

SHORT COMMUNICATION

# Microsatellite paternity analysis on captive Lake Malawi cichlids supports reproductive isolation by direct mate choice

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## Abstract

Three sympatrically occurring members of a cichlid fish species complex from Lake Malawi have previously been reported to show assortative mating in the field. Offspring from three experimental tanks each housing both sexes of all three members of this complex were screened at one to four microsatellite loci to determine paternity. Reproductive isolation was found to persist without exception under laboratory conditions, providing strong evidence for behavioural barriers to mating. Direct mate choice is suggested as the process preventing interbreeding. The results are interpreted with reference to the rapid and explosive evolution of the cichlid group.

*Keywords:* assortative mating, cichlids, Lake Malawi, microsatellites, sexual selection, speciation

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## Introduction

The staggering species richness of the haplochromine cichlid flocks of the East African Great Lakes endows them with unique status among current vertebrate groups. Lake Malawi alone houses an excess of 500 endemic species (Ribbink *et al.* 1983), considered to have collectively arisen over a mere 700 000 years (Meyer 1993). This extraordinary radiation is traditionally considered to have been brought about by a process of intralacustrine microallopatric speciation (Fryer 1959, 1977), a hypothesis which has gained widespread support (e.g. Ribbink 1994). Here, diet or microhabitat specialization (facilitated by the 'key innovation' of cichlid pharyngeal jaws, Liem 1973) in geographically isolated populations (van Oppen *et al.* 1997b) is envisaged as resulting in the separation of lineages: if returned to sympatry (via subsequent lake level changes) it is supposed that such fragments of the original population would have diverged sufficiently to prevent interbreeding. However, closely related species often display little or no ecological differentiation in either diet or habitat preference (Reinthal 1990; Turner *et al.* 1991) and some can only be distinguished by male colour. This along with other life-history traits such as a

reproductive system characterized by polygyny, female mouthbrooding and the absence of any paternal care, has prompted some authors (Dominey 1984; Turner 1994) to raise doubts as to whether a niche specialization scenario alone can suffice as an explanation for the evolution of the group, suggesting sexual selection as an alternative potentially critical mechanism. The two need not operate exclusively; indeed most authors visualize them as complementary processes (Seehausen *et al.* 1997; Galis & Metz 1998). To date, however, the evolutionary mechanisms underlying the phenomenon remain unresolved.

The mbuna are a group of small, rock-dwelling cichlids occupying the shallow fringes of the lake. At Nkhata Bay, Malawi, three members of the *Pseudotropheus* (*Maylandia*) complex coexist sympatrically: *P. zebra*, *P.* 'zebra gold' and *P. callainos*. (Note: Stauffer *et al.* (1997) have recently proposed *Metriaclima* as a new genus name for the members of this species complex.) Although morphologically very similar, males of these putative species differ strikingly in colour and pattern (*P. zebra* males are blue with black vertical bars; *P.* 'zebra gold' males are yellow with brown vertical bars; *P. callainos* males are blue with no vertical bars; see, e.g. Konings 1995). In contrast, females exist as several morphs similar in appearance, particularly those of *P. zebra* and *P.* 'zebra gold'. Considerable overlap has been found in the depth distributions of these three taxa (Holzberg 1978; Ribbink *et al.* 1983). At depths where

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these species coexist, no differences have been found in either their microhabitat or feeding preferences (M. Genner, unpublished data). As yet, however, a comprehensive study of the biology of these taxa has not been carried out in the field (critically extending over a long period and thus observing any seasonal effects).

A previous analysis of six microsatellite loci from these putative species, taken from a single rocky point, showed highly significant allele frequency differences (van Oppen *et al.* 1998), suggesting a high degree of reproductive isolation between the three taxa. The aim of this study was to investigate whether direct mate choice alone could maintain this assortative mating, screening microsatellite loci to assign paternity to offspring where parents were free to mate among all three types housed in the ecologically and environmentally uniform conditions of aquaria.

