

Variation in habitat preference and population structure among three species of the Lake Malawi cichlid genus *Protomelas*

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Abstract

Several studies have demonstrated strong population structuring over small distances in the rocky-shore mbuna cichlid fishes from Lake Malawi, suggesting the potential for allopatric speciation. However, many endemic Lake Malawi cichlids are neither mbuna, nor confined to rocky shores. Using microsatellites, we investigated the population structure in three species of the non-mbuna genus *Protomelas*. The rocky-shore *P. taeniolatus* showed high levels of population structure even over distances of less than 1 km, while the sandy-shore species *P. similis* showed no significant structure over distances up to 21 km. *Protomelas fenestratus*, which is generally found at the interface between rocks and sand, also showed low levels of population structure. Our results suggest that the model of allopatric speciation based on habitat fragmentation within the current lake basin may be equally applicable to rocky-shore non-mbuna as to mbuna, but that an alternative model is required to explain speciation among sandy-shore species as well as the deep-water and pelagic species.

Keywords: cichlid fishes, Lake Malawi, microsatellite DNA, *Protomelas*, population structure, speciation

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Introduction

Lake Malawi is believed to be inhabited by more species of fish than any other lake, the great majority of them being endemic haplochromine cichlids, which represent one of the largest and fastest known adaptive radiations (Turner 1999). The most taxon-rich Malawi cichlids are the rocky-shore 'mbuna' which are presently believed to make up about 240 of the estimated 620 endemic haplochromine species. It has been proposed that speciation in mbuna has taken place allopatrically on isolated patches of rocky habitat within the lake (Fryer 1959; Fryer & Iles 1972; Ribbink *et al.* 1983). Later studies have demonstrated fine-scale population structuring and independent local origins of colour forms, consistent with restricted movement of individuals between populations within the lake, as required

by parapatric and allopatric models (e.g. Arnegard *et al.* 1999; Markert *et al.* 1999; Danley *et al.* 2000; Rico & Turner 2002; Rico *et al.* 2003).

However, some 375 of the recent maximal estimate of 620 species of haplochromine species endemic to Lake Malawi are not mbuna. Some are specialized to live on rocky shores (Fryer 1959; Ribbink *et al.* 1983; Konings 2001), but most species are found in other habitats, such as sandy shores, deep waters or even surface offshore waters (the 'pelagic zone'). It has been suggested that just as rocky habitats are patchily distributed and the rocky-shore specialists find other habitats a barrier to dispersal, so species specialized to other habitats, such as sandy shores, will also find barriers to dispersal, in the form of rocky shores or deep-water habitats (Fryer & Iles 1972).

The inshore *Copadichromis 'virginalis kajose'* showed far less population structuring than mbuna (Taylor & Verheyen 2001), while three offshore *Diplotaxodon* species showed minimal structuring even over hundreds of kilometres

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(Shaw *et al.* 2000). Thus, a different model of speciation may be needed for these mid-water zooplankton feeders, perhaps involving sympatric speciation (Turner 1994; Shaw *et al.* 2000), or subdivision of the lake by severe drops in its water level (Sturmbauer *et al.* 2001).

To gain further insight into the generality of these studies in relation to speciation within Lake Malawi cichlids, we investigated population structure in three congeneric species of non-mbuna cichlids endemic to Lake Malawi, the rocky-shore specialist *Protomelas taeniolatus*, the sandy/weedy-shore species *Protomelas similis* and *Protomelas fenestratus*, a species generally found near the interface between rocky and sandy habitats. On the basis of the hypothesis put forward by Fryer & Iles (1972), it was predicted that all three species would show high levels of population structuring over relatively small spatial scales, as previously noted for the rocky-shore mbuna cichlids. Arguably, it would also be consistent with their prediction if the sand and rock specialists (*P. similis* and *P. taeniolatus*) both showed fine-grained population structure, while the more generalist *P. fenestratus* did not. However, should *P. taeniolatus* show considerably greater population structure than *P. similis* (and perhaps *P. fenestratus* as well), we might have to invoke a different model to explain speciation in sandy-shore cichlid species, while if none of the three species showed much population structure, it would suggest that the mbuna cichlids are probably unusual, indeed perhaps unique, among Malawi cichlids in their susceptibility to population fragmentation.

