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Reproductive isolation among deep-water cichlid fishes of Lake Malawi differing in monochromatic male breeding dress

MARTIN J. GENNER,* PAUL NICHOLS,* GARY R. CARVALHO,† ROSANNA L. ROBINSON,* PAUL W. SHAW‡ and GEORGE F. TURNER*

**Department of Biological Sciences, University of Hull, Hull HU6 7RX, UK, †School of Biological Sciences, University of Wales Bangor, Bangor, Gwynedd LL57 2UW, UK, ‡School of Biological Sciences, Royal Holloway, University of London, Egham, Surrey TW20 0EX, UK*

Abstract

Male nuptial colour hues are important for the maintenance of reproductive isolation among cichlid fish species, and environmental changes that lead to narrower light spectra can lead to hybridization. However, cichlid species can naturally co-occur in narrow light spectrum habitats, such as turbid shallow lakes and the deep benthic zones of African rift lakes. Closely related species from narrow light spectrum habitats tend to differ little in the palette of male nuptial colours, thus for these taxa differences in colour patterns may be more important than differences in colour hue for species recognition. To investigate this hypothesis we examined morphometric and genetic differentiation among males of four sympatric putative species within the deep-water genus *Diplotaxodon*. These taxa live in a narrow-light spectrum environment where only blue light is present, and males differ primarily in 'monochromatic' black, white and silver patterning of the body and fins. Significant genetic differentiation was present among taxa in both microsatellite DNA and mitochondrial DNA, including one pair with no significant morphometric differentiation. Thus, these taxa represent reproductively isolated biological species, a result consistent with male nuptial patterning being important for species recognition and assortative mating. As such, we suggest that narrow-light spectra need not always represent barriers to effective visually mediated mate recognition.

Keywords: mitochondrial DNA sequences, reproductive isolation, selection, spectral sensitivity

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Introduction

Colour is used extensively by fish for visual communication, particularly in mate choice decisions where it is used as a cue to recognize conspecifics (Seehausen & van Alphen 1998) and to estimate the fitness of potential mates (Milinski & Bakker 1992). Among the rapidly radiating cichlid fishes of African lakes, morphologically similar sympatric populations differing in male nuptial colour hues are commonplace (Ribbink *et al*. 1983; Allender *et al*. 2003). Where tested, these populations have been shown to be reproductively isolated using field observations, molecular analyses of wild fish and laboratory mate-choice experiments (Seehausen & van Alphen 1998; van Oppen *et al*. 1998; Knight & Turner 1999). This, together with evidence of intraspecific sexual selection in cichlids based on colour (Maan *et al*. 2004; Pauers *et al*. 2004), suggests that male colour traits are important in the maintenance of reproductive isolation among recently diverged species. Possible mechanisms driving divergence of male colour include male-male competition (Seehausen & Schluter 2004) and female mate choice (Knight & Turner 2004).

The range of colour hues visible to fishes is dependent not only on their optical capabilities, but also upon local environmental regimes. Some habitats have narrow ambient light spectra due to selective absorption of wavelengths. For example, in turbid rivers and shallow lakes, blue wavelengths are rapidly filtered out in surface waters, leaving only green, yellow and red light. In contrast, in the clear waters of deep lakes, green, yellow and red wavelengths are filtered out with increasing depth, leaving only

Correspondence: Martin J. Genner, Fax: +44 (0)1482 465458; E-mail: m.genner@hull.ac.uk

blue light (Seehausen *et al*. 2003). Light conditions significantly affect fish behaviour, including foraging (De Robertis *et al*. 2003) and mate choice (Seehausen *et al*. 1997), and have also been linked to spatial patterns of biodiversity (Rodríguez & Lewis 1997; Rowe *et al*. 2000). Notably, it has been shown that environments with narrow-light spectra tend to contain lower cichlid species richness than shallow clear-water broad-light spectrum lacustrine habitats (Seehausen *et al*. 1997). This may be linked to gradients that often co-vary with light spectrum breadth, such as benthic productivity and niche diversity. However, it has been proposed that low species richness in habitats with narrow-light spectra is linked to a low incidence of assortative mating based on male nuptial colour traits, which has in turn constrained speciation and/or promoted hybridization (Seehausen *et al*. 2003). This hypothesis primarily stems from evidence of introgression among a pair of species of *Pundamilia* from Lake Victoria in which males of both species share a similar body pattern but differ primarily in the distribution of red and blue colour hues on the body and fins. In this system, it is only in clear water that females can identify conspecific males consistently (Seehausen *et al*. 1997). Importantly however, while light regimes may constrain cichlid speciation by weakening assortative mating, closely related cichlid species can co-occur in deep-water or turbid habitats without apparent evidence of hybridization. Indeed, many species are found exclusively within these habitats, and since phylogenetic reconstructions of cichlid fishes clearly reveal that closely related species tend to share the same broad habitat range (Shaw *et al*. 2000; Allender *et al*. 2003), it is also possible that speciation has taken place within habitats with narrow light spectra.

