (Rambaut and Grassly 2007), using the GTR+Γ+I model of sequence evolution, identified as appropriate in MrModeltest 2.3 (http://www.ebc.uu.se/systzoo/staff/nylander.html). From these modeled sequence alignments, minimum and average maximum likelihood distances between multispecies groups were generated using a combination of PAUP* (Swofford 1999) and Excel macros. These distances represented the expected values. A median value of the 1,000 simulated expected values was calculated and compared with the observed value. We then used these 1,000 simulated expected values to calculate the one-way probability that differences between observed and expected values were significant. Next, the whole analysis was repeated assuming that the Deep-Benthic and Shallow-Benthic taxa are reciprocally monophyletic sister clades (fig. 2*c*). Finally, we repeated the whole analysis again assuming a hybridization scenario where Mbuna and Deep-Benthic groups share more recent mtDNA divergence than is shared between the Deep-Benthic and Shallow-Benthic groups (fig. 2*d*).

Morphological and Depth Distributions

A total of 159 species were classified into the five multispecies groups (see Appendix, supplementary fig. S1 and tables S4 and S5, Supplementary Material online). To capture gross shape variation, we used a landmark-based image analysis of available side profile images from 143 of the 159 species. *Astatotilapia calliptera* and *Copadichromis virginalis* were also included. The methods for quantifying morphological variation followed Genner, Nichols, Carvalho, Robinson, Shaw, Smith, et al. (2007); Genner, Nichols, Carvalho, Robinson, Shaw, and Turner (2007). Briefly, images were loaded into tpsDig2.16 (Rohlf 2010a), and 25 landmarks were marked (fig. 3). Coordinates were then aligned using Procrustes analysis in tpsRelw1.49 (Rohlf 2010b), and relative warp (RW) scores were generated. Statistical significance of among-taxa differences along RW axes was tested using analysis of variance (ANOVA) and post hoc Tukey's Honestly Significant Difference (HSD) tests in Statistica 6 (Statsoft Inc, Tulsa, OK). Depth distributions were available for 141 of the 159 species (supplementary table S5, Supplementary Material online). These data were obtained primarily from Ribbink et al. (1983), Turner (1996), Konings (2007) and enabled quantification of the proportion of species in each multispecies group occupying the depth gradient between 0 and 150 m. Significant differences between the minimum and maximum depths of species groups were tested with ANOVA and post hoc Tukey's HSD tests in Statistica 6.

Estimating Number of Species in the Deep-Benthic Group

To provide an estimate of total species richness in the Deep-Benthic group, we used lists of described species (http://malawicichlids.com/) and probable species in each genus from available literature (Konings 2007). These numbers were then multiplied by the proportion of Deep-Benthic species known to comprise each genus (supplementary table S6, Supplementary Material online).