Materials and methods

Protomelas is a genus containing about 15 described species, all endemic to Lake Malawi, its catchment and the catchment of the outflowing Shire River (Eccles & Trewavas 1989; Konings 2001). *Protomelas taeniolatus* is a specialized rocky-shore species (Ribbink *et al.* 1983). Males are seasonally territorial and females guard free-swimming fry (Robinson & Ribbink 1998). These life history traits, along with its morphological similarity to its congeners, suggest that *P. taeniolatus* has invaded the rocky habitat independently from the mbuna. *Protomelas similis* inhabits shallow water (Fryer 1959; Konings 2001), feeding largely on the leaves of higher plants (Fryer & Iles 1972). *Protomelas fenestratus* lives over rock and sand, particularly at the rock-sand interface near the shoreline (Eccles & Trewavas 1989). It often feeds by blowing loose sediment off the substrate to search for edible material concealed beneath (Konings 2001).

Although we attempted to sample all three species from the same locations, sampling was constrained by the scarcity or absence of one or more species at particular sites. Individuals of *P. taeniolatus* and *P. fenestratus* were sampled from the rocky shores at seven sites from the Lake Malawi shoreline: two from the central western shore and

five near the southern end of the lake (Fig. 1). Although *P. similis* is reported to have a lake-wide distribution (Eccles & Trewavas 1989; Konings 2001), and was studied at Nkhata Bay in the 1950s by Fryer (1959), we were unable to find this species at this location. Specimens of *P. similis* were collected from five different sandy areas separated by rocky patches around the Nankumba Peninsula (Fig. 1). Samples were collected from each site using SCUBA with monofilament nets, and fin clips preserved in 100% ethanol.

DNA was extracted following the method described by Aljanabi & Martinez (1997). Samples were screened for variation at five polymorphic microsatellite loci, all of which are perfect dinucleotide repeats: UNH001, UNH002 (Kellogg *et al.* 1995); UME002, UME003 (Parker & Kornfield 1996), and Pzeb3 (van Oppen *et al.* 1997). The 10- μ L polymerase chain reactions consisted of 1 μ L (c. 20 ng) of template DNA, 1.0 μ M each primer (one of which was dye-labelled FAM, HEX, or NED), 200 μ M of each dNTP, 0.50 units of *Taq* polymerase (Bioline), 1 μ L of 10 \times reaction buffer and 2.5 mM MgCl₂ (Bioline). Reactions were denatured for 3 min at 94 °C, followed by seven cycles of 92 °C for 20 s, A₁ °C for 20 s and 72 °C for 30 s, followed by 23 cycles of 89 °C for 20 s, A₂ °C for 20 s, 72 °C for 20 s, followed by 20 °C for 4 min. The annealing temperatures (A₁ and A₂) were 52 and 55 °C for UNH001, 53 and 55 °C for UNH002, 53 and 55 °C for UME003, 59 and 59 °C for UME002 and 49 and 51 °C for Pzeb3. The polymerase chain reaction products were then multiplexed and resolved on an ABI 377 automated sequencer (*P. taeniolatus*) or an ABI 3700 sequencer (*P. fenestratus* and *P. similis*) (Applied Biosystems) using a ROX 400 size standard. GENESCAN 3.7 and GENOTYPER 3.7 software (Applied Biosystems) were used for allele sizing.

Exact tests of linkage disequilibrium were carried out using GENEPOP 3.3 (Raymond & Rousset 1995). Allele frequencies, observed and unbiased expected heterozygosities under Hardy-Weinberg expectations were obtained with POP100GENE (<http://www.ensam.inra.fr/URLB>). Departures from Hardy-Weinberg equilibrium were tested following Weir & Cockerham (1984), implemented by GENEPOP 3.3 (Raymond & Rousset 1995). Significance levels were determined using the Markov chain method (5000 dememorizations, 100 batches, 2000 iterations per batch).

