

No evidence for parallel sympatric speciation in cichlid species of the genus *Pseudotropheus* from north-western Lake Malawi

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Abstract

To test the hypothesis of parallel speciation by sexual selection, we examined length variation at six microsatellite loci of samples from four sites of four to six putative species belonging to two subgenera of rocky shore mbuna cichlids from Lake Malawi. Almost all fixation indices were significantly different from zero, suggesting that there is presently little or no gene flow among allopatric populations or sympatric species. Analysis of variance indicated that genetic distances among allopatric populations of putative conspecifics were significantly lower than among sympatric populations of heterospecifics. The topology of trees based on distance matrices was also largely consistent with the hypothesis that the putative species are monophyletic and have thus not evolved in parallel in their present locations. If parallel speciation does occur in Malawi cichlids, it may be on a larger spatial scale than investigated in our study.

Introduction

When discussing the explosion of new forms in the fossil record of the early Cambrian Burgess Shale, Gould (1989) coined a memorable metaphor to describe his view of the primacy of contingency in biological evolution: if one could rewind the tape of life, each re-run would produce a dramatically different outcome. The implications of this view of history are profound, suggesting that the organisms that dominate the world today are largely the beneficiaries of good fortune, rather than good design. Parallel and convergent evolution of ecological adaptations has long been known. However, there is now evidence that similar environmental conditions not only produce similar forms but essentially the *same* new species can arise repeatedly in different places (reviewed by Johannesson, 2001), as may have been the case with sticklebacks in British Columbia, Canada

(Rundle *et al.*, 2000). It might seem even more surprising that parallel speciation may be driven by sexual selection, which is often considered to be more capricious and less directly influenced by the environment than ecological selection. However, we propose that it may be worth investigating this possibility in the cichlid fishes of the East African lakes.

In Lake Victoria, it is common to find two or more ecologically and morphologically similar sympatric species that differ clearly in male courtship colour (Seehausen, 1996). Strikingly, the same colour combinations re-occur in many different genera, and even in different lakes (Seehausen *et al.*, 1999). It seems that there have been repeated switches between a small number of different colour combinations during the radiation of these fishes.

Several studies have provided evidence for sympatric speciation among cichlid fishes in African Lakes (Schliewen *et al.*, 1994; Seehausen & van Alphen, 1999; Shaw *et al.*, 2000). Seehausen & van Alphen (1999) observed that the males of many Victorian species have a largely blue breeding dress, while the males of other anatomically very similar and thus probably closely related species have red and yellow courting males.

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Many of these pairs of species have largely sympatric ranges, and it was suggested that these might have speciated sympatrically *in situ* (Seehausen & van Alphen, 1999). These findings have been interpreted as evidence for repeated parallel evolution of similar male breeding colours during sympatric speciation by sexual selection (Seehausen, 2000). It seems that in some of these species pairs, male breeding colour may be entirely responsible for reproductive isolation (Seehausen *et al.*, 1997; Seehausen & van Alphen, 1998). Thus, it would not seem implausible that some of these populations may freely interbreed with other similarly coloured but independently derived populations in the event of secondary contact. This would be parallel speciation by sexual selection.

Population structure among Lake Victoria cichlids has not been studied in detail. However, the rocky shore mbuna cichlids of Lake Malawi are split by habitat discontinuities into many genetically isolated populations (van Oppen *et al.*, 1997a; Arnegard *et al.*, 1999; Markert *et al.*, 1999). This would provide opportunity for repeated parallel evolution of species. In Lake Malawi, there are many sympatric taxa that differ in male breeding colour and little else, and in many cases it has been shown that these are reproductively isolated species (van Oppen *et al.*, 1998). As in Lake Victoria, many of the same colour combinations reappear in different genera or species complexes (Ribbink *et al.*, 1983; Konings, 2001). Reinthal & Meyer (1997) suggested that mitochondrial sequences indicated that four populations of *Pseudotropheus* (*Tropheus*) spp. in the southern part of Lake Malawi formed a clade with one northern population. The other three northern populations formed a separate clade. This suggests parallel evolution of morphological and ecological traits, although not of colour patterns.

However, analysis of a larger data set has cast doubt on this conclusion (Parker & Kornfield, 1997).

In this study, we attempted to test the hypothesis that colour variation among allopatrically and sympatrically occurring populations and putative species reflects parallel speciation at four different sites separated by large habitat discontinuities along a 50-km stretch of the north-western shore of Lake Malawi.

Materials and methods

Samples

Fishes were sampled from four sites on the western shore of Lake Malawi: Ruarwe, Mara Rocks, Cape Manulo and Nkhata Bay (Fig. 1). Nkhata Bay and Cape Manulo are near to the opposite ends of a 33-km long region of rocky shore broken by a number of relatively small sandy beaches. Ruarwe and Cape Manulo are about 25 km apart: most of this distance is made up of the 20-km wide Usisya Bay which is sandy, apart from the small shallow rocky promontory in the middle. The Mara Rocks lie a few kilometres off this headland, isolated from the mainland by a deep trench. As our observations have shown that the *P. zebra* at the promontory resemble those of Ruarwe in colour, and not those of Mara Rocks, it seems that this trench is a major barrier to mbuna dispersal.