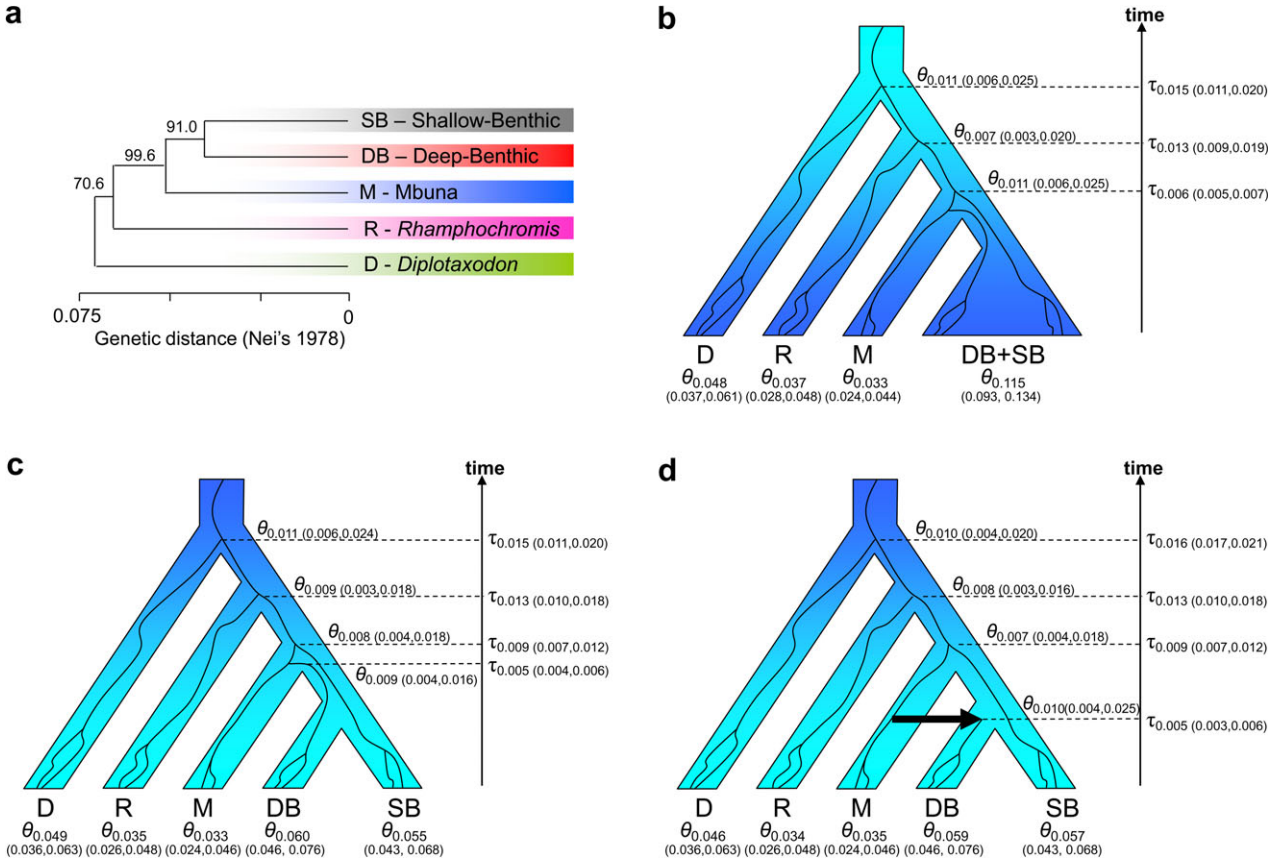

Fig. 2. (*a*) Phylogenetic hypothesis for the Lake Malawi haplochromine radiation (excluding *Astatotilapia calliptera* and *Copadichromis virginalis*) using a UPGMA tree based on Nei's genetic distances and AFLP data of sampled individuals within five multispecies groups, with percentage bootstrap percentage support estimated from 1,000 replicates. DB, Deep-Benthic; SB, Shallow-Benthic; M, Mbuna; D, *Diplotaxodon*; and R, *Rhamphochromis*. (*b*) Schematic demonstrating a hypothesis for mtDNA evolution (black line) under incomplete lineage sorting and nonmonophyly of the Shallow-Benthic and Deep-Benthic groups. (*c*) Schematic demonstrating a hypothesis for mtDNA evolution under incomplete lineage sorting and reciprocal monophyly of the Shallow-Benthic and Deep-Benthic groups. (*d*) Schematic demonstrating a hypothesis for mtDNA evolution under hybridization, the thick arrow symbolizes unidirectional mtDNA introgression between the Mbuna and Benthic groups. Labels represent results from coalescent analysis where $\theta = 4N\mu g$ and $\tau = T\mu$, and N = effective population size, μ = mutation rate per site per year, g is the generation time (assumed to be 1 year), and T = species divergence time. 95% Credibility intervals are in parentheses.

Results

MtDNA—Phylogeny

mtDNA analysis of sampled taxa (fig. 1) was consistent with evidence that Lake Malawi haplochromine radiation contains for six major mtDNA haplogroups, as previously reported (Moran and Kornfield 1993; Shaw et al. 2000; Turner et al. 2004; Joyce et al. 2011). These groups were 1) *Rhamphochromis*, 2) *Diplotaxodon*, 3) Shallow-Benthic, 4) Mbuna (including Deep-Benthic), 5) *C. virginalis*, and 6) Lake Malawi *A. calliptera*. Where multiple haplotypes were present in each clade, the clade was strongly supported as monophyletic, as shown by Shaw et al. (2000) and Joyce et al. (2011).

AFLP—Phylogeny

The phylogeny based on AFLP indicated that both *Rhamphochromis* and *Diplotaxodon* were reciprocally monophyletic (fig. 1). All other taxa were represented within a single major clade (fig. 1). Bayesian hypothesis testing supported the reciprocal monophyly of *Rhamphochromis*, *Diplotaxodon*, and the Mbuna (table 1). This approach also supported the monophyly of a single clade containing the Shallow-Benthic and Deep-Benthic taxa, but was unable to resolve the Shallow-Benthic and Deep-Benthic as reciprocally monophyletic lineages (table 1). It strongly rejected the monophyly of the putative clade comprised of the Mbuna and Deep-Benthic species group that was indicated by mtDNA (fig. 1). Reconstruction of the evolutionary relationships of the multispecies groups confirmed a closer relationship between the Shallow-Benthic and Deep-Benthic groups than between the Mbuna and Deep-Benthic groups (fig. 2*a*). Significant differences were found between the five species groups in AFLP marker frequencies in a global test ($F_{ST} = 0.190$, $P < 0.001$) and post hoc pairwise comparisons between species groups (table 2).