Narrow-light spectrum habitats not only contain fewer species than clear-water environments, but it has been shown that the range of male colours can be more restricted (Seehausen *et al*. 1997). For example, in turbid river and shallow lake systems, male haplochromines tend to be green and/or red, while in clear littoral waters of Lake Malawi, a wider variety of colours is present (Konings 2001). This may, at least in part, be linked to environmental influences on cichlid vision. Support for adaptive differences in cichlid spectral sensitivity is accruing rapidly (Sugawara *et al*. 2002, 2005; Carleton *et al*. 2005; Parry *et al*. 2005; Spady *et al*. 2005). Critically, there is also strong evidence for phylogenetic conservation of optically related genetic and phenotypic traits. For example, in a study of a wide range of cichlid fishes, species from the closely related deep-water Lake Malawi genera, *Pallidochromis* and *Diplotaxodon*, share a mutation at a rhodopsin gene *RH1* not shared by any other taxa sampled (Sugawara *et al*. 2005). Taken together, this evidence suggests that adaptations to narrow-light spectra have evolved prior to speciation within narrow-light spectrum environments. Thus, how can assortative mating evolve and be maintained among cichlids in habitats where both the visible light range and the male nuptial colour range are strictly limited? It may be that auditory or olfactory cues are used (Amorim *et al*. 2004; Plenderleith *et al*. 2005), but it is also possible that some cichlids have overcome hue range limitations by preferentially selecting mates on the basis of differences in body patterns within a limited visible colour palette.

The cichlid fishes of genus *Diplotaxodon* are mid-water zooplanktivores and piscivores endemic to Lake Malawi. All species breed near the bottom at depths between 40 m and 200 m (Turner *et al*. 2004), where only blue light will be present. Seven species of *Diplotaxodon* have been formally described, delimited almost exclusively using morphometric procedures (Turner *et al*. 2004). However, several morphologically similar putative species have been recorded, differing primarily in 'monochromatic' male nuptial patterning of black, white and silver on body and fins. These taxa represent an ideal case to examine the roles of male patterning in the maintenance of reproductive isolation in a narrow-light spectrum environment. We studied four sympatric putative species of *Diplotaxodon* differing in male breeding patterning (Fig. 1; Table 1). We used multivariate assessment of direct morphometric measurements and landmark-based image data to test for differences in body shape, while genetic differentiation among putative species was tested using nuclear microsatellite DNA and mitochondrial (mt)DNA control region sequences.

Methods

Sampling

Samples were collected from Ngara (10°13′59 S, 34°06′27 E) on the northwestern shore of Lake Malawi between 20 and 23 July 2004. Four sympatric putative species, as diagnosed on the basis of male breeding pattern (Table 1), were collected from deep-water gill nets. All taxa were reproductively active during the sampling period as indicated by mature gonads. Nets were set by artisanal fishermen overnight in offshore waters. The fishermen reported that nets were set between 50 m and 100 m depth. From 20 to 51 individuals of each morph were collected. Prior to preservation in formalin (paraformaldehyde ~30 g per 20 L), each fish was labelled, photographed and a sample of fin tissue was taken from the right pectoral fin and placed in absolute ethanol for later molecular analyses. Preserved whole fish were later placed in 70% ethanol.

Morphometric analyses 1 — direct measurements and counts

Twenty-two measurements were made of each fish according to protocols described in Barel *et al*. (1977) and Snoeks (2004): total length (TL), standard length, head

Table 1 Summary characteristics of focal taxa

Variable	*D.* 'macrops ngulube'	*D.* 'macrops black dorsal'	*D.* 'macrops offshore'	*D.* 'limnothrissa black pelvic'
Dorsal fin	white, fading black anterior	black, white lappets	white, fading black anterior	white, fading black anterior
Dorsal body	white	black	silver, black near dorsal	white
Dorsal body striations	slight, anterior only	absent	absent	conspicuous, entire back
Ventral Body	black	silver	silver	black
Pelvic Fin	black, white leading edge	hyaline	black, white leading edge	black, white leading edge
Anal fin	black, white leading edge	hyaline	hyaline	black, white leading edge
Head ventrally	black	silver	silver	black
Head Dorsally	white above eye	black snout and above eye	black snout and above eye	white above eye
Caudal fin Dorsal	white	black	hyaline	white
Caudal fin Ventral	black	hyaline	hyaline	white
n morphometrics	20	50	43	51
Mean SL (mm)	116.8	119.0	116.3	132.7
Range SL (mm)	108.1–130.0	105.5–139.1	97.5–130.5	117.8–145.6
Mean dorsal spines	14.2	13.6	13.7	15.0
Range dorsal spines	13–15	12–15	12–15	12–17
Mean dorsal rays	11.0	11.1	10.9	11.5
Range dorsal rays	10–12	9–13	9–14	9–14
Mean anal rays	10.5	10.4	10.1	10.1
Range anal rays	9–12	8–12	9–11	8–12
n mtDNA sequenced	20	26	27	25
n unique haplotypes	16	26	27	25

