Evolution of a cichlid fish in a Lake Malawi satellite lake

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Allopatric divergence in peripheral habitats may lead to rapid evolution of populations with novel phenotypes. In this study we provide the first evidence that isolation in peripheral habitats may have played a critical role in generation of Lake Malawi's cichlid fish diversity. We show that Lake Chilingali, a satellite lake 11.5 km from the shore of Lake Malawi, contains a breeding population of *Rhamphochromis*, a predatory genus previously thought to be restricted to Lake Malawi and permanently connected water bodies. The Lake Chilingali population is the smallest known *Rhamphochromis*, has a unique male nuptial colour pattern and has significant differentiation in mitochondrial DNA from Lake Malawi species. In laboratory mate choice trials with a candidate sister population from Lake Malawi, females showed a strong tendency to mate assortatively indicating that they are incipient biological species. These data support the hypothesis that isolation and reconnection of peripheral habitats due to lake level changes have contributed to the generation of cichlid diversity within African lakes. Such cycles of habitat isolation and reconnection may also have been important in evolutionary diversification of numerous other abundant and wide-ranging aquatic organisms, such as marine fishes and invertebrates.

Keywords: peripatric speciation; adaptation; sexual selection; morphometrics; mitochondrial DNA; growth rates

1. INTRODUCTION

Species with pelagic life histories typically have large population sizes, high dispersal and little population subdivision within their present distributional ranges (Palumbi 1994). As such, speciation within these taxa is widely believed to have been initiated by large-scale historic population subdivision caused by the formation of land-mass or hydrographic barriers (Norris 2000), or perhaps by behavioural mechanisms such as natal homing (Beheregaray & Sunnucks 2001). However, there is evidence that isolation within peripheral basins as a consequence of water level changes may also lead to evolutionary diversification of species with pelagic life histories. For example, several marine species groups including fish and crustacea show molecular phylogeographic patterns consistent with peripheral isolation during Pleistocene sea level changes (Barber *et al.* 2002; McKinnon & Rundle 2002; Liu *et al.* 2007). Moreover, jellyfish confined to Holocene marine lakes on the island of Palau have undergone extensive allopatric diversification in molecular and morphological traits (Dawson & Hamner 2005). Taken together this evidence suggests that peripheral isolated water bodies may be a significant source of pelagic biodiversity.

Suggestions for a role of peripheral environments in evolutionary divergence of abundant and widely ranging

species echo an early proposal that haplochromine cichlid fish diversity of Lake Victoria may, at least in part, have been seeded by repeated climate-driven cycles of lake level change and allopatric divergence in peripheral water bodies (Brooks 1950, Greenwood 1965). This proposal was based primarily on evidence from the satellite Lake Nabugabo, estimated to have been separated from Lake Victoria around 5000 years ago (Stager *et al.* 2005) and containing five endemic haplochromine species delimited mainly using morphological characters (Trewavas 1933; Greenwood 1965). Although discussed in many reviews, and for years a textbook case of allopatric speciation, more recent studies have tended to focus on the conservation biology of cichlids of the Lake Victoria satellite lakes (Mwanja *et al.* 2001; Abila *et al.* 2004), and critically there have been no explicit tests of population genetic differentiation or behavioural reproductive isolation between main lake cichlids and satellite lake congeners. Moreover, the generality of a role for satellite lakes in haplochromine cichlid diversification has been uncertain because Lake Malawi also contains a species-rich and ecologically diverse haplochromine cichlid fauna, but no endemics have been recorded from peripheral water bodies.

In this study we report evidence indicating that satellite lakes may have been of substantial importance in the speciation of Lake Malawi's abundant and wide-ranging offshore haplochromine cichlids. Owing to the absence of genetic population subdivision and physical barriers within the main water body, these cichlids have been proposed as a potential example of sympatric speciation (Shaw *et al.* 2000). We found that Lake Chilingali, a

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Figure 1. (a) Lake Malawi, (b) Lake Chilingali is connected to Lake Malawi by a seasonal outflow, the Kaombe river, during the rainy season and (c) Lake Chilingali from the shoreline. Lake Malawi is approximately 474 m above sea level.

satellite lake 11.5 km inland of Lake Malawi, contains a permanent breeding population of the pelagic genus *Rhamphochromis*. Previously, species within this genus were thought to breed exclusively within the main lake body in offshore habitats (Turner *et al.* 2002). The purpose of this study was to test whether the Lake Chilingali population is genetically and phenotypically differentiated from congeneric species in Lake Malawi. Our results are consistent with proposals that isolation in satellite lakes can result in genetic differentiation, phenotypic diversification and speciation.