Incomplete Lineage Sorting or Hybridization

To distinguish between incomplete lineage sorting and hybridization as an explanation for the shared mtDNA

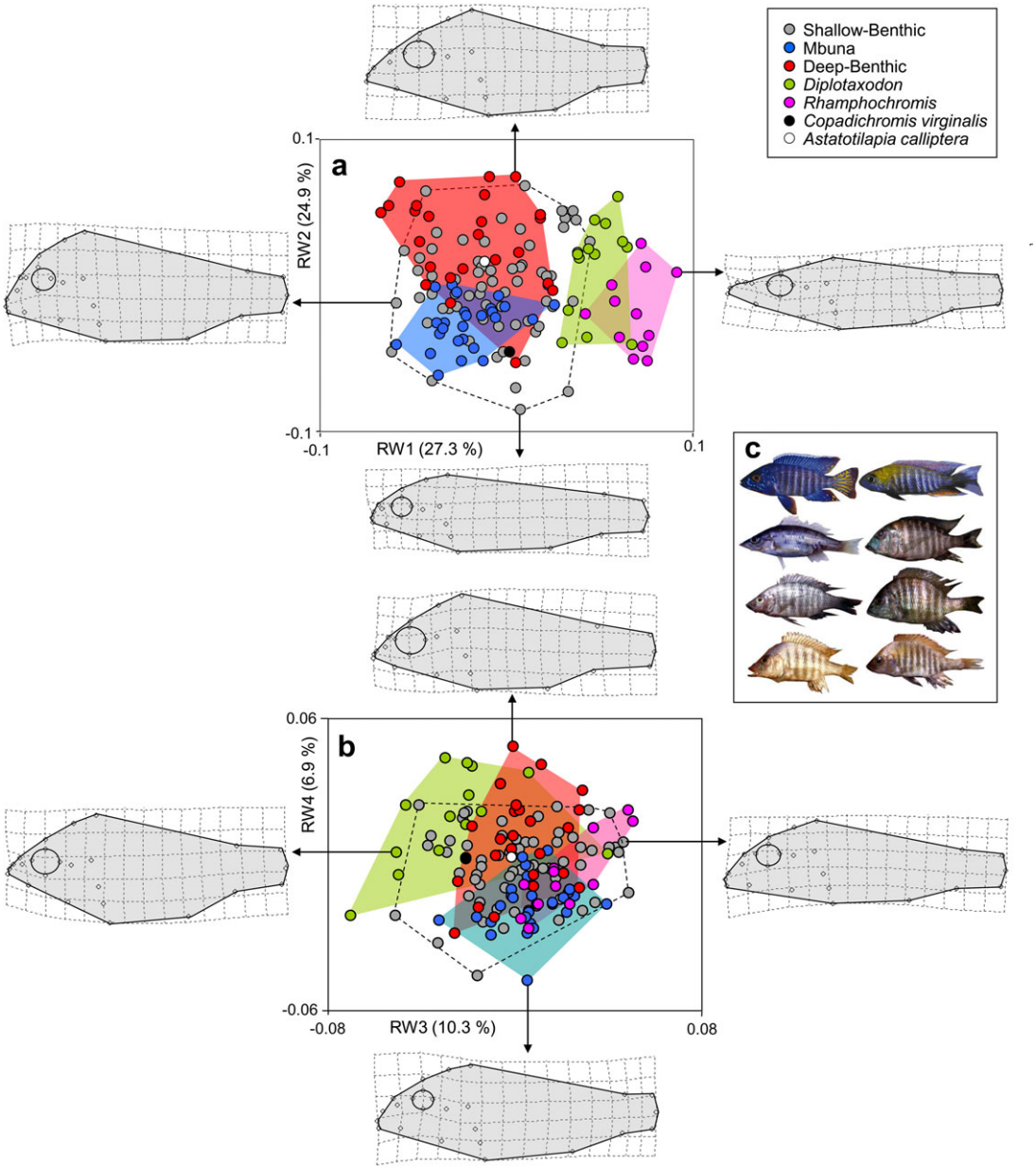

Fig. 3. (*a*, *b*) Ordination of gross head and body morphology of species in the Lake Malawi cichlid flock generated using RW analysis. Extreme morphologies on each axis are illustrated with deformation grids. (*c*) Morphological diversity in the Deep-Benthic species group, from top left clockwise, *Aulonocara stuartgranti*, *Aulonocara jacobfreibergi*, *Lethrinops* sp. "deep water albus," *Lethrinops longimanus*, *Alticorpus peterdaviesi*, *Alticorpus macrocleithrum*, *Placidochromis polli*, and *Placidochromis platyrhynchos*.