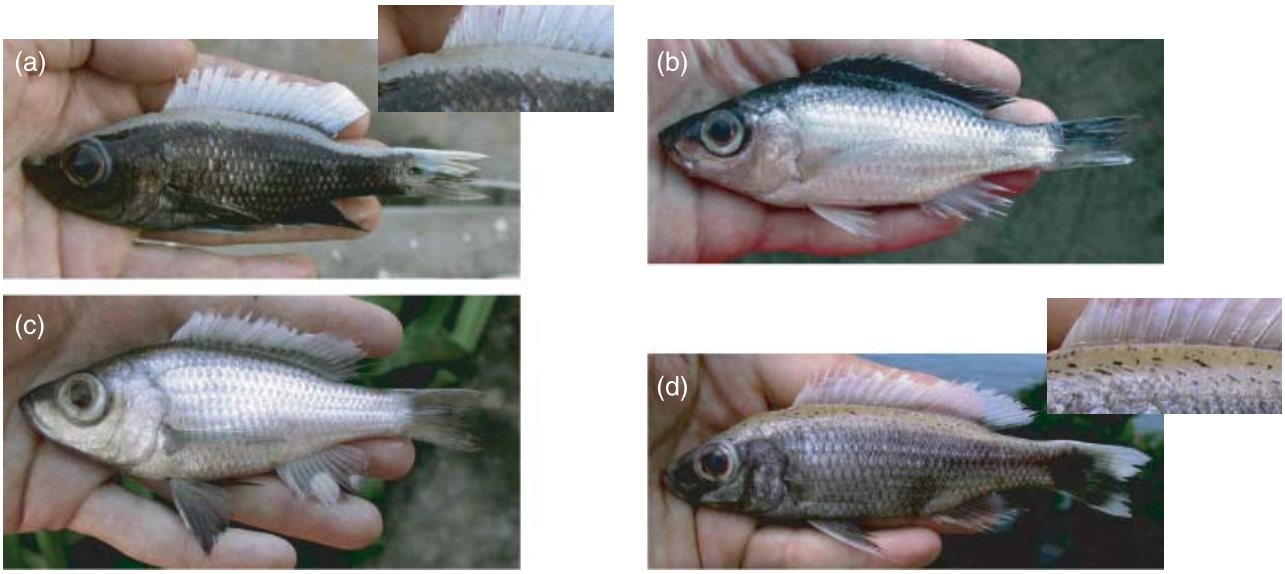

Fig. 1 Males of the four putative species of *Diplotaxodon;* (a) *D*. 'macrops ngulube'; (b) *D*. 'macrops black dorsal'; (c) *D*. 'macrops offshore'; (d) *D*. 'limnothrissa black pelvic', Expanded portions show the absence and presence of dorsal body striations on *D*. 'macrops ngulube' and *D*. 'limnothrissa black pelvic', respectively.

length, body depth, caudal peduncle length, caudal peduncle depth, snout length, predorsal distance, prepelvic distance, dorsal fin base length, anal fin base length, pre-orbital head length, pre-orbital depth, lower jaw length, interorbital eye width, pre-orbital width, cheek depth, head depth, horizontal eye diameter, vertical eye diameter, pectoral fin length and pelvic fin length. Measurements were made using digital callipers. To account for effects of body size, data were regressed against TL, and residuals of the remaining 21 variables were entered into a principal component analysis. Statistical significance of among-taxa differences along principal component (PC) axes were tested using ANOVA in STATISTICA 5. Post-hoc Tukey's honestly significant difference (HSD) tests identified putative species differing significantly along axes, and variables contributing the most to axes were identified from PC loadings greater or less than 0.25. Numbers of dorsal fin spines, dorsal fin rays, anal fin spines and anal fin rays were counted. Significance of differences in count frequency distributions were tested using Kolmogorov-Smirnov 2-sample tests in STATISTICA 6. Significance of all multiple comparisons in this study was tested using the sequential Bonferroni procedure (Sokal & Rohlf 1995).

Morphometric analyses 2 — image analysis

Landmark-based morphometrics offer an advantage over linear measurements because they also capture variation in geometric relationships between points, and as such are becoming increasingly used in fish morphometric studies (e.g. Taylor *et al*. 2006). However, they can have limitations. At present two-dimensional images are most commonly used, and when examining lateral profiles measurements such as body and head width are unable to be incorporated. Due to this, we chose to use both landmark-based and standard measurements. Here, all individuals were photographed in a standardized orientation with the head pointing left. Images were calibrated to scale using TPSDIG 1.37 (Rohlf 2001), and 25 landmarks were marked (Fig. 2). To account for bending of specimens during preservation, four landmarks (6, 10, 15, 24; Fig. 2) were aligned using the 'unbend specimens' option in TPSUTIL 1.33 (Rohlf 2004). Coordinates were then aligned using Procrustes analysis in TPS RELATIVE WARPS 1.31 (Rohlf 2003) and size-corrected relative warp (RW) scores were generated. Statistical significance of among-taxa differences along RW axes were tested using ANOVA and post-hoc Tukey's HSD tests in STATISTICA 6.

Molecular analyses 1 — microsatellite DNA allele frequencies

DNA was extracted from ethanol preserved fin tissue using the Promega Wizard DNA extraction kit. Samples were screened at eight polymorphic microsatellite loci: UNH154 (Kellogg *et al*. 1995), Pzeb3, Pzeb5 (van Oppen *et al*. 1997), TmoM5 (Zardoya *et al*. 1996), Ppun5, Ppun7, Ppun21 and Ppun32 (Taylor *et al*. 2002) on a Beckman CEQ sequencer. The 10-μL polymerase chain reactions (PCRs) consisted of 1 μL (~20 ng) of template DNA, 1.0 μm each