2. MATERIAL AND METHODS

(a) Study site

Lake Chilingali (southern limit 12°57'46" S, 34°12'49" E), also known as Lake Chikukutu, is approximately 5 km long, 1 km wide and had a maximum depth of 5.1 m when surveyed on 4 May 2005. The lake has a single seasonal outflow at the northern end, a tributary of the Kaombe river that flows directly into Lake Malawi during the rainy season (figure 1). Linear distance between Lake Chilingali (northern limit 12°55'30" S, 34°12'31" E), and the Kaombe river mouth (12°52'47" S, 34°18'05" E) to the east is 11.5 km. The present Lake Chilingali was formerly two separate lakes, Lake Chikukutu to the north and Lake Chilingali to the south. A weir constructed across the outflow around 1992 led to a rise in water level forming a continuous water body (Malawi Government Department of Surveys, M. J. G. Nkhotakota, 2004 personal communication). Both former lakes are on early European exploration maps of the region (Stewart 1883; Money & Kellett-Smith 1897), indicating an age of at least 124 years, but probably much older.

(b) Study taxa

Rhamphochromis is a monophyletic genus of haplochromine cichlid fishes (Moran *et al.* 1994) that are endemic to Lake Malawi and its catchment, including Chia Lagoon, Lake Malombe and the Shire River upstream of the Kapichira Falls. There are eight formally described *Rhamphochromis* species, but some may be synonymous. Formal descriptions are based on morphological criteria alone, often on poorly preserved material (Turner *et al.* 2004). All *Rhamphochromis* are elongate streamlined predators of fish and arthropods. They are maternal mouthbrooders: females spawn with territorial males, take eggs away in their buccal cavity and

brood clutches for periods of three to four weeks before releasing free-swimming offspring.

In April 2004, within Lake Chilingali we discovered a population of small *Rhamphochromis* hereafter referred to as *R. 'chilingali'*. We compared it with eleven *Rhamphochromis* taxa from Lake Malawi identified using morphological traits and mature male nuptial colour (table 1; figures 2 and 3). When combined, these characters have been found to be reliable indicators of biological species status in sympatric haplochromines (van Oppen *et al.* 1998; Genner *et al.* 2007). For simplicity we subsequently refer to these taxa as species, although recognize their provisional status. We were unable to collect sufficient samples of mature males of a further three species due to their rarity (*R. 'stripe'*, *R. 'longsnout north'* and *R. 'bigtooth'*), although all differ in male nuptial colour from *R. 'chilingali'* (electronic supplementary material), and maximum lengths (292, 230 and 221 mm SL, respectively) are considerably greater than that of *R. 'chilingali'* (106 mm SL). Collections were made between 6 May 2004 and 14 September 2005 from artisanal fisheries and Lake Malawi trawler catches.

(c) Morphological analyses

Adult males in breeding coloration were photographed in a standard orientation with the head pointing left. Images were analysed using a Relative Warp analysis, a landmark-based geometrical morphometric procedure to quantify relative shape variation among individuals. Images were calibrated with TPS DIG v. 1.37 (Rohlf 2001) and 25 landmarks were marked (electronic supplementary material). To account for bending of specimens, four landmarks (6, 10, 15 and 24; electronic supplementary material) were aligned using the 'unbend specimens' option in TPSUtil v. 1.33 (Rohlf 2004). Coordinates were aligned using Procrustes analysis, and size-corrected Relative Warp (RW) scores were generated in TPS Relative Warps v. 1.31 using $\alpha = 0$ (Rohlf 1993, 2003). This Relative Warp analysis was thus equivalent to a principal component analysis of the residual distances of coordinates from those of the Procrustes consensus configuration. Counts were made of dorsal fin spines, dorsal fin rays and anal fin rays. In total, 800 individuals of the 12 putative species were sampled for morphology (sample sizes are listed in table 1).