We sampled species belonging to two subgenera of the genus *Pseudotropheus*. The classification of these fishes is rather confused (see for example Stauffer *et al.*, 1997; Condé & Géry, 1999; Konings, 2001), and we follow Eschmeyer's on-line catalogue of fishes (<http://www.calacademy.org/research/ichthyology/catalog/fishcatsearch.html>). The subgenus *Maylandia*, which is sometimes

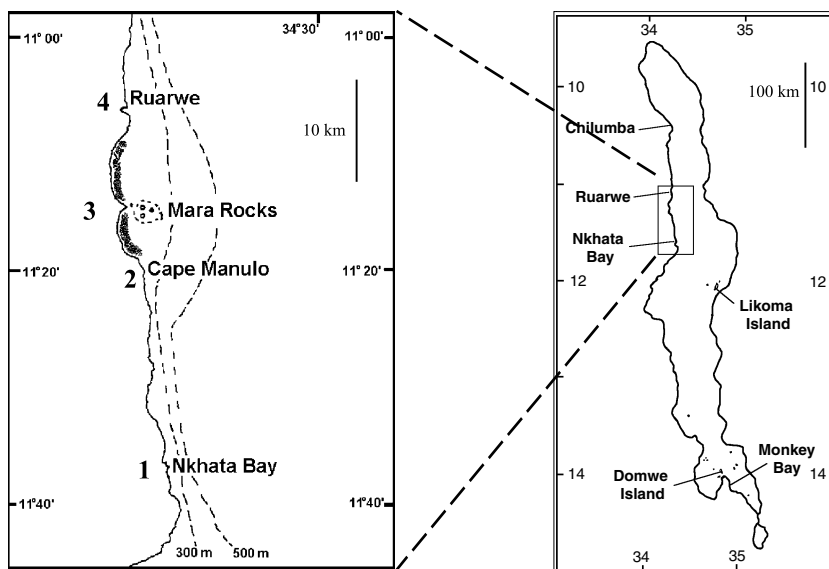


Fig. 1 Study area, showing the four sample areas. Mara Rocks is a group of islands about 1 km off the shore. The coasts are largely rocky and suitable habitats for the study species, apart from the large sandy beaches (shaded areas) to the north and south of Mara Rocks. The broken lines are 300 and 500 m depth contours. Scale bars are approximate.

treated as a full genus, contains the species of the *P. zebra* complex, also known by the junior synonym of *Metriaclima*. These species have terminal mouths and long slender bicuspid and tricuspid teeth, which they use to comb loose material from among filamentous algae growing on rock surfaces, although they also feed on plankton. The species of the subgenus *Tropheops* are also known as the *P. tropheops* complex or the genus *Tropheops*. The outer teeth of *P. (Tropheops)* are set together in the underslung mouth to form a cutting blade, which is used to shear off filamentous algae from the rock surface. By contrast to the clearly adaptive morphological differences between mbuna genera or

subgenera, species within a genus or subgenus vary only slightly in morphology but are usually clearly different in colour. We sampled three species of each subgenus (Table 1): several of these show geographical variation, particularly in male colour and in presence or absence of rarer female colour morphs (Table 2).

In July 1995 and 1996, we collected 24–120 individuals of each species from each site (Table 1), as described by van Oppen *et al.* (1997a, 1998). We were unable to collect enough *P.* 'gold' and *P.* 'band' from the two least accessible sites, Mara Rocks and Cape Manulo. We did not include *P.* 'mauve' from Ruarwe in the analysis, because we discovered during the second collecting trip

Table 1 Sample size and collection site for *P. (Maylandia)* and *P. (Tropheops)* species. Abbreviations for each site and species are used hereafter in tables, figures and text. See 'Methods' for details.

Site	Sample size					
	<i>P. (M.) zebra</i> (Mz)	<i>P. (M.) 'gold'</i> (Mg)	<i>P. (M.) callainos</i> (Mc)	<i>P. (T.) 'olive'</i> (To)	<i>P. (T.) 'band'</i> (Tb)	<i>P. (T.) 'mauve'</i> (Tm)
Nkhata Bay (N)	104	50	121	110	72	119
Cape Manulo (C)	53	Not collected	65	30	Not collected	37
Mara Rocks (M)	80	Not collected	61	53	Not collected	53
Ruarwe (R)	59	40	57	61	24	Not analysed

Table 2 Geographical variation in colour for putative species of *P. (Maylandia)* and *P. (Tropheops)*. Abbreviations are given in Table 1. Absence of a morph from a sample site does not necessarily mean that it does not occur there, rather that no positive record exists.

Site	Putative species					
	<i>P. zebra</i> (Mz)	<i>P. 'gold'</i> (Mg)	<i>P. callainos</i> (Mc)	<i>P. 'olive'</i> (To)	<i>P. 'band'</i> (Tb)	<i>P. 'mauve'</i> (Tm)
Nkhata Bay	<i>Male</i> : blue with dark bars, blue dorsal fin, black chin (BB), occasionally OB or O morphs <i>Female</i> : brown with dark bars (BB); orange with dark blotches (OB); bright orange (O)	<i>Male</i> : yellow with dark bars <i>Female</i> : brown with dark bars (BB); orange with dark blotches (OB)	<i>Male</i> : blue (B), occasionally W morph <i>Female</i> : blue (B), white (W) or blotched (OB)	<i>Males</i> : yellow-brown <i>Females</i> : silvery grey with rows of black spots	<i>Males</i> : yellow and blue <i>Females</i> : sandy/grey with rows of dark spots	<i>Males</i> : blue <i>Females</i> : yellow
Cape Manulo	BB, OB as Nkhata Bay	Not collected	B, W as Nkhata Bay	As Nkhata Bay	Not collected	As Nkhata Bay
Mara Rocks	<i>Male</i> : blue with faint bars, blue dorsal fin, yellow chin (BB)* <i>Female</i> : BB as Nkhata Bay; O dull orange	Not collected	B, W morphs recorded	As Nkhata Bay	Not collected	<i>Males</i> : blue with yellow chest <i>Females</i> : yellow
Ruarwe	<i>Male</i> : blue with darkbars, dark horizontal band in dorsal fin, yellow chin (BB)* <i>Female</i> : BB, OB as Nkhata Bay	As Nkhata Bay	Both sexes all white†	As Nkhata Bay, although males recorded as slightly more intensely coloured	As Nkhata Bay, but males recorded as less intensely coloured‡	Not analysed

*Populations considered as heterospecific by Ribbink *et al.* (1983: *P. 'zebra chilumba'*) and Konings (2001: *Metriaclima fainzilberi*).