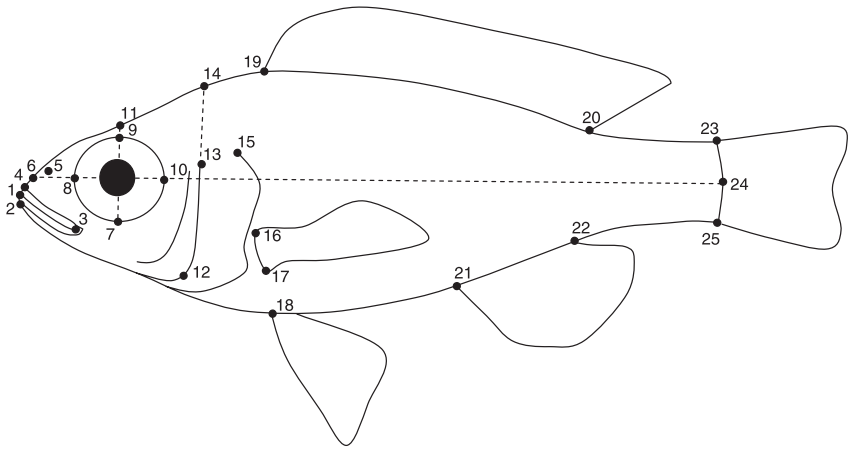

Fig. 2 Twenty-five landmarks used in the landmark-based geometric morphometric analyses.

Table 2 Genetic variability at eight microsatellite loci in populations of focal taxa. N_a, number of alleles; H_E, expected heterozygosity; H_O, observed heterozygosity

Locus	Variable	*D.* 'macrops ngulube'	*D.* 'macrops black dorsal'	*D.* 'macrops offshore'	*D.* 'limnothrissa black pelvic'
	n individuals	20	49	102	51
UNH154	N_a	18	22	33	36
	H_E	0.954	0.963	0.953	0.965
	H_O	1.000	0.815	0.717	0.956
PZEB3	N_a	4	7	8	12
	H_E	0.664	0.588	0.617	0.637
	H_O	0.737	0.602	0.489	0.698
PPUN5	N_a	15	18	25	27
	H_E	0.946	0.936	0.923	0.941
	H_O	1.00	0.957	0.917	0.955
PPUN7	N_a	20	36	47	34
	H_E	0.953	0.977	0.975	0.967
	H_O	0.947	0.907	0.896	0.977
PZEB5	N_a	1	2	5	5
	H_E	—	0.124	0.0410	0.196
	H_O	—	0.109	0.0208	0.188
PPUN32	N_a	4	7	9	8
	H_E	0.583	0.624	0.518	0.701
	H_O	0.850	0.650	0.542	0.625
TMOM5	N_a	11	12	18	16
	H_E	0.857	0.772	0.700	0.887
	H_O	0.706	0.740	0.652	0.886
PPUN21	N_a	17	21	26	20
	H_E	0.941	0.943	0.943	0.946
	H_O	1.000	0.929	0.979	1.000

— monomorphic.

primer (one of which was dye-labelled), 200 μm of each dNTP, 0.5 U of *Taq* polymerase (Bioline), 1 μL of 10× reaction buffer and 2.5 mm $MgCl_2$ (Bioline). Thermocycling conditions for particular loci were as described in source publications.

Descriptive statistics including number of alleles (N_a), expected heterozygosity (H_E) and observed heterozygosity (H_O) were compiled using ARLEQUIN 2.0 (Schneider *et al.* 2000) (Table 2). Deviations from Hardy–Weinberg equilibrium were examined using a Markov chain method exact tests in ARLEQUIN 2.0, with significance levels determined using a chain length of 100 000 and 1000 dememorization steps. The null hypothesis of genetic homogeneity among putative species was tested using three methods with differing properties (see Balloux & Lugon-Moulin 2002).

First, analysis of molecular variance (AMOVA) (Excoffier *et al*. 1992) in ARLEQUIN 2.0 was used to estimate global and pairwise F_{ST} (Weir & Cockerham 1984) under the infinite allele model with significance estimated using 1023 permutations. Next, unbiased R_{ST} (that uses the stepwise mutation model) was estimated using r_{st}CALC (Goodman 1997) and significance was estimated with 5000 permutations. Finally, Exact tests were used to test for differences in allele frequencies among putative species using GENEPOP 3.4 (Raymond & Rousset 1995).

Molecular analyses 2 — mtDNA sequencing

As a comparison to nuclear genome (microsatellite) variation, we utilized mtDNA sequence variation among the putative species. This approach that has been successfully employed for examining population structuring in other lacustrine cichlid flocks (Abila *et al*. 2004; Barluenga & Meyer 2004; Salzburger *et al*. 2005). DNA was isolated from ethanol-preserved fin tissue using the cetyltrimethyl ammonium bromide (CTAB)-chloroform method and a ~900-bp section of the mtDNA control region was amplified using forward primer HapThr-2+4 (5′-CCTACTCCCAAAGCTAGGATC-3′) and reverse primer Fish12s (5′-TGCGGAGACTTGCATGTGTAAG-3′) following Joyce *et al*. (2005). All 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 U *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 a Beckman CEQ sequencer using the forward primer HapThr-2+4 and Quickstart cycle sequencing kits (Beckman-Coulter), according to manufacturer's protocol.