(d) Molecular analyses

DNA was isolated from ethanol-preserved fin tissue of adult males in full breeding coloration using the Promega Wizard

Table 1. Summary characteristics of samples of *Rhamphoichthys* 'chilingali' and 11 species from Lake Malawi. (All sampled individuals were adult males in breeding colour. Colour patterns: Y, yellow; H, hyaline (pale, translucent); W, white; G, grey; B, blue; +, present; -, absent; SL_s, standard length.)

male nuptial colour pattern																														
taxon	collection site(s)	anal										n mor-						n					n hap-							
		anal fin	egg-spots	dorsal fin	pelvic fin	gular	cheek	pectoral fin	caudal fin	caudal fin	dorsal body	dorsal body	ventral body	body stripe	dorsal striations	photo-metrics	mean SL (mm)	min SL (mm)	max SL (mm)	dorsal spines (mean)	dorsal spines (range)	dorsal rays (mean)		dorsal rays (range)	anal rays (mean)	anal rays (range)	mtDNA sequences (h)			
<i>R. 'chilingali'</i>	Lake Chilingali	Y	-	G	Y	Y	G	G	Y	G	G	Y	G	G	G	Y	-	-	20	93	76	106	18.2	18-19	11.8	11-12	9.3	9-10	29	16
<i>R. c.f. brevis</i>	Nkhata Bay; Southeast Arm	Y	-	G	Y	Y	G	G	G	G	G	Y	-	-	83	303	210	377	18.0	17-19	11.5	10-13	10.0	8-11	37	35				
<i>R. cf. ferox</i>	Southeast Arm	Y	-	G	Y	W	G	G	G	G	G	G	-	-	48	212	112	285	18.2	17-19	11.6	11-12	9.6	8-10	20	14				
<i>R. esox</i> (Boulenger)	Nkhata Bay; Southeast Arm	H-Y	+	G	Y	W	G	G	G	G	G	G	+	-	77	300	164	384	19.2	18-21	11.8	11-12	9.8	8-10	36	25				
<i>R. cf. woodi</i>	Nkhata Bay; Southeast Arm	Y	-	G	Y	W	G	G	G	G	G	G	-	-	62	325	266	411	17.7	16-19	11.5	10-13	9.7	8-11	36	30				
<i>R. 'grey'</i>	Nkhata Bay	Y	-	Y	Y	Y	G	G	Y-G	G	G	G	-	-	42	284	234	333	18.7	17-21	11.4	10-13	9.2	8-11	20	13				
<i>R. 'longfin yellow'</i>	Southeast Arm	Y	-	Y	Y	Y	Y	Y	Y-G	G	G	Y	-	-	31	195	156	233	17.3	16-18	11.5	10-13	9.3	9-11	18	17				
<i>R. longiceps</i> (Günther)	Nkhata Bay; Salima; Southeast Arm	H	+	G	Y	W	G	G	G	G	B/G	G	-	-	156	148	114	205	17.7	16-19	11.8	10-13	9.7	7-12	63	60				
<i>R. 'longiceps grey back'</i>	Nkhata Bay; Salima; Southeast Arm	H	+	G	Y	W	G	G	G	G	G	G	-	+	164	158	132	214	18.5	17-20	11.7	10-13	9.4	8-11	55	54				
<i>R. 'longiceps yellow belly'</i>	Salima	Y	-	Y	Y	Y	G	G	Y-G	G	G	Y	-	+	23	181	151	206	18.8	18-20	11.4	11-12	9.8	9-11	21	17				
<i>R. 'longsnout south'</i>	Southeast Arm	Y	-	G	Y	W	G	G	G	G	G	W	-	-	52	276	231	321	17.8	17-19	11.3	10-12	10.0	9-11	21	21				
<i>R. 'maldeco yellow'</i>	Southeast Arm	Y	-	Y	Y	Y	G	Y	Y-G	G	B/G	Y	-	-	42	267	202	316	18.0	17-19	11.6	10-13	9.6	9-10	20	18

Figure 2. Mean similarity of adult male body shape of *R. 'chilingali'* and 11 congeneric species from Lake Malawi. Error bars indicate one standard deviation from the mean. Deformation grids illustrate body shape differences in four different taxa.