†Population considered as heterospecific (*P. 'zebra pearly'*) by Ribbink *et al.* (1983).

‡Population considered as heterospecific by Ribbink *et al.* (1983: *P. 'tropheops weed'*) and Konings (2001: *Tropheops 'weed'*).

that this taxon actually represented a cryptic species pair at this site, which we had not distinguished initially. In the present study, the samples from Nkhata Bay are the same as those reported by van Oppen *et al.* (1998).

Data analysis

Samples were screened for variation at six polymorphic microsatellite loci as described by van Oppen *et al.* (1997b). The six loci (Kellogg *et al.*, 1995; van Oppen *et al.*, 1997b) include four perfect dinucleotide repeats (*Pzeb1*–3 and *UNH002*) and two imperfect dinucleotide repeats, *Pzeb4* and *Pzeb5*. Allele frequencies, expected (H_e) and observed (H_o) heterozygosities were calculated using GENEPOP 3.1 (Raymond & Rousset, 1995). Samples were tested for linkage disequilibrium and departure from Hardy–Weinberg equilibrium by the Markov chain method. Heterogeneity in genotype distribution for all loci and all pairwise comparisons, was tested based on an assumption of no differentiation. Nonamplifying ‘null’ alleles were found from pedigree analysis of six pairs of *P. zebra* adults from Nkhata Bay (van Oppen *et al.*, 1998). Null alleles were found in four of the six loci. We therefore assumed that null alleles were present in other species as well. The frequency of null alleles was estimated following the approach of Markert *et al.* (1999). Further, to assess the effects of the loci that have null alleles on the significance of population differentiation estimates, weighted F_{ST} statistics over the two loci with no null alleles were obtained and tested as described below.

As both drift and mutation probably influence differences between putative species pairs, and those between populations are probably mostly influenced by drift (Slatkin, 1995), we followed two approaches in investigating the amplitude of genetic differentiation between populations and taxa. First, the computer program Arlequin 1.1 (Schneider *et al.*, 1997) was used to calculate pairwise fixation indices, based on allele frequency variation using an AMOVA framework to estimate weighted F_{ST} statistics (θ) over all loci (Weir & Cockerham, 1984; Excoffier *et al.*, 1992). Secondly, we calculated ρ , which is based on the stepwise mutation model (SMM) and takes into account the differences in sample size between populations and differences in variance between loci (Goodman, 1997). Indeed, it has been empirically shown that calculating ρ using this approach is unaffected by the differences in sample size and variability as long as samples are moderately large (i.e. $n \geq 50$) (Ruzzante, 1998). Thus, we only calculated pairwise ρ estimates for comparisons where each sample comprised at least 50 individuals.

The significance of genetic subdivision was assessed using 1000 permutations in both Arlequin and R_{ST} CALC. To correct for multiple simultaneous comparisons, sequential Bonferroni corrections were applied to all pairwise tests using a global significance level of 0.05 (Rice, 1989).

Genetic divergence among populations and taxa was estimated by two approaches because of recent debate on model-specific distance estimators for microsatellite loci (e.g. Takezaki & Nei, 1996; Goodman, 1997; Angers & Bernatchez, 1998). Therefore, we first quantified Cavalli-Sforza & Edwards’s (1967) chord distance (D_{CE}) and Nei’s distance using GENEDIST. Secondly, we calculated Goldstein *et al.*’s (1995) delta-mu squared ($\delta\mu^2$) pairwise distances using R_{ST} CALC (Goodman, 1997). Phenograms based on Cavalli-Sforza and Edwards’ distance (D_{CE}), Nei’s distance and $\delta\mu^2$ were constructed using the NEIGHBOUR program with the neighbour-joining algorithm (Saitou & Nei, 1987). A further phenogram was estimated using a maximum likelihood algorithm (CONTML). Support for the tree nodes was assessed by 1000 bootstraps of gene frequencies using the SEQBOOT program and compiled using the CONSENSE program. All these programs are included in the PHYLIP computer package, version 3.5c (Felsenstein, 1993).

From the matrix of pairwise genetic distances, comparisons were classed according to whether they were between conspecifics in allopatry, and heterospecifics in sympatry or allopatry. Heterospecific comparisons were further divided into comparisons among members of the same subgenus and species of different subgenera. These five classes of comparisons were then tested for differences in mean genetic distances, using one-way analysis of variance (ANOVA) (SPSS). For this analysis, we used Nei’s distance and also Slatkin’s (1995) linearization transformation of F_{ST} applied to θ , as this is believed to render its behaviour more clock-like.

Results

Genetic diversity and tests of disequilibrium

All species and populations compared were highly genetically diverse showing high heterozygosities at most loci (Table 3). A total of 60, 44, 43, 22, 17, and 10 alleles were observed at *Pzeb1*, *UNH002*, *Pzeb2*, *Pzeb3*, *Pzeb4*, and *Pzeb5*, respectively. Significant deviations from Hardy–Weinberg equilibrium in the form of heterozygote deficits were present in some populations for most loci. Heterozygote deficiencies were assumed to be mostly as a result of nonamplifying alleles, because in a pedigree test using *P. zebra*, true-breeding null alleles were found in four of the six loci (van Oppen *et al.*, 1998). Few significant heterozygote deficits were found in any species at the loci that had no null alleles in the pedigree test, whereas the four loci where null alleles were detected exhibited frequent heterozygote deficiencies. The analysis carried out only with the loci that had no null alleles (*Pzeb4* and 5) showed that only 11 θ estimates of 171 were nonsignificant: Cape Manulo *P. zebra* vs. *P. callainos* from both Nkhata Bay and Cape Manulo and *P. zebra* from Ruarwe; Nkhata Bay *P. callainos* and *P. zebra* vs. *P. zebra* from Ruarwe; Ruarwe *P. ‘gold’* vs. Mara Rocks

Table 3 Number of alleles (NA), observed (H_o) and expected (H_e) heterozygosity. Significant deviations from Hardy–Weinberg equilibrium are under $H_o = HWE$ and $H_1 =$ heterozygote deficit.