FSTAT 2.9.3 (Goudet 2001) was used to estimate population differentiation using the *F*-statistic θ , taking into account departures from Hardy-Weinberg equilibrium, and *R*_{ST} CALC 2.2 (Goodman 1997) to estimate unbiased *R*_{ST}, which corrects for variance among loci and sample sizes among populations. *G*-statistics were calculated with 2000 permutations and 2000 bootstraps to estimate the significance of these analyses. *F*-statistics may be underestimated because of the high mutation rates of microsatellites (Balloux *et al.* 2000). Consequently, Fisher's exact tests using GENEPOP 3.3 (Raymond & Rousset 1995), were

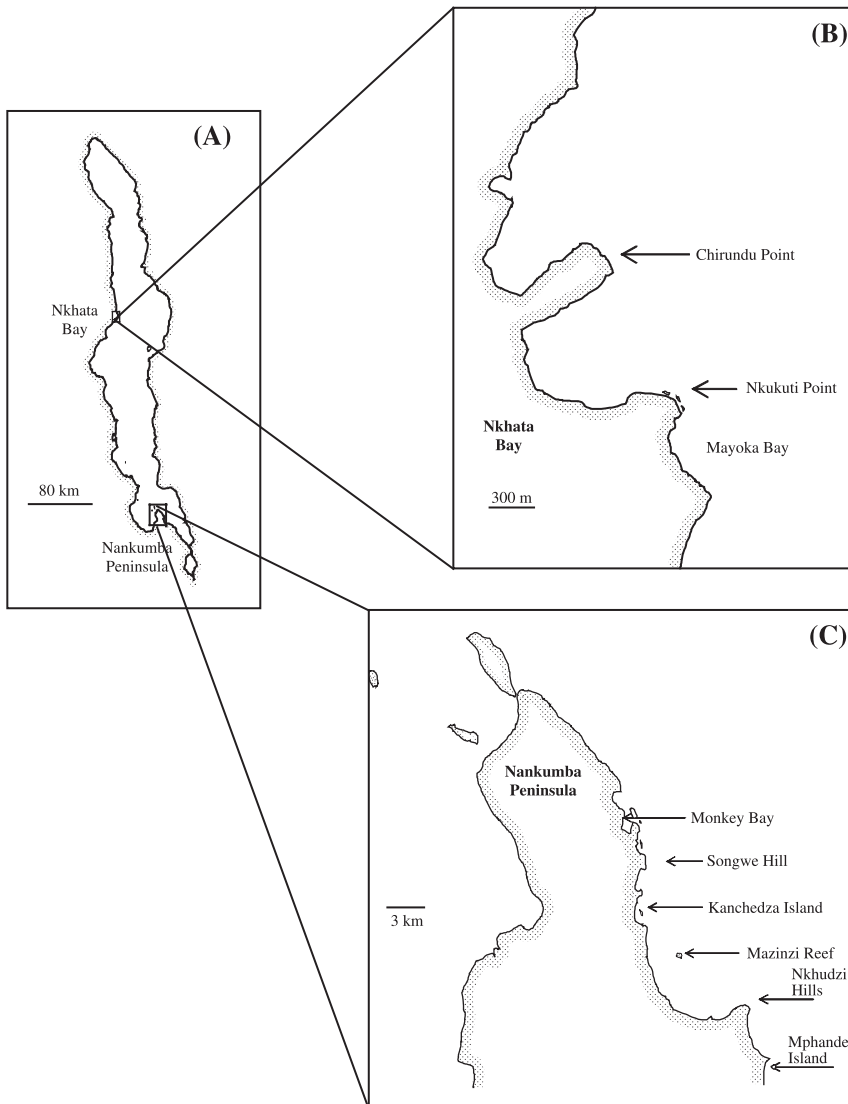


Fig. 1 Sampling sites, showing (A) the overall location in Lake Malawi, (B) the sample sites on the central west coast of the lake and (C) those in the south of the lake. Over the entire sampling area, the distance is approximately 280 km, mostly sandy beaches or muddy bays with occasional rocky headlands and islands.

performed to test for differences in allele frequencies between populations as another indicator of population subdivision, with significance levels determined using the Markov chain method (5000 dememorizations, 100 batches, 2000 iterations per batch). Results (data not shown) were very similar to those for F_{ST} .