DNA extraction kit. An approximately 1000 base pair section of the mtDNA control region was amplified using primers HapThr-2+4 and Fish12S (Joyce *et al.* 2005). 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 MgCl₂ (25 mM stock), 0.5 units *Taq* and 14.9 µl double-distilled water. 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 on Beckman–Coulter CEQ sequencers using the forward primer HapThr-2+4 and Quickstart cycle sequencing kits (Beckman–Coulter). In total, 376 *Rhamphochromis* were sequenced from the 12 putative species (sample sizes are listed in table 1). Sequences were checked by eye and aligned using CLUSTALW in DAMBE (Xia & Xie 2001); they ranged from 479 to 482 base pairs in length, with a final alignment of 482 base pairs. Analysis of molecular variance (AMOVA) in ARLEQUIN v. 2.000 (Schneider *et al.* 2000) was used to test for significant genetic structure between sympatric species. The null hypothesis of genetic homogeneity was tested using uncorrected *p*-distances, the *F*_{ST} estimator Φ_{ST} and 1023 permutations of sequences among species. For the phylogenetic analysis, sequences were aligned against those of outgroups, *Diplotaxodon greenwoodi* Stauffer & McKaye (GenBank AY911752) and *Nimbochromis linni* (Burgess & Axelrod) (GenBank AY913941), resulting in an alignment of 483 base pairs. The best-fitting model of sequence evolution was found using MRMODELTEST v. 2.1 (<http://www.ebc.uu.se/systzoo/staff/nylander.html>) in PAUP* v. 4.0b10 (Swofford 2002). The GTR+Γ+I model was selected and used in PhyML (Guindon & Gascuel 2003) to construct a rooted phylogram. New sequences generated have GenBank accession numbers EF683164 to EF683539.

(e) *Mate choice and growth trials*

Several lines of evidence suggest *Rhamphochromis longiceps* (Günther) as a candidate sister species to *R. 'chilingali'*. *Rhamphochromis longiceps* was the only Lake Malawi species found to share a mitochondrial haplotype with *R. 'chilingali'* (see §3). It is also the only species of the genus known to migrate to Lake Malawi's lagoons, where mouthbrooding females release young (M. J. Genner & G. F. Turner, 2004 unpublished data), providing a possible mechanism for colonization of a peripheral lake prior to its isolation. It is also the smallest known *Rhamphochromis* in Lake Malawi (table 1) and has a similar body shape to *R. 'chilingali'* (figures 2 and 3).

To determine whether *R. 'chilingali'* and *R. longiceps* females discriminated between males of these populations, we evaluated mate choice under laboratory conditions by allowing females to select males, and then determining paternity of offspring using microsatellite DNA allele sizing. Trials were divided into three experiments, each with two replicates and a minimum of five spawnings in each replicate. Each replicate used a different set of males. In experiment 1, wild-caught female *R. 'chilingali'* chose among two wild-caught males of each population. As wild-caught males differ considerably in size, they could not be size matched. A 240 cm×80 cm×40 cm aquarium was partially partitioned into four compartments by a plastic mesh grid, restricting movement of the larger *R. longiceps* and enabling the smaller *R. 'chilingali'* males to establish territories. After each spawning, *R. longiceps* males were moved to different compartments. In experiment 2, wild-caught female *R. 'chilingali'* were given a choice among two wild-caught adult *R. 'chilingali'* males and two first generation laboratory-reared male *R. longiceps*. Males were size matched; laboratory-bred *R. longiceps* matured at a smaller size than in the wild, while adult *R. 'chilingali'* attained larger sizes than

Figure 3. Maximum-likelihood phylogram of the 376 *Rhamphochromis* mtDNA control region sequences and two outgroups. Tree based on GTR + Γ + I model suggested by MrModeltest and parameters estimated by PhyML. *Rhamphochromis* 'chilingali' sequences occur at four places in the tree, indicated with light green and arrows. Note terminal nodes tend to be shared by the same species, indicating that lineage sorting is underway. All photographs are of males in nuptial coloration.

in nature when in laboratory conditions. A 240 cm $\times$ 80 cm $\times$ 40 cm aquarium was again partially partitioned into four compartments by a plastic mesh grid. This delimited territorial spaces, but enabled both males and females to pass freely among compartments. In experiment 3, first generation laboratory-reared female *R. longiceps* were given a

choice of a wild-caught adult *R. 'chilingali'* male or a size-matched first generation laboratory-reared male *R. longiceps*. In this experiment, mesh partitions could not be used owing to the larger size of the females, instead five potential territories within the 300 cm $\times$ 80 cm $\times$ 40 cm aquarium were delimited by solid, approximately 10 cm high partitions

Table 2. Statistical comparisons of genetic and morphological differentiation between *R.* 'chilingali' and Lake Malawi taxa. (*Significant at $\alpha=0.05$ after sequential Bonferroni correction for multiple comparisons. n.s., non-significant.)