Site/species	Pzeb1				Pzeb2				Pzeb3				Pzeb4				Pzeb5				UNH002			
	NA	H_o	H_e	Null	NA	H_o	H_e	Null	NA	H_o	H_e	Null	NA	H_o	H_e	Null	NA	H_o	H_e	Null	NA	H_o	H_e	Null
McN	46	0.81*	0.97	0.17	38	0.77*	0.95	0.16	11	0.61*	0.77	0.21	4	0.50	0.59	n.s.	2	0.53	0.49	n.s.	29	0.81*	0.96	0.15
MzN	47	0.79*	0.96	0.17	45	0.77*	0.95	0.19	12	0.48*	0.74	0.34	10	0.73	0.77	n.s.	6	0.30	0.34	n.s.	34	0.80*	0.95	0.16
MgN	8	0.20*	0.70	0.41	28	0.77*	0.94	0.17	5	0.61	0.67	n.s.	3	0.41	0.46	n.s.	4	0.42	0.49	n.s.	27	0.88	0.95	n.s.
ToN	28	0.71*	0.93	0.23	39	0.84*	0.95	0.11	6	0.08*	0.27	0.48	3	0.20	0.22	n.s.	4	0.09	0.09	n.s.	24	0.61*	0.88	0.30
TmN	45	0.87	0.96	n.s.	39	0.79*	0.94	0.15	10	0.55	0.63	n.s.	3	0.36	0.32	n.s.	4	0.14	0.15	n.s.	21	0.64*	0.89	0.28
TbN	40	0.71*	0.97	0.26	25	0.82*	0.96	0.14	7	0.45*	0.65	0.31	7	0.43	0.52	n.s.	4	0.41	0.41	n.s.	25	0.83*	0.95	0.13
McC	40	0.77*	0.96	0.19	20	0.75*	0.92	0.18	9	0.84	0.80	n.s.	5	0.61	0.66	n.s.	5	0.10*	0.21	0.25	22	0.55*	0.92	0.40
MzC	36	0.76*	0.96	0.21	25	0.69*	0.95	0.27	8	0.65*	0.75	0.13	9	0.82	0.76	n.s.	4	0.11	0.11	n.s.	25	0.64*	0.94	0.32
ToC	18	0.81*	0.92	0.12	16	0.75*	0.94	0.20	2	0.40	0.40	n.s.	5	0.26*	0.39	0.20	2	0.04	0.04	n.s.	11	0.44*	0.81	0.45
TmC	26	0.77*	0.91	0.16	20	0.69*	0.93	0.26	4	0.42	0.32	n.s.	5	0.35	0.41	n.s.	2	0.03	0.03	n.s.	11	0.91	0.86	n.s.
McM	34	0.83*	0.97	0.14	23	0.65*	0.91	0.28	13	0.75	0.82	n.s.	7	0.46	0.48	n.s.	3	0.23	0.21	n.s.	21	0.49*	0.91	0.47
MzM	41	0.77*	0.96	0.25	28	0.80*	0.95	0.24	11	0.64*	0.80	0.22	10	0.68	0.77	n.s.	5	0.25	0.26	n.s.	30	0.65*	0.92	0.44
ToM	20	0.84	0.91	n.s.	19	0.60*	0.92	0.35	5	0.09	0.12	n.s.	5	0.47*	0.57	0.16	1	0	0	n.s.	11	0.40*	0.85	0.53
TmM	15	0.52*	0.76	0.24	21	0.75*	0.93	0.10	4	0.32	0.31	n.s.	7	0.67	0.64	n.s.	3	0.60	0.57	n.s.	18	0.47*	0.86	0.25
McR	35	0.82*	0.97	0.15	19	0.66*	0.90	0.26	11	0.56*	0.66	0.15	7	0.49*	0.64	0.23	2	0.15	0.14	n.s.	25	0.73*	0.94	0.22
MzR	44	0.76*	0.98	0.22	24	0.76*	0.95	0.20	16	0.74	0.77	n.s.	13	0.78	0.86	n.s.	4	0.15	0.17	n.s.	29	0.78*	0.96	0.18
MgR	25	0.83*	0.94	0.13	22	0.67*	0.94	0.24	6	0.57*	0.69	0.15	10	0.78	0.81	n.s.	6	0.60	0.70	n.s.	17	0.71*	0.93	0.24
ToR	22	0.64*	0.93	0.20	20	0.88	0.93	n.s.	5	0.10	0.14	n.s.	6	0.47*	0.65	0.27	5	0.31	0.32	n.s.	13	0.52*	0.82	0.34
TbR	26	0.80*	0.90	0.19	20	0.85*	0.95	0.21	4	0.51	0.50	n.s.	6	0.42*	0.73	0.43	2	0.73	0.59	n.s.	22	0.83*	0.96	0.21

*Sequential Bonferroni adjusted P for F_{IS} significantly different from zero. Null = estimated frequency of null alleles calculated for cases with significantly nonzero F_{IS} . For other abbreviations see Table 1.