Sequences were checked by eye and a 489-bp alignment was generated using CLUSTAL W in DAMBE (Xia & Xie 2001) including an outgroup sequence of *Rhamphochromis esox* (Boulenger) (GenBank AF298913). Analysis of molecular variance (AMOVA) (Excoffier *et al*. 1992) in ARLEQUIN 2.0 was used to test for significant genetic structure among sympatric putative 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 putative species. This method tests the hypothesis that there is overall higher genetic similarity among individuals within populations than between populations. F_{ST} was not calculated from mtDNA haplotype frequencies due to the high number of unique haplotypes. Evolutionary relationships among all haplotypes were examined using a distance matrix approach in PAUP* (Swofford 2002). The neighbour-joining algorithm was used to construct a rooted phylogram. Sequences have GenBank Accession nos DQ991517–DQ991614.

Results

Morphometric analyses 1 — standard measurements and meristic counts

Principal component axes 1–3 comprised 50.8%, 6.1%, and 5.5% of total variation, respectively. All other axes comprised less than 5% of the variation among individuals each. Global tests indicated significant variation among putative species along PC1 ($F_{3,171}$ = 38.31; $P < 0.001$) and PC3 ($F_{3,171}$ = 4.19; $P = 0.007$), but no significant differentiation along PC2 ($F_{3,171}$ = 1.22; $P = 0.31$). PC1 scores for *Diplotaxodon* 'limnothrissa black pelvic' were significantly greater than all other putative species (Fig. 3a; Table 3), which taken together with the PC1 loadings indicated that *D*. 'limnothrissa black pelvic' had shorter head measurements, smaller eye size, and narrower body and head depths than all other taxa. PC3 scores for *Diplotaxodon* 'macrops black dorsal' were significantly greater than *D*. 'macrops ngulube', which taken together with PC3 loadings indicated that *D*. 'macrops

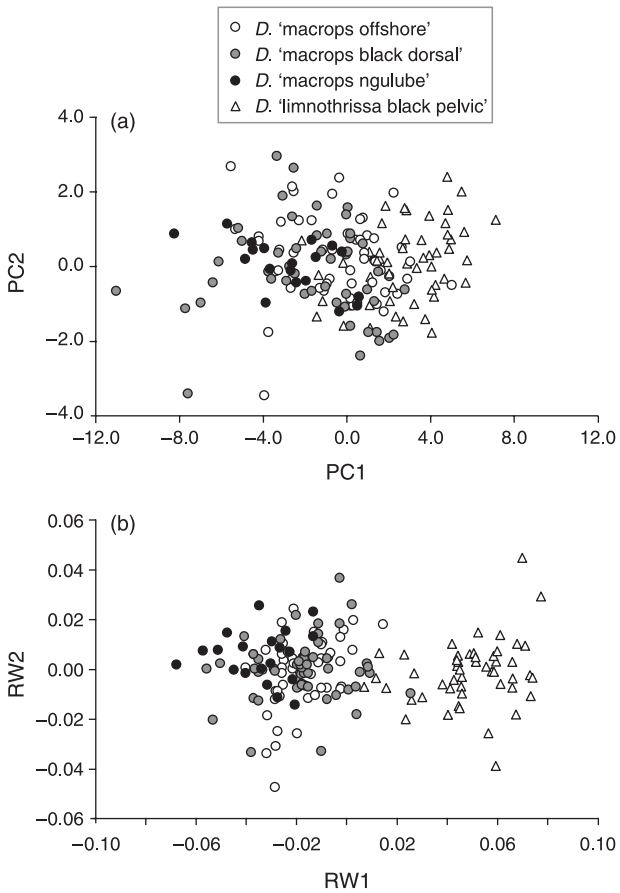

Fig. 3 Size-standardized body shape variation among the four *Diplotaxodon* putative species captured using (a) standard measurements and Principal Component (PC) analysis and (b) landmark-based Relative Warp (RW) morphometric analyses.

Table 3 Summary of pairwise statistical comparisons between focal taxa. MN, *D.* 'macrops ngulube'; MBD, *D.* 'macrops black dorsal'; MO, *D.* 'macrops offshore'; LBP, *D.* 'limnothrissa black pelvic'. Microsatellite results are across all eight loci. Sequential bonferroni corrections were applied to each test independently

Character	MN:MBD	MN:MO	MBD:MO	MN:LBP	MBD:LBP	MO:LBP
Morphometric						
PC1	NS	$P = 0.014$*	NS	$P < 0.001$*	$P < 0.001$*	$P < 0.001$*
PC2	NS	NS	NS	NS	NS	NS
PC3	$P < 0.005$*	NS	NS	NS	NS	NS
RW1	$P < 0.001$*	$P < 0.001$*	NS	$P < 0.001$*	$P < 0.001$*	$P < 0.001$*
RW2	NS	NS	NS	NS	NS	NS
RW3	NS	NS	NS	NS	NS	NS
Meristic						
Dorsal spines	NS	NS	NS	$P < 0.005$*	$P < 0.001$*	$P < 0.001$*
Dorsal rays	NS	NS	NS	NS	NS	$P < 0.005$*
Anal rays	NS	NS	NS	NS	NS	NS
Microsatellite DNA						
F_{ST}	0.034	0.031	0.007	0.036	0.020	0.004
F_{ST} significance	$P < 0.001$*	$P < 0.001$*	$P = 0.003$*	$P < 0.001$*	$P < 0.001$*	$P = 0.021$*
R_{ST}	0.057	0.064	0.012	0.029	0.021	0.016
R_{ST} significance	$P < 0.001$*	$P < 0.001$*	$P = 0.016$*	$P = 0.024$*	$P = 0.009$*	$P = 0.007$*
Exact test	$P < 0.001$*	$P < 0.001$*	$P = 0.028$*	$P < 0.001$*	$P < 0.001$*	$P < 0.001$*
Mitochondrial DNA						
Φ_{ST}	0.367	0.140	0.082	0.037	0.290	0.082
Φ_{ST} significance	$P < 0.001$*	$P < 0.001$*	$P < 0.001$*	$P = 0.042$*	$P < 0.001$*	$P < 0.001$*