taxon	taxon	genetic (mtDNA) ^a		morphometrics ^b		meristics ^c		
		Φ_{ST}	p	RW1, p	RW2, p	spines, p	dorsal rays, p	anal rays, p
<i>R.</i> 'chilingali'	<i>R. c.f. brevis</i>	0.282*	<0.001*	<0.001*	n.s.	n.s.	n.s.	<0.001*
<i>R.</i> 'chilingali'	<i>R. cf. ferox</i>	0.543*	<0.001*	n.s.	<0.001*	<0.001*	n.s.	0.043
<i>R.</i> 'chilingali'	<i>R. esox</i>	0.613*	<0.001*	n.s.	<0.001*	n.s.	n.s.	0.002*
<i>R.</i> 'chilingali'	<i>R. cf. woodi</i>	0.527*	<0.001*	<0.001*	n.s.	0.003*	n.s.	0.004*
<i>R.</i> 'chilingali'	<i>R. 'grey'</i>	0.364*	<0.001*	n.s.	<0.001*	<0.001*	0.006	n.s.
<i>R.</i> 'chilingali'	<i>R. 'longfin yellow'</i>	0.362*	<0.001*	<0.001*	n.s.	0.006*	n.s.	n.s.
<i>R.</i> 'chilingali'	<i>R. longiceps</i>	0.558*	<0.001*	n.s.	n.s.	0.028	n.s.	0.008
<i>R.</i> 'chilingali'	<i>R. 'longiceps grey back'</i>	0.293*	<0.001*	n.s.	n.s.	0.014	n.s.	n.s.
<i>R.</i> 'chilingali'	<i>R. 'longiceps yellow belly'</i>	0.416*	<0.001*	<0.001*	0.003*	n.s.	n.s.	n.s.
<i>R.</i> 'chilingali'	<i>R. 'longsnout south'</i>	0.413*	<0.001*	<0.001*	<0.001*	n.s.	0.002*	<0.001*
<i>R.</i> 'chilingali'	<i>R. 'maldeco yellow'</i>	0.406*	<0.001*	<0.001*	<0.001*	0.018	n.s.	n.s.

^a Pairwise permutation tests in ARLEQUIN following global AMOVA.^b Pairwise Tukey's honest significant differences, following global ANOVA.^c Pairwise Mann-Whitney *U*-tests.

placed across the base of aquarium. Only a single male from each population was used to alleviate within-population male–male aggression. In these experiments, offspring collection and paternity testing protocols followed Knight & Turner (2004), except that loci Ppun5 and Ppun7 were used (Taylor *et al.* 2002), and allele sizes were resolved on a Beckman CEQ sequencer against 400 base pair size standards (Beckman–Coulter).

To determine whether the comparatively small size of *R.* 'chilingali' was environmentally induced or possibly had a genetic basis, we compared the growth rates with those of *R. longiceps* in adjacent aquaria of 75 cm × 75 cm × 40 cm (for days 0–174) and 150 cm × 75 cm × 40 cm (for days 174–223). Each replicate used eight size-matched first generation laboratory-reared juveniles of each population fed ad libitum. All individuals were weighed and measured at the start and thereafter on days 54, 116, 174 and 223. At the end of this period, sexually mature males and mouthbrooding females of both populations were observed in both replicates. Females that are mouthbrooding during the trial were periodically manually stripped of eggs.

3. RESULTS

(a) Morphology and male nuptial coloration

Rhamphochromis males exhibited a limited range of colours, mainly black, white, silver and orange. The males of Lake Malawi species differed primarily in distributions of markings on the body and fins, and all differed from *R.* 'chilingali' in at least one aspect of male colour distribution (table 1). All species from Lake Malawi also differed from each other in male colour distribution except one sympatric pair (*R. cf. woodi* and *R. cf. ferox*) that differed considerably in shape (figure 2).

Relative Warp 1 captured 39.72% of total variation in adult male body shape among the 800 individuals. Greater RW1 scores were associated with comparatively shorter total head lengths, shorter snout lengths, greater body

depths and greater body lengths. Relative Warp 2 captured 20.74% of total variation and greater RW2 scores were associated with relatively smaller eyes and deeper bodies (figures 2 and 3). All other axes captured less than 7% of variation each. Global ANOVA revealed highly significant differences in both RW1 ($F_{11,788}=318.18$; $p<0.001$) and RW2 ($F_{11,788}=119.94$; $p<0.001$) between species. In *post hoc* comparisons, only *R. longiceps* and *R. 'longiceps grey back'* did not differ significantly in body shape from *R. 'chilingali'* in RW1 or RW2 (figures 2 and 3; table 2). Only *R. longiceps*, *R. 'longiceps grey back'*, *R. 'longiceps yellow belly'* and *R. 'maldeco yellow'* did not differ significantly from *R. 'chilingali'* in dorsal spine counts, dorsal ray counts or anal ray counts (table 2).