P. zebra and both populations of *P. 'band'*; Cape Manulo *P. 'olive'* vs. conspecifics at Nkhata Bay and also *P. 'band'* at Ruarwe; and finally the two populations of *P. 'band'*. As 93% of significant θ estimates remained significant when loci with null alleles were excluded from the analysis, the presence of null alleles in four of the loci clearly had a negligible effect on the estimates of population differentiation. Exact tests for genotypic linkage disequilibrium confirmed the absence of physical linkage at these loci, as previously reported (van Oppen *et al.*, 1997a).

At locus *Pzeb5*, allele 129 occurred at high frequencies in several populations or was fixed. Statistically significant differences ($P < 0.05$) in genotype frequency distributions were demonstrated in most pairwise comparisons for all other loci (data not shown), whether comparing sympatric putative species or allopatric putative conspecific populations. Allele frequency distributions could be very different. Take, for example, *T. 'mauve'* at two different sites for locus *UNH002*. The three most abundant alleles in the Mara Rocks sample, 199, 215 and 217, had a combined frequency of almost 45% in that site while they were amplified at a frequency of <3% from the same putative species at Cape Manulo.

Microsatellite genetic differentiation

All comparisons between pairs of allopatric populations of putative conspecifics gave fixation indices significantly different from zero (Table 4). This was irrespective of

whether the index was based on the infinite alleles model (θ) or the SMM (ρ). Not surprisingly comparisons of allopatric populations of different species were all different as well. These figures indicate that there is little or no gene flow between these allopatric populations.

All comparisons of between sympatric species also differed significantly from zero using θ , whereas for ρ , only one comparison was nonsignificant, that between *T. 'band'* and *T. 'mauve'* at Nkhata Bay ($\rho = 0.005$, $P > 0.05$). This supports previous findings (van Oppen *et al.*, 1998) that sympatrically occurring putative species of *Pseudotropheus* are reproductively isolated, even when distinguished on little more than the colour of mature males and in some cases females. In addition, fixation indices were significantly different from zero for all comparisons between allopatric populations of different putative species, indicating that gene flow among them is very low or does not occur at all.

Population relationships

Contrary to the predictions of the parallel speciation model, comparisons of pairs of allopatric populations of putative conspecifics showed significantly lower mean genetic distances than pairs of sympatric species of the same subgenus (Fig. 2; Table 5). This pattern was evident irrespective of whether the analysis was carried out with Slatkin's linearized F_{ST} or Nei's distance. Also, there was no indication that sympatric congeneric populations were more similar than allopatric populations of different

Table 4 Pairwise fixation indices for all putative species pairs and conspecific populations in allopatry. Fixation indices were computed following the infinite allele model (θ) lower diagonal, and the stepwise mutation model standardized Slatkin R_{ST} (ρ) (Goodman, 1997) upper diagonal. When information from all six loci were used in the analysis, all indices were significantly different from zero ($P < 0.05$, after Bonferroni correction), apart from the comparison of R_{ST} for *P. (Tropheops)* 'band' and 'mauve' at Nkhata Bay (asterisked). Figures in bold typeface are comparisons of allopatric conspecifics. For abbreviations see Table 1.

	McN	McC	McM	McR	MzN	MzC	MzM	MzR	TmN	TmC	TmM	ToN	ToC	ToM	ToR	MgN	MgR	TbN	TbR
McN		0.037	0.268	0.105	0.141	0.110	0.292	0.046	0.190	NC	0.585	0.221	NC	0.416	0.334	0.619	NC	0.273	NC
McC	0.077		0.161	0.029	0.058	0.086	0.193	0.010	0.122	NC	0.530	0.161	NC	0.337	0.260	0.548	NC	0.191	NC
McM	0.137	0.070		0.123	0.019	0.128	0.010	0.099	0.045	NC	0.341	0.066	NC	0.094	0.071	0.390	NC	0.044	NC
McR	0.077	0.066	0.063		0.050	0.090	0.164	0.019	0.129	NC	0.483	0.145	NC	0.278	0.226	0.541	NC	0.181	NC
MzN	0.087	0.074	0.081	0.057		0.065	0.029	0.023	0.028	NC	0.302	0.020	NC	0.117	0.055	0.346	NC	0.045	NC
MzC	0.067	0.027	0.089	0.058	0.024		0.146	0.039	0.051	NC	0.387	0.114	NC	0.253	0.151	0.505	NC	0.092	NC
MzM	0.088	0.038	0.036	0.095	0.028	0.036		0.121	0.035	NC	0.249	0.042	NC	0.059	0.014	0.306	NC	0.020	NC
MzR	0.068	0.048	0.087	0.032	0.025	0.022	0.042		0.075	NC	0.410	0.069	NC	0.225	0.143	0.458	NC	0.119	NC
TmN	0.145	0.085	0.068	0.151	0.085	0.083	0.048	0.096		NC	0.255	0.048	NC	0.134	0.042	0.359	NC	0.004*	NC
TmC	0.149	0.111	0.067	0.168	0.091	0.100	0.048	0.105	0.030		NC	NC	NC	NC	NC	NC	NC	NC	NC
TmM	0.109	0.123	0.129	0.189	0.131	0.112	0.092	0.137	0.106	0.098		0.379	NC	0.219	0.258	0.283	NC	0.230	NC
ToN	0.161	0.194	0.206	0.168	0.167	0.166	0.137	0.207	0.174	0.206			NC	0.130	0.045	0.438	NC	0.042	NC
ToC	0.151	0.202	0.203	0.186	0.166	0.176	0.165	0.154	0.202	0.201	0.183	0.030		NC	NC	NC	NC	NC	NC
ToM	0.151	0.155	0.152	0.160	0.136	0.139	0.121	0.118	0.155	0.124	0.154	0.053	0.030		0.065	0.284	NC	0.110	NC
ToR	0.120	0.154	0.148	0.166	0.132	0.138	0.115	0.124	0.146	0.129	0.127	0.067	0.059	0.031		0.363	NC	0.022	NC
MgN	0.154	0.116	0.080	0.177	0.088	0.113	0.051	0.119	0.081	0.086	0.130	0.232	0.218	0.159	0.165		NC	0.351	NC
MgR	0.064	0.093	0.091	0.119	0.061	0.073	0.036	0.074	0.113	0.114	0.105	0.178	0.176	0.145	0.121	0.103		NC	NC
TbN	0.097	0.073	0.036	0.129	0.066	0.071	0.066	0.072	0.038	0.030	0.075	0.152	0.138	0.102	0.082	0.049	0.040		NC
TbR	0.059	0.154	0.084	0.145	0.084	0.085	0.050	0.088	0.090	0.084	0.057	0.147	0.141	0.114	0.072	0.088	0.031	0.023	