## Materials and methods

### *Experimental procedure*

One male (with one exception, see below) and 3–8 females belonging to each of the three putative species were introduced into each of three experimental tanks (153 × 61 × 46 cm). Female numbers varied due to an initial lack of stock. In expt 1, six *P. callainos* males were present. A fin clip was taken from the soft-rayed region at the posterior end of the dorsal fin from each male before introduction and preserved in NaCl–DMSO buffer (Seutin *et al.* 1991). Several shelters (either rocks or terracotta pots) were placed in each tank to provide suitable spawning sites.

Each community was left to breed freely. Mouthbrooding females were caught and isolated in small tanks (30 × 22 × 20 cm). Initially all females were kept individually until the fry were released (a period of 28 days), although subsequently eggs were stripped from the mouth of the female after a period of 4 days and either developed in a hatchery or preserved immediately in buffer. A fin clip was taken from each female before re-introduction into the experimental tank. Each brood was kept isolated until fry had grown to a size of ≈ 5 cm, after which a fin clip was taken from each individual and preserved (see above).

The experiments were left running for a period of 7–9 months. A total of 61 broods were produced over the three experimental tanks although only a subset of 25 of these broods (selected at random) were analysed for paternity (due to time and space limitations).

### *Fish maintenance*

All fish used were wild caught as adults from Nkhata Bay, Malawi, and had been maintained in the laboratory prior to the experiment for a minimum of 6, maximum of 30,

months. Tanks were given weekly water changes (tap water, pH 8.0–8.3), illuminated with 58 W white fluorescent bulbs on a 12:12 light–dark cycle and fed daily with a mixture of trout pellets and fish flake. Water was filtered through an external Eheim™ wet–dry canister filter in one experiment and through a 1500 gallon recirculating system in the other two.

### *Microsatellite analysis*

Of 313 original offspring from 25 broods, 308 were analysed: five fry were lost before a fin clip could be taken (see Table 1). Brood sizes ranged from two to 29. Paternity of all individuals was assigned by screening one to four polymorphic microsatellite loci, all perfect dinucleotide repeats (*Pzeb1–3* [van Oppen *et al.* 1997a]; *UNH002* [Kellogg *et al.* 1995]). Samples were extracted following the method used by Rico *et al.* (1992) and run on an ALF sequencer (Pharmacia) with internal standards as described in van Oppen *et al.* (1997b). All samples were screened at *Pzeb1*, the most polymorphic of the four loci. In any case where ambiguity arose (due to there being only a 2 bp difference between potential fathers and/or the mother, or where offspring allele lengths were found to differ by 2 bp from any potential parent), three further loci were screened to ensure correct paternity assignment. All samples were amplified at least twice and in all cases PCR was reproducible. Amplified DNA extract from the offspring and potential parents for each brood were run on the same gel.

### *Spawning sequence*

Despite having been kept in captivity for some time, it was possible that there was a remnant seasonal 'clock' operating within individuals which could result in differences in breeding time and therefore maintain reproductive isolation temporally between the three putative species even under laboratory conditions. The sequence of spawning events with respect to female type was therefore examined by considering all broods produced from each experimental tank separately (expt 1, 16 broods; expt 2, 26 broods; expt 3, 19 broods; 61 broods in total). These data were analysed using a runs test for three categories (Zar 1984), testing whether there was a significant deviation from a random sequence of events in any of the three experimental tanks.

## Results

### *Paternity analysis using microsatellite loci*

Data from the most polymorphic microsatellite locus, *Pzeb1*, are presented in Table 1. All broods were found to be the product of complete assortative mating among the three putative species.