(b) Molecular differentiation

In the 376 *Rhamphochromis* sequenced, there were 311 haplotypes of which only eight were shared between more than one species (electronic supplementary material). A highly significant component of variation among the 376 mitochondrial sequences was explained by species (Global $\Phi_{ST}=0.383$; $p<0.001$; figure 3). In *post hoc* comparisons following sequential Bonferroni correction, all Lake Malawi species differed significantly from each other (mean $\Phi_{ST}=0.316$, range 0.013–0.668; electronic supplementary material) and also from *R. 'chilingali'* (mean $\Phi_{ST}=0.434$, range 0.282–0.612; table 3). Average divergence in mtDNA of *R. 'chilingali'* from Lake Malawi species was of the same magnitude as found among Lake Malawi species, but was significantly greater than the mtDNA divergence between allopatric conspecific populations (electronic supplementary material). *R. 'chilingali'* were present at four places in the maximum-likelihood phylogram (indicated by arrows in figure 3). Out of 29 specimens of *R. 'chilingali'*, 26 were resolved as falling into a single clade, and only one *R. 'chilingali'* haplotype (GenBank EF683260, EF683265) was shared with another taxon, *R. longiceps* (GenBank EF683416).

Table 3. Summary of results of laboratory mate choice trials between *R. longiceps* and *R. 'chilingali'*.

experiment	replicate	<i>R. 'chilingali'</i> ♂1 TL (mm)	<i>R. 'chilingali'</i> ♂2 TL (mm)	<i>R. longiceps</i> ♂1 TL (mm)	<i>R. longiceps</i> ♂2 TL (mm)	female population	female mean size brooding TL (mm)	female clutches analysed	<i>n</i> females spawned with <i>R. 'chilingali'</i> ♂	<i>n</i> females spawned with <i>R. longiceps</i> ♂	binomial test <i>p</i>
1	1	119	130	205	194	<i>R. 'chilingali'</i>	122	5	4	1	
	2	123	126	204	211	<i>R. 'chilingali'</i>	129	6	4	2	
	Combined							11	8	3	0.113
2	1	127	126	126	129	<i>R. 'chilingali'</i>	129.8	6	6	0	
	2	138	141	141	136	<i>R. 'chilingali'</i>	139.7	6	6	0	
	Combined							12	12	0	<0.001
3	1	174	—	177	—	<i>R. longiceps</i>	179.0	5	0	5	
	2	166	—	163	—	<i>R. longiceps</i>	171.2	5	0	5	
	Combined							10	0	10	<0.001

(c) Mate choice and growth trials

At least eight offspring from each clutch were genotyped, with the exception of three smaller clutches. In total, 243 offspring from 33 clutches were genotyped. The combination of alleles derived from both loci revealed no multiple paternity and left no possibility of ambiguity. In non-size-matched trials of experiment 1, female *R. 'chilingali'* preferentially mated with males from their own population on 8 out of 11 occasions. In size-matched trials of experiments 2 and 3, both female *R. 'chilingali'* and *R. longiceps* mated completely assortatively (table 3). *Rhamphochromis longiceps* grew significantly larger than *R. 'chilingali'* in experimental conditions (repeated measures ANOVA: total length, $F_{4,112}=9.35$, $p<0.001$; total mass: $F_{4,112}=14.00$, $p<0.001$; electronic supplementary material). *Rhamphochromis longiceps* females also matured at comparatively larger sizes, the smallest brooding female being 143 mm in total length, compared with 118 mm for *R. 'chilingali'*.