haplogroup between the Mbuna and Deep-Benthic groups, we compared genetic distances between lineages with those expected from simulations of mtDNA sequence evolution. The observed genetic distances (both average and minimum) between the Deep-Benthic and Mbuna were significantly lower than would be expected under the incomplete lineage sorting model where Deep-Benthic and Shallow-Benthic were not reciprocally monophyletic (fig. 2*b* and table 3) and also when they were considered reciprocally monophyletic (fig. 2*c* and table 3). By contrast, these distances were not significantly different to those expected using the hybridization model (fig. 2*d* and table 3). Our fitted models enabled us to identify the putative divergence times of the major multispecies groups. Using a "Gondwana"-derived calibration where the origin of the Lake Malawi species flock dates to 4.63 Ma (Genner, Seehausen, et al. 2007), we estimated the timing of the hybridization event between the Mbuna and Deep-Benthic groups to be approximately 1.29 Ma (supplementary table S3, Supplementary Material online). Using a "cichlid fossil"–derived calibration where the origin of the Lake Malawi species flock dates minimally to 2.43 Ma (Genner, Seehausen, et al. 2007), we estimated the timing of the hybridization event between the Mbuna and Deep-Benthic groups to be a minimum of 0.68 Ma (supplementary table S3, Supplementary Material online).

Table 1. Comparisons of Mean Likelihoods of the Unconstrained and Constrained Topologies of Trees Based on AFLP Data.

Hypothesis/Constraint(s) Employed	Mean LnL: Constrained	Bayes Factor	Phylogenetic Inference
Mbuna monophyly	−16123.12	0.88	Supported
Deep-Benthic monophyly	−16169.57	93.78	Not supported
Shallow-Benthic monophyly	−16193.67	141.98	Not supported
Diplotaxodon monophyly	−16123.24	1.11	Supported
Rhamphochromis monophyly	−16122.56	−0.24	Supported
Shallow-Benthic and Deep-Benthic combined monophyly	−16122.45	−0.47	Supported
Shallow-Benthic and Deep-Benthic reciprocal monophyly	−16177.35	109.34	Not supported
Mbuna, Shallow-Benthic, and Deep-Benthic combined monophyly	−16121.53	−2.30	Supported
Mbuna, Shallow-Benthic, and Deep-Benthic reciprocal monophyly	−16171.07	96.77	Not supported
(Mbuna plus Deep-Benthic)(Shallow-Benthic) = as mtDNA	−16193.56	141.76	Not supported
(Mbuna)(Shallow-Benthic plus Deep-Benthic)	−16123.92	2.47	Supported

NOTE.—Bayes factors are twice the difference in harmonic means of log-likelihoods between constrained and unconstrained trees. Harmonic mean unconstrained LnL = −16122.68.

Morphology and Depth

In total, the first 4 RW axes captured 69.4% of observed shape variation within the 143 species. RW axis 1 captured 27.3% of variation and reflected a change from deep to shallow body shape, with the head size roughly proportional across the axis (fig. 3*a*). RW axis 2 captured 24.9% of variation and reflected a change from species with a large head and eye to species with a small head and eye, relative to body size (fig. 3*a*). RW axis 3 captured 10.3% of variation and reflected variation in jaw shape from a short upturned jaw to a longer downturned jaw (fig. 3*b*). RW axis 4 captured 6.9% of variation and reflected in the size and position of the eye within the head (fig. 3*b*). Other axes each captured less than 6% of the variation. Overall, there were highly significant global differences between the multispecies groups in each of the first 4 RW axes (RW1, $F_{4,138} = 47.67$, $P < 0.001$; RW2, $F_{4,138} = 13.40$, $P < 0.001$; RW3, $F_{4,138} = 9.27$, $P < 0.001$; RW4, $F_{4,138} = 15.32$, $P < 0.001$). Post hoc pairwise comparisons demonstrated that all groups differed significantly on at least one of the RW axes (table 2). Some members of the Deep-Benthic group had a distinct morphology not represented within the Shallow-Benthic or the Mbuna groups, namely a deep body and large eyes.

The major multispecies groups differed significantly in their depth distributions (minimum depth, $F_{4,134} = 47.33$, $P < 0.001$; maximum depth $F_{4,134} = 52.71$, $P < 0.001$). Post hoc analyses only showed nonsignificant differences in depth between the Mbuna and Shallow-Benthic species (table 2 and fig. 4*a*). Despite overlap between Deep-Benthic and Shallow-Benthic species for much of the depth range, this overlap was restricted to only a small fraction of the diversity within each. Most (>50%) of Deep-Benthic species have been recorded between 30 and 120 m, whereas most Shallow-Benthic species were recorded at depths between 0 and 30 m (fig. 4*b*).