species of the same subgenus, which would be expected if there was any significant gene flow among sympatric species now or in the recent past. Likewise, sympatric populations of different subgenera showed similar pairwise distances to allopatric populations of different subgenera.

A similar analysis carried out on R_{ST} values found no significant differences among any of the categories (ANOVA: $F_{4,100} = 1.86$, $P = 0.123$). The analysis of R_{ST} figures had lower statistical power than the analyses of F_{ST} and Nei's D , as the four population samples with 40 or fewer individuals were omitted from this analysis, reducing the number of comparisons by almost 40%. However, the rank order of the means of the categories was roughly the same, with allopatric conspecifics having the lowest distance values and sympatric heterospecifics of the same subgenus the second lowest.

Although poorly resolved, the phenograms constructed with the neighbour-joining algorithm using chord distance (D_{CE}) or Nei's distance (Fig. 3a,b) or the maximum likelihood phenogram (Fig. 3c) depicted several clusters of conspecific populations, within the subgenus *Tropheops*, supported by nodes with relatively high (>75%) bootstrap values. Only the tree based on Nei's distance (Fig. 3b) exhibited clusters of sympatric heterospecific populations: two nodes clustering pairs of sympatric taxa, and two further nodes joining groups including sympatric heterospecific taxa. In all cases, these clusters comprised populations of *P. zebra* and *P. callainos*. However, bootstrap support for these clusters was weak, ranging from 18 to 45%. If each of the species

had a single origin, a fully resolved tree would contain 13 nodes uniting only conspecific populations. The neighbour-joining phenogram based on D_{CE} recovered 10 such nodes, and the maximum likelihood phenogram 9. On the other hand, if all speciation had taken place *in situ* in the present habitats, a maximum of 15 nodes should unite exclusively sympatric populations. More conservatively, if it is assumed that parallel speciation had occurred exclusively within subgenera, up to 11 nodes could be expected to unite clusters of all-sympatric populations. The neighbour-joining phenogram based on Nei's distance recovered two such nodes but with low bootstrap support, whereas neither of the other methods produced any at all.

Trees constructed from the $\delta\mu^2$ distance matrix depicted clusters that were supported by very low bootstrap values and generally did not support either the parallel or monophyletic model. In fact, they resolved several nodes that made little biological sense, clustering allopatric populations of *P. (Maylandia)* with *P. (Tropheops)* species that are morphologically and ecologically distinct. Exclusion of *Pzeb4*, which was known *a priori* not to conform to SMM (van Oppen *et al.*, 2000), did not improve the resolution of the trees (data not shown).

Discussion

Our results provide no support for the parallel sympatric speciation hypothesis for the surveyed taxa and at the geographical scale under consideration. Although the tree nodes are mostly poorly resolved, probably as a

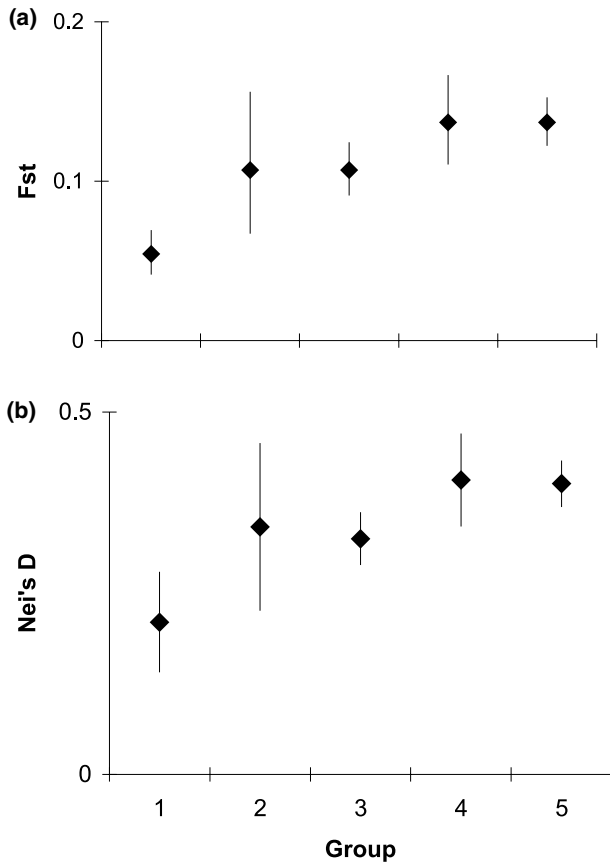


Fig. 2 Contrary to the predictions of the parallel speciation model, comparisons of allopatric populations of putative conspecifics (group 1) show lower genetic distances than sympatric populations of species of the same subgenus (group 2). Also, there is no indication that sympatric consubgeneric populations (group 2) show lower distances than allopatric populations of different species of the same subgenus (group 3), which would be expected if there was any significant gene flow among sympatric species now or in the recent past. Likewise, sympatric populations of different subgenera (group 4) or allopatric populations of different subgenera (group 5) show very similar distances. Similar results are found for comparisons of Slatkin's linearized F_{ST} (a) or Nei's genetic distance (b). Shown are means and 95% confidence intervals. For Slatkin's F_{ST} , means and confidence intervals were calculated on square-root transformed data. Statistical analyses are presented on Table 5.