4. DISCUSSION

A role of satellite lakes in cichlid diversification was proposed in the pioneering studies of the haplochromines from Lake Nabugabo, a satellite lake of Lake Victoria (Trewavas 1933; Greenwood 1965). Subsequently, the role of satellite lakes in diversification of cichlid fish other than in Lake Victoria has rarely been considered. Most studies have focused on scenarios of sympatric speciation (Shaw *et al.* 2000), allopatric speciation through large-scale subdivision of the lake into independent basins during lake level changes (Verheyen *et al.* 1996) or allopatric speciation through small-scale intralacustrine fragmentation of shoreline habitat during lake level changes (van Oppen *et al.* 1997). Reviews of speciation in Lake Malawi have omitted the possibility of speciation in satellite lakes (Fryer & Iles 1972; Dominey 1984), dismissed it as inapplicable (Turner 1994; 1999) or suggested it would be unlikely to have been significant (Shaw *et al.* 2000). Nevertheless, by invoking a role for satellite lakes in speciation, we may be able to explain how high species richness can evolve in offshore cichlids that typically show little or no intraspecific population subdivision over lake-wide spatial scales (Shaw *et al.* 2000; Taylor & Verheyen 2001). Our discovery of *R. 'chilingali'* provides the first evidence of a population of an endemic and radiating Malawi haplochromine genus within a satellite lake. Moreover, the distinctiveness of this population indicates that it has undergone substantial evolutionary change consistent with a relatively long period of geographical isolation.

Employing morphological traits and male nuptial colours, we delimited 14 putative species of *Rhamphochromis* within Lake Malawi. All of these were distinct in male colour traits from *R. 'chilingali'*, and we have never encountered *Rhamphochromis* individuals from the main Lake Malawi basin that share male breeding coloration with *R. 'chilingali'*, despite extensive survey. Support for the Lake Malawi putative species representing biological species was provided by significant mtDNA genetic differentiation between sympatric taxa. However, because mitochondrial lineage sorting was far from complete, mtDNA should not be considered a reliable marker for reconstructing species-level phylogenies of the genus. It is

possible that nuclear markers such as amplified fragment length polymorphisms may be more informative (Allender *et al.* 2003). Nevertheless, on the basis of shared mtDNA haplotypes, similar morphology and known inshore stages to its life history, we identified *R. longiceps* as a candidate sister taxon to *R. 'chilingali'*. Our results show that these populations have developed a high degree of assortative mating; indeed, among size-matched individuals this was complete. As such, it seems that *R. 'chilingali'* is likely to be an incipient biological species. At the very least it can be regarded as a phylogenetic species on the basis of distinct adult morphology and male breeding colours. Moreover, its smaller size at maturity than *R. longiceps* suggests that it has evolved life-history adaptations within the peripheral lake.

Lake Chilingali presently lies some 30 m above the level of Lake Malawi, and although it is also possible that the ancestors of the *Rhamphochromis 'chilingali'* population gained access to the lake through a riverine connection, it was probably formerly connected to Lake Malawi. Evidence for historic high stands is provided by raised beaches (Moore 1897; Dixey 1926; Desmond-Clark 1966) and lacustrine molluscan fossil deposits above present lake levels (M. J. Genner, 2004 personal observations). For speciation and ecomorphological diversification in peripheral isolation to have been a major force in the evolution of the species flocks, satellite lake formation and loss must have been common processes since formation of major African rift basins. However, quantification of the rates of satellite lake formation and loss depends upon more accurate reconstructions of palaeoclimates, lake levels and basin morphology than are currently available. Where records of lake level change have been studied, it is clear that these have been both frequent and of substantial magnitude: changes as great as 100–200 m have occurred during the last 15 700–23 000 years (Johnson *et al.* 2002). As such, it seems probable that many now-extinct satellite lakes are likely to have existed around Lake Malawi, as well as other African lakes.

Here we have provided evidence consistent with the evolution of a novel phenotype and reproductive isolation within a satellite lake of Lake Malawi. Thus, isolation in satellite lakes provides a plausible mechanism for generating faunal diversity within large lakes, although only alongside other proposed evolutionary scenarios including sympatric speciation and allopatric speciation within the main lake basins. Diversification in peripheral water bodies may also partially explain speciation in other pelagic taxa that are typically abundant and free ranging within their primary habitat, but occasionally become isolated in peripheral environments, such as marine fish and invertebrates (Dawson & Hamner 2005). Finally, small lakes such as Lake Chilingali are vulnerable to overfishing and stocking with non-native species, particularly when it is not appreciated that they contain unique species. This threat adds to risks of increased sediment load, eutrophication and water extraction due to changes in agricultural land use practices in sub-Saharan Africa. Such water bodies require careful monitoring and management to ensure sustainable use and preservation of their unique biodiversity.

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