Species Richness of Multispecies Groups

We screened available mtDNA data from this study and previously published work (sequences and restriction fragment length polymorphism) to estimate the proportions of Deep-Benthic species within genera (supplementary table S4 and fig. S1, Supplementary Material online). All *Aulonocara* and *Alticorpus* species screened were Deep-Benthic (nine and four species, respectively), as were most *Lethrinops* (10 of 13 species) and half of the *Placidochromis* (three of six species). If these proportions hold for the remainder of species in these genera, then we estimate the Deep-Benthic group comprises at least 66 described species and 141 probable species (supplementary table S6, Supplementary Material online). Within the *Placidochromis* and *Lethrinops*,

Table 2. Tests for Significance Genetic (AFLP), Morphological (RW 1–4), and Depth (Minimum and Maximum Recorded) Differences between the Species Groups.

Group 1	Group 2	Genetic Distance (AFLP)	Genetic Distance (AFLP)	Morphology RW1	Morphology RW2	Morphology RW3	Morphology RW4	Minimum Depth	Maximum Depth
		F_{ST}	*P*	*P*	*P*	*P*	*P*	*P*	*P*
DB	R	0.2377	<0.001	<0.001	<0.001	0.056	0.080	0.005	0.906
DB	D	0.1908	<0.001	<0.001	0.455	0.001	0.378	0.517	0.001
DB	SB	0.0851	0.004	0.088	<0.001	1.000	0.066	<0.001	<0.001
DB	M	0.1751	<0.001	1.000	<0.001	0.944	<0.001	<0.001	<0.001
R	D	0.2120	<0.001	0.216	0.049	<0.001	0.001	<0.001	0.001
R	SB	0.2077	<0.001	<0.001	0.354	0.020	0.907	0.083	<0.001
R	M	0.2429	<0.001	<0.001	0.950	0.202	0.181	0.001	<0.001
D	SB	0.1931	<0.001	<0.001	0.436	<0.001	<0.001	<0.001	<0.001
D	M	0.2142	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
SB	M	0.1276	<0.001	0.105	0.002	0.875	<0.001	0.059	0.074

NOTE.—Global tests revealed significant differences among species groups in all response variables, see main text for details. Letters represent species groups: R, *Rhamphochromis*; D, *Diplotaxodon*; M, Mbuna; DB, Deep-Benthic; and SB, Shallow-Benthic.

Table 3. Observed and Expected Genetic Distances (GTR+Γ+I model) between Major Multispecies Groups, Under Hypothesized Scenarios where Incomplete Lineage Sorting and Hybridization Explain the High Similarity between Mbuna and Deep-Benthic mtDNA Sequences (fig. 2*b*–*d*).

	Minimum Genetic Distances				Average Genetic Distances			
Groups	Observed	Expected (median)	*P*	Interpretation	Observed	Expected (median)	*P*	Interpretation
No-hybridization (incomplete lineage sorting) scenario (SB and DB combined monophyly)								
R-SB	0.041	0.020	0.024	O > E	0.056	0.033	0.041	O > E
D-M	0.020	0.023	0.326	O = E	0.033	0.039	0.162	O = E
D-DB	0.022	0.024	0.335	O = E	0.037	0.039	0.389	O = E
R-D	0.033	0.023	0.077	O = E	0.050	0.039	0.146	O = E
SB-D	0.029	0.024	0.249	O = E	0.044	0.039	0.291	O = E
M-DB	0.001	0.007	0.003	O < E	0.012	0.022	0.020	O < E
M-R	0.038	0.018	0.025	O > E	0.049	0.033	0.080	O = E
M-SB	0.025	0.007	0.004	O > E	0.038	0.022	0.047	O > E
SB-DB	0.022	0.000	< 0.001	O > E	0.041	0.011	0.007	O > E
DB-R	0.039	0.020	0.032	O = E	0.051	0.033	0.071	O = E
No-hybridization (incomplete lineage sorting) scenario (SB and DB reciprocal monophyly)								
R-SB	0.041	0.019	0.026	O > E	0.056	0.034	0.040	O > E
D-M	0.020	0.024	0.301	O = E	0.033	0.040	0.162	O = E
D-DB	0.022	0.024	0.352	O = E	0.037	0.040	0.428	O = E
R-D	0.033	0.023	0.105	O = E	0.050	0.040	0.154	O = E
SB-D	0.029	0.024	0.293	O = E	0.045	0.040	0.315	O = E
M-DB	0.001	0.012	< 0.001	O < E	0.012	0.025	< 0.001	O < E
M-R	0.038	0.019	0.022	O > E	0.049	0.034	0.078	O = E
M-SB	0.025	0.011	0.024	O > E	0.038	0.025	0.081	O = E
SB-DB	0.022	0.004	0.001	O > E	0.041	0.017	0.005	O > E
DB-R	0.039	0.020	0.031	O = E	0.051	0.033	0.064	O = E
Hybridization scenario (DB and SB reciprocal monophyly)								
R-SB	0.041	0.019	0.012	O > E	0.056	0.034	0.031	O > E
D-M	0.020	0.025	0.206	O = E	0.033	0.040	0.161	O = E
D-DB	0.022	0.025	0.298	O = E	0.037	0.040	0.312	O = E
R-D	0.033	0.024	0.131	O = E	0.050	0.040	0.152	O = E
SB-D	0.029	0.024	0.234	O = E	0.045	0.040	0.329	O = E
M-DB	0.001	0.004	0.123	O = E	0.012	0.017	0.078	O = E
M-R	0.038	0.020	0.029	O > E	0.049	0.034	0.080	O = E
M-SB	0.025	0.011	0.007	O > E	0.038	0.025	0.075	O = E
SB-DB	0.022	0.011	0.010	O > E	0.041	0.025	0.041	O > E
DB-R	0.039	0.020	0.030	O > E	0.051	0.034	0.047	O > E