Results

Significant overall population structuring was detected for *Protomelas taeniolatus* (overall $\theta = 0.026$, $P < 0.001$; $R_{ST} = 0.1082$, $P < 0.001$). Pairwise F_{ST} (θ) values indicated significant differentiation among all pairs of populations, although the comparison between the neighbouring Kanchedza Island and Mazinzi Reef was non-significant after Bonferroni correction ($P = 0.016$; Table 1). Two further pairwise comparisons were not significantly different for the R_{ST} -based

fixation index, namely Songwe Hill vs. Kanchedza Island ($P = 0.454$) and Nkukuti Point vs. Chirundu Point ($P = 0.066$), but deviated significantly from zero with θ estimates ($P < 0.001$ for both). Finally, fixation indexes between Chirundu Point and Nkhudzi Hills, the northernmost sampled population in Nkhata Bay and the southernmost in the Nankumba Peninsula respectively, also indicated significant differences between regions (both $P < 0.001$).

Estimates of population structure (θ) also revealed significant differentiation ($\theta = 0.012$, $P < 0.001$) over all *Protomelas fenestratus* populations (Table 2). Although there was a clear difference between northern and southern regions in both θ and R_{ST} , Mphande Island was differentiated from other southern populations only by θ (Table 1).

Estimates of population subdivision revealed no significant differences either over all *P. similis* populations (Table 2), or between individual populations (Table 1).

Table 1 Estimates of population differentiation between adjacent samples taken from *Protomelas* populations in Nankumba Peninsula (first six comparisons) and Nkhata Bay (Nkukuti-Chirundu) and for populations representing the whole sampled range (Nkhudzi-Chirundu)

Comparison	Distance (km)	Intervening substrate	<i>P. taeniolatus</i>		<i>P. fenestratus</i>		<i>P. similis</i>	
			θ_{ST}	R_{ST}	θ_{ST}	R_{ST}	θ_{ST}	R_{ST}
Mphande–Nkhudzi	5.6	Shallow sandy bay	N/A	N/A	0.013*	0.021	< 0.001	< 0.001
Nkhudzi–Mazinzi	6.4	Shallow sandy bay	0.015*	0.046*	< 0.001	< 0.001	N/A	N/A
Mazinzi–Kanchedza	4.3	Shallow sandy bay	0.006*	0.008	0.002	< 0.001	N/A	N/A
Nkhudzi–Kanchedza	10.7	Shallow sandy bay	0.017*	0.054*	0.004	0.011	< 0.001	< 0.001
Kanchedza–Songwe	2.4	Shallow sandy bay	0.007*	0.001	0.001	0.008	0.002	< 0.001
Songwe–Monkey Bay	2.8	Sand & rock	N/A	N/A	N/A	N/A	< 0.001	0.028
Nkukuti–Chirundu	0.7	Sandy/deep trough	0.024*	0.020	0.001	0.018	N/A	N/A
Nkhudzi–Chirundu	c. 270	Sand, rock, river mouths and deep water	0.036*	0.208*	0.018*	0.051*	N/A	N/A

For each pairwise comparison the table indicates the distance between the localities in kilometers, θ_{ST} , R_{ST} . Significance values after Bonferroni correction are indicated with an asterisk. N/A = not applicable, as one of the localities was not sampled for this species.

Table 2 Comparison of levels of multilocus microsatellite estimates of population differentiation among Lake Malawi cichlid species with different habitat preferences

Species	Habitat	Distance (km)	θ	<i>P</i>	Source
<i>Pseudotropheus zebra</i>	Rock (mbuna)	60	0.025	< 0.05	Rico <i>et al.</i> (2003)
<i>Pseudotropheus callainos</i>	Rock (mbuna)	60	0.077	< 0.05	Rico <i>et al.</i> (2003)
<i>P. (Tropheops) 'mauve'</i>	Rock (mbuna)	60	0.106	< 0.05	Rico <i>et al.</i> (2003)
<i>P. (Tropheops) 'olive'</i>	Rock (mbuna)	60	0.067	< 0.05	Rico <i>et al.</i> (2003)
<i>Melanochromis auratus</i>	Rock (mbuna)	42	0.151	< 0.001	Markert <i>et al.</i> (1999)
<i>Labeotropheus fuelleborni</i>	Rock (mbuna)	42	0.079	< 0.001	Arnegard <i>et al.</i> (1999)
<i>Protomelas taeniolatus</i>	Rock (non-mbuna)	280	0.026	< 0.001	Present study
<i>P. fenestratus</i>	Rock/sand	280	0.012	< 0.001	Present study
<i>P. similis</i>	Sand	20	0.001	0.198	Present study
<i>Copadichromis 'virginalis kajose'</i>	Midwater inshore	400	0.004	0.006	Taylor & Verheyen (2001)
<i>Diplotaxodon macrops</i>	Deep pelagic	75	< 0.001	0.261	Shaw <i>et al.</i> (2000)
<i>Diplotaxodon 'offshore'</i>	Deep pelagic	175	< 0.001	0.291	Shaw <i>et al.</i> (2000)
<i>Diplotaxodon limnothrissa</i>	Pelagic	460	0.001	0.017	Shaw <i>et al.</i> (2000)