*significant following sequential Bonferroni correction for multiple comparisons; NS, nonsignificant.

black dorsal' had shorter pelvic and pectoral fin lengths and greater caudal peduncle depth and preorbital distances than *D.* 'macrops ngulube'. *D.* 'limnothrissa black pelvic' had significantly more dorsal spines than all other taxa, and significantly more dorsal rays than *D.* 'macrops offshore'. There were no significant differences in anal ray counts among putative species (Tables 1 and 3).

Morphometric analyses 2 — image-based morphometrics

Relative warp axes 1–3 comprised 56.1%, 7.5% and 6.9% of total shape variation, respectively. All other axes comprised less than 5% of the variation among individuals each. Global tests indicated significant variation along RW1 ($F_{3,171} = 251.97$; $P < 0.001$), but not along RW2 ($F_{3,171} = 1.82$; $P = 0.15$) or RW3 ($F_{3,171} = 1.24$; $P = 0.30$). In post-hoc comparisons, *D.* 'limnothrissa black pelvic' had significantly greater RW1 scores than all other putative species while *D.* 'macrops ngulube' had significantly lower RW1 scores (Fig. 3; Table 3). Visualization of the mean RW1 scores of putative species on deformation grids revealed greater RW1 scores corresponded to a narrowing of body depth and contractions of head and eye size (Fig. 4). Overall RW1 scores correlated significantly with PC1 scores ($r = 0.73$, $P < 0.001$), but there were no significant correlations between PC2 and RW2 ($r = 0.10$, $P = 0.19$), or between RW3 and PC3 ($r = -0.01$, $P = 0.87$).

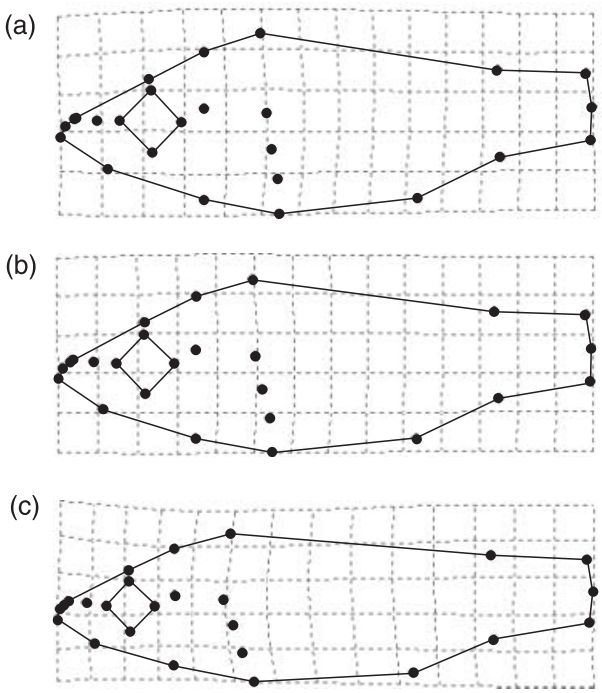

Fig. 4 Deformation grids showing body shape along Relative Warp (RW) Axis 1. Illustrations represent mean RW1 values for (a) *D.* 'macrops ngulube'; (b) *D.* 'macrops offshore' and *D.* 'macrops black dorsal' combined; (c) *D.* 'limnothrissa black pelvic'.

Microsatellite DNA differences among putative species

When pooled across putative species, significant departures ($P < 0.05$) from Hardy–Weinberg equilibrium were detected at two of the eight loci (UNH154, Ppun7), but none was significant following sequential Bonferroni correction for multiple comparisons ($\alpha = 0.05$). A global AMOVA revealed a small yet highly significant ($P < 0.001$) component of total variation present between putative species (global $F_{ST} = 0.014$; 1.39% of total variation). Post-hoc pairwise comparisons revealed significant differentiation between all putative species using F_{ST}, R_{ST} and Exact tests (Table 3). Notably F_{ST} values between putative species were lower than those observed among sympatric Lake Malawi rock cichlid species ($F_{ST} \sim 0.10$; Rico *et al*. 2003), but similar to a previous study quantifying interspecific differentiation among *Diplotaxodon* taxa (F_{ST} 0.011 to 0.015; Shaw *et al*. 2000). Low observed F_{ST} between *Diplotaxodon* putative species may, at least in part, be consequential of their large population sizes (Kanyerere *et al*. 2005). Larger populations are known to possess greater microsatellite allele size homoplasy potentially leading to underestimates of genetic differentiation among populations (Estoup *et al*. 2002).