NOTE.—R, *Rhamphochromis*; D, *Diplotaxodon*; M, Mbuna; DB, Deep-Benthic; SB, Shallow-Benthic; O, Observed; and E, Expected. Underlined are the only cases where observed genetic distances were significantly lower than expected under the model.

deep-water species possessed mtDNA typical of Deep-Benthic species, whereas shallow-water species possessed mtDNA typical of Shallow-Benthic species (supplementary table S5, Supplementary Material online).

Discussion

Congruence and Discordance between mtDNA and AFLP

Using a combination of nuclear and mtDNA, we identified five major multispecies groups in the Lake Malawi cichlid flock. Representatives of the groups were, on average, ecologically and morphologically different, but there was nevertheless considerable overlap. Typically, Mbuna are rock cichlids, present in shallow waters to depths of approximately 40 m. *Rhamphochromis* are midwater piscivores, and the genus is present over a range of habitats and has a broad depth range from surface waters to depths of over 140 m. *Diplotaxodon* are piscivores and zooplanktivores, found almost exclusively in deep waters below 50 m. The Shallow-Benthic species are ecologically diverse, encompassing piscivores, zooplanktivores, and benthic invertebrate eaters, but most species are found in shallow waters up to 30 m depth. The Deep-Benthic species tend to be benthic invertebrate eaters and piscivores and are found primarily in deep waters below 30 m. Notably, the few species that we classified as Deep-Benthic that are found in shallower waters tend to inhabit dark environments, such as in caves and under rocky overhangs (Konings 2007).

The *Rhamphochromis*, *Diplotaxodon*, and Shallow-Benthic groups were found to be reciprocally monophyletic in mtDNA. However, consistent with earlier studies (Moran et al. 1994; Turner et al. 2004), we found that the Deep-Benthic species and Mbuna share an mtDNA haplogroup, but importantly no haplotypes. In contrast to these phylogenetic relationships suggested by mtDNA, our results from nuclear markers strongly supported closer relationships between the Shallow-Benthic species and Deep-Benthic species than between the Mbuna and Deep-Benthic species, as also suggested in a recent analysis of nuclear single nucleotide polymorphisms (SNPs) (Loh et al. 2008). The results of our simulations suggest that the discordance

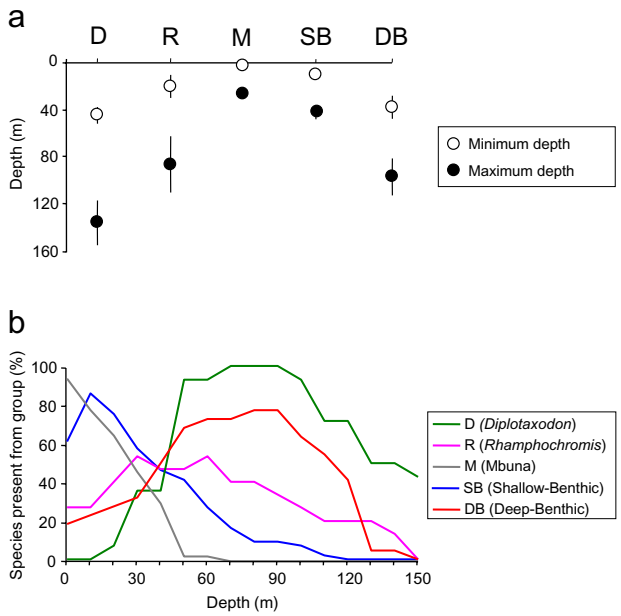

FIG. 4. (*a*) Average maximum and minimum depth of species in the five multispecies groups. (*b*) Depth distribution of species studied from each multispecies group.

between the nuclear and mitochondrial placements of the Deep-Benthic is most likely to be the result of hybridization rather than incomplete lineage sorting. Thus, our data suggest that cichlids in then Deep-Benthic group possess mtDNA that was inherited from Mbuna, before radiation into the darker and deeper environments of the lake.