P-values given are probabilities that the genetic structure estimate (θ) is zero. Shown are values for the maximum distances analysed for each species.

Because of the constraints on sampling, the clearest comparisons between species are obtained by viewing across rows of Table 1. At all six pairs of sites where *P. taeniolatus* and *P. fenestratus* were both sampled, θ and R_{ST} were always lower in *P. fenestratus*, indicating a weaker population structuring than in *P. taeniolatus*. In comparison to *P. taeniolatus* and *P. fenestratus*, *P. similis* showed the lowest values of both structure estimators for all comparable pairs of sites, with the exception of the θ -values for the comparison between Kanchedza and Songwe, which were marginally lowest for *P. fenestratus*. To account for differences in the numbers of individuals sampled from different sites, the θ calculations were repeated three times with 22 individuals randomly subsampled from each species from each site.

The broad picture was unchanged: in comparisons of populations from the southern part of the lake only, the mean θ -values for were 0.014 for *P. taeniolatus* (the same as for the full data set); 0.005 for *P. fenestratus* (compared to 0.004) and 0.002 for *P. similis* (compared to 0.001).

Discussion

Our study indicates that closely related Malawian haplochromine species can show very different patterns of population structure, in a manner that can be related to their habitat preferences. The rocky shore species *Protomelas taeniolatus* showed relatively high levels of population structuring, similar to those reported for mbuna (Table 2).

With *P. fenestratus*, genetic differentiation was limited to comparisons between the northern and southern populations (over 270 km apart) and between the Mphande Island and other southern populations (Table 1 and other data not shown). The Mphande Island population lies at the southern extreme of this group of rocky-shore patches, and thus might be expected to show more divergence than those populations where gene flow along the coast from both north and south would be possible. The lack of population structure among the other southern populations and between the two headlands at Nkhata Bay indicates somewhat greater ability to cross sandy beaches or open bottom than is the case for mbuna or *P. taeniolatus*. *Protomelas similis* showed no significant population structuring over distances and habitat barriers that have been shown to cause population structuring both in mbuna and in more rock-bound species of *Protomelas*. Contrary to the suggestion by Fryer & Iles (1972; p. 543), our results seem to suggest that shallow-water, sandy-shore species do not exist as finely subdivided populations in the manner of rocky-shore species, and so the potential for allopatric speciation in this group might be lower than it is presumed to be for mbuna. However, the spatial scale of our study is rather smaller than those of studies of benthic and pelagic zooplanktivorous species (*Copadichromis*, *Diplotaxodon*), which showed low levels of population structure over hundreds of kilometres (Table 2). It may be worth investigating the potential of more significant habitat barriers, such as long stretches of sheer rocky coasts, as along the northeastern shores, or the northwestern shore north of Nkhata Bay, and deep-water barriers isolating offshore islands such as Likoma and Chisumulu. An alternative explanation for allopatric speciation in taxa with low levels of population structuring might be past population fragmentation caused by extreme environmental changes. The water level of Lake Malawi has certainly fluctuated considerably (Sturmbauer *et al.* 2001).