Mitochondrial DNA differentiation among putative species

The aligned 489-bp matrix of 98 sequences contained 118 polymorphic sites. In total, there were 94 unique haplotypes, none of which was shared between putative species. Only *D*. 'macrops ngulube' had haplotypes shared by more than one individual (Table 1). The neighbour-joining phylogram showed incomplete lineage sorting across all four taxa (Fig. 5). A global AMOVA revealed a highly significant ($P < 0.001$) component of total variation between putative species (17.59%), with a global between-species Φ_{ST} of 0.176. Post-hoc tests showed significant differences between all putative species. Taken together, the mtDNA are consistent with the interpretation that all four taxa are reproductively isolated species.

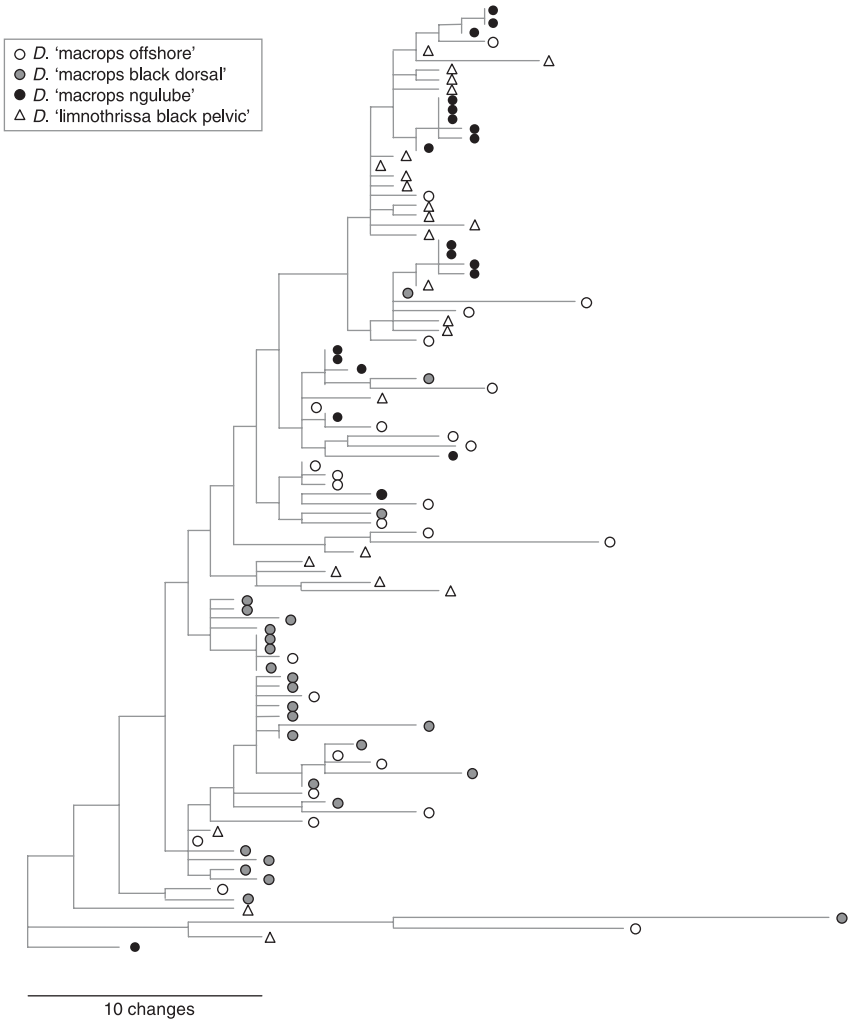

Fig. 5 Neighbour-joining phylogram of *Diplotaxodon* sequences. The tree was rooted using a sequence of the Lake Malawi endemic haplochromine *Rhamphochromis esox*. Note that the most recently diverged haplotypes tend to be shared by individuals of the same putative species, indicating lineage sorting has begun.

Discussion

Male breeding dress, mate recognition and species diagnosis

Our results showed that all four sympatric and breeding *Diplotaxodon* taxa studied possessed distinct and significant divergence in both nuclear and mitochondrial DNA markers. Thus, the results support the concept that these taxa represent reproductively isolated species, and that species within this genus differ in monochromatic patterning on the bodies and fins of males. Hence nuptial pattern information can sometimes be a valuable tool for distinguishing biological species within cichlid taxa, including *Diplotaxodon*. During recent field trips (2004 and 2005), we identified at least 14 *Diplotaxodon* taxa on the basis of body shape and male nuptial pattern. In the light of the present study, it seems likely that most of these represent biological species and that the species richness of *Diplotaxodon* and other offshore and deep-water cichlid fishes is currently underestimated.

Pattern variation in fishes

Cichlid colour patterning is dependent on the size, sex and social dominance of individuals. In haplochromine cichlids, two categories of patterns can be distinguished: (i) nuptial patterns of adult reproductively active courting males and (ii) 'markings' of nonreproductively active males, females and juveniles. This latter category is prominent among cichlids living in open water or over sand, where most nonreproductively active fish have prominent dark pigment markings such as spots or stripes over counter-shaded sand coloured or silver backgrounds. In previous studies such nonbreeding markings have been examined in relation to ecological and behavioural characters or phylogenetic relationships, and it has been shown these melanin markings are phylogenetically conservative (Eccles & Trewavas 1989) and influenced by the preferred habitat of the taxon (Seehausen *et al*. 1999).