result of the small number of loci used in this study (Takezaki & Nei, 1996), we believe the study has provided evidence for the monophyletic origin for all of the *P.* 'olive' populations studied and for the *P.* 'mauve' populations south of Usisya Bay. The overall support for monophyly of species emerges from the ANOVA on linearized F_{ST} and on Nei's distances, and also from the neighbour-joining phenogram based on chord distances and the maximum likelihood tree. Only the neighbour-joining tree based on Nei's distances is more ambiguous, but even here, the strongly supported nodes are consis-

tent with monophyletic species origins among *P.* (*Tropheops*), while nodes suggesting parallel *in situ* speciation among *P.* (*Maylandia*) are much more weakly supported. These results therefore do not suggest that parallel sympatric speciation has been a common event in the diversification of the cichlid populations we have investigated, at least not within the habitat patches they occupy in north-western Lake Malawi.

Can our results rule out parallel sympatric speciation in these taxa? One possible interpretation might be that parallel speciation has occurred *in situ*, but that gene flow among allopatric populations of conspecifics has been sufficiently high that all traces of their independent origin have been erased. This is impossible to refute, but it seems less parsimonious than the alternative, particularly considering the high fixation indices among allopatric conspecific populations. Rocky shore mbuna are generally sedentary, both as adults and juveniles, avoid open water and only reluctantly cross open sand (see Ribbink *et al.*, 1983; Rico & Turner, 2002; references therein). Previous studies have indicated that significant population structuring may be caused by sandy bays of a few hundred (van Oppen *et al.*, 1997a) or even, when a cold inflowing stream is present, tens of metres wide (Rico & Turner, 2002). In the present study, most populations were separated by sandy bays many kilometres wide. The Nkhata Bay and Cape Manulo populations, although more than 25 km apart, lie at opposite ends of largely unbroken rocky coast. Gene flow is more likely between these two sites, which are the only ones where we have no records of differences in colour among the sampled taxa (Table 2).

We cannot rule out the possibility that a few of the taxa in our study could have diverged *in situ*, or at least have exchanged genes with sympatric heterospecific populations relatively recently. The best candidates for this might be among the *Maylandia* species, where there are hints of similarities among sympatric *P. zebra* and *P. callainos* in the tree based on Nei's distances. Furthermore, investigation of relationships on a large spatial scale may yet reveal cases of parallel speciation in Lakes Malawi and Victoria.

Had we found evidence of apparent monophyly of sympatric species pairs, could we have made a strong case for parallel speciation? One alternative explanation might be introgression rather than common ancestry. Contradictory claims have been made for the geographical mode of origin of sympatric species pairs of sticklebacks (*Gasterosteus* spp.) in recently deglaciated lakes in British Columbia, Canada (Taylor & McPhail, 2000). A mitochondrial DNA (mtDNA)-based phylogeny provided what appeared to be evidence for monophyly of some species pairs within lakes and thus for sympatric speciation (Taylor & McPhail, 1999). However, a microsatellite phylogeny appears to suggest an allopatric 'double invasion' mode of speciation. The apparent

Table 5 Statistical comparisons among categories of genetic distance measures, expressed as probabilities of accepting the null hypothesis given by *post-hoc* pairwise comparisons using Tukey's HSD. Means and confidence intervals of these data are presented in Fig. 2. Numerical designations of columns indicate the same categories as are given in full on row titles. *Below diagonal*: square-root transformed values of Slatkin's linearized F_{ST} . This transformation was required to eliminate significant heteroscedasticity (transformed data: Levene's test: $F_{4,166} = 1.70$; $P = 0.152$, n.s.). Testing the null hypothesis of no difference in F_{ST} among categories of comparisons was carried out by one-way analysis of variance on the transformed data ($F_{4,166} = 11.85$; $P < 0.001$). *Above diagonal*: Nei's genetic distances. Overall ANOVA: $F_{4,166} = 9.05$; $P < 0.001$. Data not significantly heteroscedastic: Levene's test: $F_{4,166} = 1.51$; $P = 0.202$, n.s. *: $P < 0.05$; ***: $P < 0.001$.

Population comparison	1	2	3	4	5
1. Allopatric, same species	–	0.049*	0.014*	<0.001***	<0.001***
2. Sympatric, different species, same subgenus	0.012*	–	0.996	0.660	0.605
3. Allopatric, different species, same subgenus	<0.001***	>0.999	–	0.11	0.045*
4. Sympatric, different subgenus	<0.001***	0.576	0.295	–	>0.999
5. Allopatric, different subgenus	<0.001***	0.432	0.076	>0.999	–