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Appendix I

Genetic variability of 18 populations of three species of *Protomelas* at five microsatellite loci. Number of alleles (NA), observed (H_O) and expected (H_E) heterozygosity. Significant deviations from Hardy–Weinberg equilibrium after sequential Bonferroni correction (by species) are asterisked

	UME002			Pzeb3			UNH002			UME0003			UNH001			Mean			
	<i>n</i>	NA	H_O	H_E	NA	H_O	H_E	NA	H_O	H_E	NA	H_O	H_E	NA	H_O	H_E	NA	H_O	H_E
<i>P. taeniolatus</i>																			
Nkhudzi	70	26	0.829*	0.930	12	0.757	0.786	25	0.857	0.925	30	0.757*	0.947	18	0.887	0.938	22.6	0.817	0.905
Mazinzi	47	28	0.957	0.954	9	0.745	0.782	21	0.872	0.912	32	0.723*	0.956	22	0.766*	0.933	22.4	0.813	0.907
Kanchedza	50	32	0.840*	0.957	11	0.720	0.812	22	0.700*	0.929	36	0.760*	0.961	24	0.860	0.948	25.3	0.776	0.921
Songwe	58	33	0.897	0.940	8	0.862	0.805	25	0.845*	0.932	39	0.845*	0.966	25	0.879*	0.940	26.0	0.866	0.920
Nkukuti	50	29	0.980	0.956	9	0.500	0.531	19	0.800	0.863	32	0.900	0.958	27	0.880	0.916	21.4	0.802	0.845
Chirundu	41	34	0.976	0.973	9	0.683	0.771	24	0.878	0.941	32	0.976	0.976	26	0.902	0.943	24.2	0.883	0.920
Overall/Mean	316	30.3	0.924	0.954	9.6	0.722	0.743	22.6	0.825	0.917	33.5	0.873	0.960	23.6	0.862	0.937	23.7	0.826	0.903
<i>P. fenestratus</i>																			
Mphande	48	30	0.938	0.948	9	0.813	0.758	20	0.896	0.919	28	0.917	0.951	21	0.792	0.913	21.6	0.871	0.901
Nkhudzi	47	30	0.915	0.948	7	0.766	0.797	18	0.936	0.898	30	0.936	0.921	25	0.957	0.930	22.0	0.902	0.899
Kanchedza	46	30	0.935	0.948	7	0.717	0.794	15	0.978	0.897	37	0.953	0.949	20	0.957	0.913	21.8	0.917	0.900
Mazinzi	50	36	0.940	0.942	8	0.680	0.828	19	0.920	0.898	30	0.880	0.917	23	0.800*	0.931	23.2	0.844	0.903
Songwe	47	28	0.894*	0.955	10	0.745	0.829	17	0.809	0.896	34	0.894*	0.950	23	0.872	0.923	22.4	0.843	0.911
Nkukuti	49	37	0.918	0.971	8	0.735	0.773	26	0.959	0.942	30	0.857*	0.943	31	0.918*	0.960	26.4	0.878	0.918
Chirundu	30	36	0.933	0.981	7	0.800	0.775	24	0.900	0.942	31	0.867*	0.975	26	0.900	0.958	24.6	0.880	0.926
Overall/Mean	317	32.4	0.925	0.956	7.8	0.751	0.793	19.8	0.914	0.913	31.4	0.907	0.944	24.1	0.885	0.933	23.1	0.876	0.907
<i>P. similis</i>																			
Mphande	46	41	0.957	0.942	10	0.848	0.835	25	0.957	0.945	34	0.739	0.942	31	0.957	0.965	28.2	0.891	0.932
Nkhudzi	32	41	0.938	0.981	9	0.750	0.810	23	0.938	0.938	35	0.938	0.977	27	0.969	0.960	27	0.906	0.935
Kanchedza	47	43	0.979	0.970	12	0.809	0.843	28	0.979	0.955	37	0.894	0.973	27	0.936	0.955	29.4	0.919	0.939
Songwe	22	27	0.864	0.922	11	0.737	0.885	21	0.909	0.953	23	0.737	0.926	22	0.904	0.955	21.2	0.827	0.941
Monkey Bay	22	30	0.727*	0.974	9	0.727	0.866	18	0.909	0.955	20	0.773*	0.952	20	0.909	0.944	19.4	0.809	0.938
Overall/Mean	169	36.4	0.893	0.958	10.7	0.772	0.848	23	0.938	0.949	30.0	0.814	0.954	25.6	0.936	0.956	25.0	0.870	0.937