In *Diplotaxodon*, despite being monochromatic, male nuptial pattern differences appear to be decoupled from phylogenetic and selective influences on the nonterritorial body pattern. All known female and juvenile individuals of this genus have plain silver bodies, largely translucent fins and lack any conspicuous melanic markings. Unlike many other many other Malawi cichlids, such as the rocky habitat 'mbuna', male *Diplotaxodon* only develop conspicuous nuptial patterning during a breeding season that for most known taxa extends from February to June (Kanyerere *et al*. 2005; M.J.G. personal observation). Throughout the remainder of the year, there is minimal sexual dimorphism, and males of different taxa are virtually indistinguishable. Such seasonality, taken together with the variety of male monochromatic patterns that are present in sympatry in this narrow light spectrum environment, suggests the evolution of a sexually selected mate recognition system based on male nuptial patterning. It would thus appear that closely related Lake Malawi cichlid species can diverge on both male nuptial colour hue and nuptial pattern axes, but also that visual adaptive and environmental constraints operate on the evolution of male phenotypes.

Other fish species groups are believed to use body patterns for mate attraction and recognition in narrow spectrum or low light conditions. For example, the seven species of deep-water predatory *Bathybates* in Lake Tanganyika were originally diagnosed on the basis of subtle morphological differences, but it is now known that reproductive males of these species differ strikingly in vertical and horizontal body patterning of black and silver/yellow (Konings 1998). Ponyfishes (Leiognathids), which occupy turbid nearshore marine and estuarine habitats in the IndoPacific, exhibit sexually dimorphic species-specific patterns of lateral bioluminescence (Sparks *et al*. 2005). Some deep-sea fishes also show extreme sexual dimorphism in the distribution of bioluminescence on the body, which may indicate a function in attracting mates (Herring 2000) and a role in species recognition. Thus, it seems likely that pattern variation may have widespread importance in mate recognition in fishes, but the importance relative to hue variation, olfaction, auditory and behavioural traits, is likely to be highly habitat dependent.

Speciation in deep and shallow waters of African lakes

There is considerable evidence for localized divergence of male colouration among allopatric populations of shallow-water cichlid fishes in the African Great Lakes (Seehausen 1996; Konings 1998, 2001; Genner *et al*. 2004). Laboratory mate-choice trials showed that females from these populations generally preferred to mate with males from their own population (Knight & Turner 2004), suggesting at least incipient species status. This provides support for the longstanding hypothesis that population subdivision, promoted by strong preference for discontinuously distributed habitats, has played an important role in intralacustrine speciation among inshore cichlid fishes (Fryer 1959). However, a study of microsatellite markers of three species of *Diplotaxodon* provided no evidence of genetic population structure over lakewide spatial scales (Shaw *et al*. 2000). Thus, *Diplotaxodon* has been proposed as a candidate genus in which to investigate the possibility of sympatric speciation (Shaw *et al*. 2000). Our study suggests that, despite living and breeding in habitats with ambient light spectra of restricted width, reproductive isolation of *Diplotaxodon*, like that of many shallow water cichlids, is likely to be maintained, at least in part, by differences in male breeding dress.

Recent reviews have cast doubt on the plausibility of models of sympatric speciation driven by sexual selection (e.g. Arnegard & Kondrashov 2004; Coyne & Orr 2004). Only minimal differences in diet, trophic morphology and habitat preference are known among *Diplotaxodon* species, suggesting that ecological selection is unlikely to have played a major role in speciation within this group. By contrast, ecological selection is believed to have played a major role in the divergence of other lake fish radiations where sympatric speciation is implicated, including cichlid fishes in small tropical lakes in Nicaragua (Barluenga *et al*. 2006) and Cameroon (Schliewen & Klee 2004), as well as salmonids, sticklebacks and whitefish in postglacial lakes (Schluter 1996) and cyprinids in Lake Tana (Berrebi & Valiushok 1998). Thus, while it might be premature to rule out sympatric speciation of *Diplotaxodon* driven either by ecological or sexual selection, it might also be fruitful to consider whether allopatric divergence in female preferences and male body patterns may have evolved during periods of restricted gene flow between geographically separated populations. Possible allopatric speciation scenarios for deep water cichlids could include subdivision of the lake basin, which has been proposed for Lake Tanganyika offshore cichlids (Koblmüller *et al*. 2005) or homing to natal breeding grounds, as reported for many marine and anadromous fishes (Thorrold *et al*. 2001; Maes & Volckaert 2002).

Concluding remarks

Here we have provided evidence consistent with the hypothesis that differences in nuptial patterning are important for species recognition in cichlids from a deep-water environment where the range of visible colour hues is strictly limited. Similar differences in body patterning may help to explain how closely related fish species can co-exist without introgression in other narrow-light spectra habitats, such as turbid waters. However, in such habitats the visual range will be reduced thus influencing encounter rates and plausibly mate choice decisions. Comparative analyses of the spatial patterns of co-occurrence of species differing in nuptial patterning and hue under contrasting light regimes may well provide further insights into their roles for reproductive isolation. In any case, this study suggests that the relevance of sexually selected traits in fishes must be considered within the context of the environments in which they have evolved, and that light-limited narrow-spectrum environments need not necessarily provide barriers to effective visually mediated mate recognition. Moreover, where such traits exhibit strong seasonality as is the case here, it is evident that tests of genetic structuring or reproductive isolation must be undertaken on spawning, rather than mixed feeding aggregations.

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