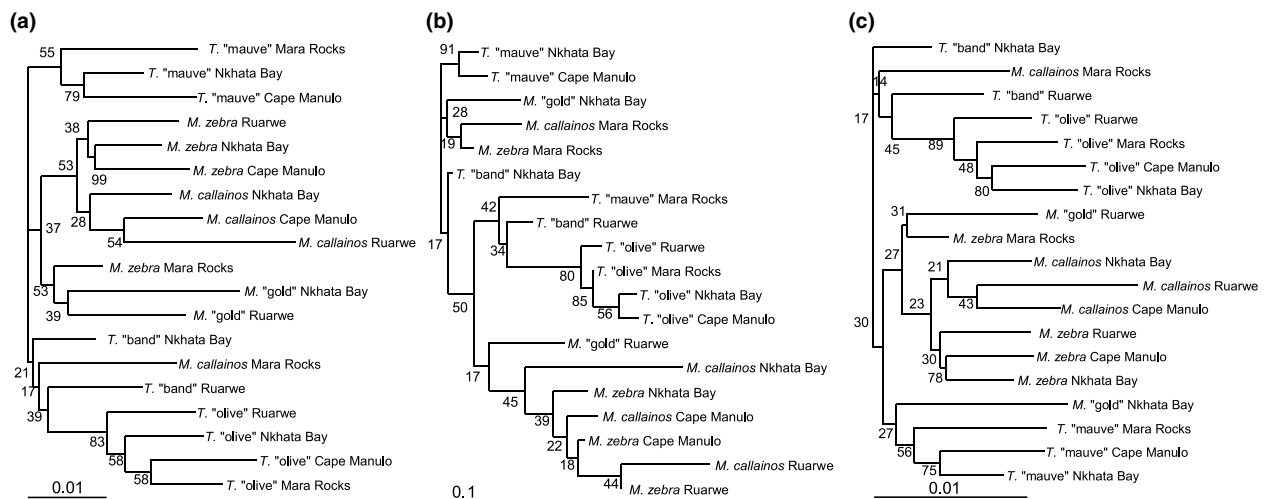


Fig. 3 Neighbour-joining and maximum-likelihood unrooted phenograms estimating relationships among 19 putative species and/or conspecific populations of Lake Malawi mbuna cichlids based on (a) Cavalli-Sforza and Edwards' chord distance (D_{CE}), (b) Nei's distance and (c) maximum-likelihood algorithm. See Table 1 for key to site and species names. Percentages of a thousand bootstrap values are given along branches for nodes with at least 75% support.

monophyly suggested by mtDNA is now believed to be the result of introgression among these young taxa, which can often leave a strong imprint on mtDNA which is a single locus with a four-fold smaller effective population size than the nuclear genome. It is therefore conceivable that other studies that have reported evidence for sympatric speciation based on mtDNA data alone (e.g. Schlieffen *et al.*, 1994; Shaw *et al.*, 2000) may give a different picture if based on multilocus nuclear markers. There have been many reports of fertile and viable cichlid hybrids in laboratory studies (e.g. Crapon de Caprona & Fritzsche, 1984; Seehausen *et al.*, 1997) and there is morphological evidence for hybridization among wild Lake Victoria cichlid species (Seehausen *et al.*, 1997) and molecular evidence for ancient introgression among Lake Tanganyika cichlids (Rüber *et al.*, 2001). Incomplete lineage sorting of mitochondrial and other markers has

been well-documented in Lake Malawi cichlids (e.g. Parker & Kornfield, 1997; Takahashi *et al.*, 2001), as would be expected if introgression had been common among divergent lineages.

What if introgression had led to apparent monophyly of sympatric taxa when assessed from many unlinked nuclear loci? This could only mean that introgression was so frequent as to cause massive gene flow across the entire genome. Might this mean that these taxa could be considered conspecific irrespective of their prior history? Given enough time, the gene pool should become homogenized, unless there was strong disruptive selection against intermediates (Clarke *et al.*, 1996). However, nearly neutral alleles might persist for a considerable time at high frequencies in a large population, whereas they might be less likely rise to high frequency *de novo*. Such populations could retain polymorphisms at both

male courtship trait loci and female preference loci. Under conditions of poor water clarity, differences in male colour might not be easily perceived either by conspecifics or predators, and they might be nearly neutral. Under appropriate conditions, such as increased water clarity, disruptive sexual selection could be restored (Seehausen *et al.*, 1997). Re-establishment of the same reproductive isolating barriers following a period of introgression has been termed 'respeciation' (Turner, in press). This would mimic the conditions assumed by several models of speciation by sexual selection, where populations initially contain several common alleles for male courtship traits and for female preferences for these traits in linkage equilibrium (Wu, 1985; Higashi *et al.*, 1999). Although these assumptions have been criticized as unrealistic and more stringent assumptions suggested (e.g. Turner & Burrows, 1995), this may have been unduly pessimistic.

Finally, it is appropriate to mention that several genetic measures, which take allele size into account and are linear with time, have been developed for phylogenetic inferences based on microsatellite-generated distances. However, Angers & Bernatchez (1998) empirically showed that, as suggested by Takezaki & Nei (1996), the use of distances that are independent of mutation models and that have lower sampling errors should be more efficient in depicting tree topologies among closely related taxa. Our results provide further empirical support that the use of methods such as the chord distance is more reliable than $\delta\mu^2$ distance in resolving population relationships, providing higher confidence values in tree topology, better clustering concordance and higher components of genetic variance among population groups. This is particularly relevant when a small number of loci have been used and the divergence among taxa is recent.

Conclusions

This study of genetic differentiation among sympatric putative species and allopatric populations of mbuna cichlids from Lake Malawi provides evidence for the following conclusions. (1) Heterogeneity in gene frequencies and highly significant fixation indices were observed among almost all populations tested, suggesting minimal or no gene flow. (2) Colour similarities of allopatric taxa in the study area generally reflect recent-shared ancestry, rather than *in situ* parallel origin. (3) The use of a genetic distance that makes no assumption of mutational process (such as D_{CE}) is more appropriate in depicting tree topologies when the number of loci used is small, and their mutational properties are unknown. (4) The fact that only 7% of the significant θ estimates became nonsignificant when loci with null alleles were excluded from the analysis suggests that null alleles have negligible effects on population structure estimates.

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