Importantly, analyses of the AFLP data were unable to conclusively resolve the monophyly of either the Deep-Benthic or Shallow-Benthic groups. The restricted directional introgression of typically Mbuna mtDNA to only four Benthic genera with closely allied ecological traits is suggestive of close evolutionary affinities of those taxa with the Benthic lineage. Thus, the monophyly of the Deep-Benthic cichlids is a plausible hypothesis that deserves further exploration. The data from this study are at least partially consistent with the division of the Deep-Benthic and Shallow-Benthic species representing a genuine dichotomy within this species-rich group. Tests of differences in AFLP allele frequencies demonstrated significant differences between the groups, and there were also significant morphological and ecological (depth) differences between them. It is plausible that the failure of AFLPs to resolve reciprocal monophyly may in part be due to the retention of ancestral polymorphisms within both the Shallow-Benthic and Deep-Benthic group (Moran and Kornfield 1993) or perhaps a lack of truly homologous parsimony informative polymorphisms within the data set, given the AFLP analysis relies on fragment presence–absence only. However, at present, we cannot rule out the possibility that the Deep-Benthic cichlids are a polyphyletic group containing multiple lineages that have experienced convergent evolution to adapt to deep-water habitats or are a group that have undergone complex introgression with Shallow-Benthic cichlids during their evolutionary history. Clearly, this aspect of the Lake Malawi cichlid flock phylogeny deserves further investigation. Analyses based on next-generation sequencing of homologous fragments may help to improve resolution of phylogenetic relationships both among and within these Lake Malawi cichlid species groups. Importantly, however, the absence of evidence for Deep-Benthic monophyly does not conflict with the hypothesis that ancient hybridization occurred prior to diversification of the taxa within this group.

Adaptations and Species Richness of Deep-Benthic Species

Given the evidence in support of an ancient hybridization event before diversification of the Deep-Benthic cichlids, we compared the diversity present with that of other species groups in the Lake Malawi radiation. We estimated that the Deep-Benthic species group contains between 66 and 141 species. However, species richness is almost certainly higher given the deep-water regions of the lake remain so poorly sampled (Turner 1996; Snoeks 2004; Konings 2007). Generally, the taxa comprising the Deep-Benthic group are relatively little known, apart from a group of shallow-water species of the genus *Aulonocara* which mainly hide among rocks, but emerge at dusk, detecting movements of invertebrate prey hidden in soft sediments using their expanded cephalic lateral line pores (Bassett and Webb 2009). The remaining members of this group appear to live near the bottom over muddy habitats, mainly at depths of 50 m or more. It is certainly plausible that current maximum depth distributions of many Deep-Benthic species are underestimated due to the paucity of accurate information on depth distributions throughout the lake.

Our multivariate analysis of head and body shapes indicated that species assigned to the Deep-Benthic group are morphologically diverse and include body forms not exhibited by either the Mbuna or Shallow-Benthic groups (fig. 3). Extreme morphology is typically characterized by deep body forms and ventrally positioned mouths that are presumably adaptations for feeding on invertebrates or small fish near or in the sediment (Turner 1996; Snoeks 2004). Moreover, these species have adaptations for feeding in low-light environments, in the form of expanded cephalic lateral line pores and large eyes. Together, these Deep-Benthic genera dominate the deep-water areas of the lake, along with the midwater feeding members of the smaller *Rhamphochromis* and *Diplotaxodon* clades (fig. 4). Thus, the Deep-Benthic group represents a major component of the adaptive radiation, exhibiting high species richness and morphological diversity, which has evolved subsequent to one or more hybridization events that left a detectable mitochondrial legacy.

Polyphyletic Genera?

Evidence suggested that nearly all Lake Malawi genera were present within only one of the multispecies groups, demonstrating evidence of lineage sorting toward the tips of the Lake Malawi phylogeny. However, two morphologically defined genera, *Placidochromis* and *Lethrinops*, were

found to be polyphyletic with respect to their mtDNA, with deep-water species belonging to the Deep-Benthic group and shallow-water forms to the Shallow-Benthic group (supplementary tables S4 and S5, Supplementary Material online). Further work is required to resolve the question of whether these taxa are truly polyphyletic, but if they are, it would not really be surprising: The last major taxonomic work on *Placidochromis* described the genus as "problematic" and a "catch-all genus" of species that "do not fit into other genera with the same melanin pattern" (Hanssens 2004), although the deep-water species seem to form a reasonably coherent group. Similarly, *Lethrinops* is defined on the basis of shared possession of a single morphological trait (shape of the dental arcade) and absence of other morphological traits, and taxonomists have drawn a distinction between shallow-water and deep-water *Lethrinops* species, congruent with mtDNA classification (Ngatunga and Snoeks 2004).

Hybridization and Transgressive Phenotypes

The results of this study are consistent with homoploid hybridization at the base of an adaptive radiation. Nevertheless, it is not the first study to suggest ancient hybridization in cichlids from the Lake Malawi region. Recently, Joyce et al. (2011) used similar techniques to demonstrate that a clade of riverine cichlids found to the southeast of the Lake Malawi catchment possessed mtDNA that fell within the Mbuna haplogroup. Simulation analyses were consistent with hybridization rather than incomplete lineage sorting, and it was proposed that the hybridization event may have seeded the diversity within the Mbuna. However, neither that study nor the present study have shown that an adaptive shift to novel habitat was a direct consequence of hybridization. This process has, however, been demonstrated in sunflowers, where synthetic hybrids were created with adaptive phenotypes that closely resembled natural hybrid species, and showed significant similarities to them in analyses of quantitative trait loci (Rieseberg et al. 1996, 2003). Equivalent identification of cichlid genomic regions associated with deep-water habitats may help to identify if hybridization has led to novel gene combinations that enabled the colonization of this challenging habitat before widespread radiation began. Such traits are most likely associated with sensory ecology and diet. Thus, this group of deep-water cichlids represents a strong candidate system to test if hybridization can promote evolution of novel ecological characters that can in turn provide opportunity for rapid speciation.

Concluding Remarks

Is it possible that hybridization is at the base of other species-rich adaptive radiations? There are frequent reports of hybrid speciation among recently diverged species pairs (Seehausen 2004; Mallett 2007; Mavárez and Linares 2008). Although it is true that this pattern could be the result, rather than the cause, of the existence of many closely related and genetically weakly differentiated species generated by an adaptive radiation (Wiens et al. 2006), it nonetheless demonstrates the relative ease of hybridization among taxa many of which show striking differences in ecomorphological or sexually selected traits. Furthermore, there are many cases where nuclear and mtDNA trees are not entirely congruent, even in relation to relatively deep structures (Seehausen 2004). Although some of these incongruities may be due to lack of data and thus poor phylogenetic resolution, acquisition of further nuclear DNA information and more rigorous hypothesis testing may reveal additional cases of basal hybridization in major adaptive radiations. With indications that geographically restricted radiations may seed diversity on (sub)continental scales (Joyce et al. 2005; Salzburger et al. 2005), it seems possible that local radiations initiated through hybridization may make a major contribution to the generation of adaptive novelty and species richness.

Supplementary Material

Supplementary figure S1 and tables S1–S6 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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Appendix

Defining the Multispecies Groups

Five multispecies groups were delimited on the basis of mtDNA and nuclear data from this study and previous work: 1) *Diplotaxodon* contains all taxa in *Diplotaxodon* and *Pallidochromis* and was resolved as monophyletic using mtDNA (Shaw et al. 2000) and AFLPs (this study). 2) *Rhamphochromis* has been found to be monophyletic in mtDNA (Shaw et al. 2000; Genner, Nichols, Carvalho, Robinson, Shaw, Smith, et al. 2007) and AFLPs (this study). 3) Mbuna contains the genera *Abactochromis*, *Cynotilapia*, *Cyathochromis*, *Genyochromis*, *Gephyrochomis*, *Iodotropheus*, *Labeotropheus*, *Labidochromis*, *Metriaclima* (=*Maylandia*), *Melanochromis*, *Petrotilapia*, *Pseudotropheus*, and *Tropheops*. Representative taxa have been supported as monophyletic here using AFLPs and distinct from Deep-Benthic and Shallow-Benthic taxa in nuclear SNPs (Loh et al. 2008). They share an mtDNA haplogroup with Deep-Benthic, but not mtDNA haplotypes. 4) Deep-Benthic contains all *Alticorpus* and *Aulonocara* and many species of *Lethrinops* and *Placidochromis* (supplementary fig. S1, Supplementary Material online). Further analyses (this study) revealed that they share a mtDNA haplogroup

with Mbuna, but not mtDNA haplotypes. Representative taxa were not supported as being monophyletic with respect to the Shallow-Benthic group using AFLPs (this study), and they were not distinct from the Shallow-Benthic group using SNPs (Loh et al. 2008). However, they were significantly different from the Shallow-Benthic species group in permutation tests of AFLP data, supporting them as diverging lineages (this study). 5) Shallow-Benthic contains all remaining taxa within the radiation, with the possible exceptions of *A. calliptera* and *C. virginalis*. Shallow-Benthic have an mtDNA haplogroup distinct from both Mbuna and Deep-Benthic. Representative taxa were not monophyletic with respect to Deep-Benthic in AFLPs (this study) or SNPs (Loh et al. 2008) but were statistically significantly different from both Deep-Benthic and Mbuna in permutation tests of AFLP data (this study).

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