Table 1 *Pzeb1* locus genotypes for all individuals (paternally inherited alleles in bold)

Expt	Brood ID number	Maternal phenotype	n	Maternal genotype M1	Maternal genotype M2	Offspring genotypes						Paternal genotype		Paternal phenotype		
						1	2	3	4	5	6	7	8		P1	P2
1	1	callainos <sup>d</sup>	12 (+1) <sup>b</sup>	220	252	174/220	3	174/252	3	174/252	3	210/252	1 (+1) <sup>e</sup>	174	210	callainos
1	2	callainos	11 (+4) <sup>b</sup>	170	210	170/174	3 (+1) <sup>f</sup>	174/210	(1) <sup>f</sup>	174/210	(1) <sup>f</sup>	210/210	1	174	210	callainos <sup>f</sup>
1	3	callainos <sup>d</sup>	13 (+3) <sup>a</sup>	220	252	174/220	2 (+1) <sup>g</sup>	174/252	2 (+1) <sup>f</sup>	174/252	2 (+1) <sup>f</sup>	210/252	1	174	210	callainos <sup>f</sup>
1	4	zebra	9	170	184	164/170	(±2) <sup>h</sup>	164/184	3 (±2) <sup>h</sup>	164/184	3 (±2) <sup>h</sup>	170/184	4	164	170	zebra
1	5	zebra	21 (+1) <sup>b</sup>	194	228	164/194	3	164/228	3 (+1) <sup>i</sup>	164/228	3 (+1) <sup>i</sup>	170/228	8 (+1) <sup>i</sup>	164	170	zebra
1	6	gold	7	152	156	150/152	(±4) <sup>j</sup>	150/156	3 (±4) <sup>j</sup>	150/156	3 (±4) <sup>j</sup>	-	-	150	150	gold
1	7	gold	6 (+2) <sup>b</sup>	148	148	148/150	2 (+1) <sup>k</sup>	150/150	3 <sup>k</sup>	150/150	3 <sup>k</sup>	-	-	150	150	gold
1	8	gold	13	150	150	150/150	13	150/150	-	150/150	-	-	-	150	150	gold
2	9	callainos	7	162	210	162/190	2	162/204	5	190/210	0	204/210	0	190	204	callainos
2	10	callainos <sup>d</sup>	4	194	210	190/194	1	194/204	1	190/210	1	204/210	1	190	204	callainos
2	11	callainos <sup>d</sup>	2	194	210	190/194	0	194/204	1	190/210	0	204/210	1	190	204	callainos
2	12	zebra	11 (+1) <sup>b</sup>	176	198	176/210	5	176/214	3	198/210	1	198/214	2	210	214	zebra
2	13	zebra	11 (+2) <sup>c</sup>	158	164	158/210	5	158/214	1 (±1) <sup>l</sup>	164/210	0	164/214	4 (±1) <sup>l</sup>	210	214	zebra
2	14	zebra	12 (+2) <sup>c</sup>	168	188	168/210	4 <sup>m</sup>	168/214	2	188/210	1 (+3) <sup>m</sup>	188/214	2	210	214	zebra
2	15	gold	4	148	148	148/160	4	148/160	-	-	-	-	-	160	160	gold
2	16	gold	27 (+2) <sup>c</sup>	146	150	148/160	11	148/160	-	150/160	16	-	-	160	160	gold
2	17	gold	13 (+2) <sup>a</sup>	150	158	150/160	7	150/160	-	158/160	6	-	-	160	160	gold
3	18	callainos	14 (+1) <sup>b</sup>	168	240	162/168	2	168/198	5	162/240	3	198/240	3 (+1) <sup>n</sup>	162	198	callainos
3	19	callainos	12	164	198	162/164	1	164/198	4	162/198	3	198/198	4	162	198	callainos
3	20	callainos	9	164	238	162/164	4	164/198	2	162/238	1	198/238	2	162	198	callainos
3	21	zebra	9	180	184	154/180	2	168/180	2	154/184	1	168/184	4	154	168	zebra
3	22	zebra	14	164	200	154/164	5 (±1) <sup>p</sup>	164/168	0	154/200	6 (±1) <sup>p</sup>	168/200	2	154	168	zebra
3	23	zebra	14	146	164	146/154	3	146/168	5	154/164	3	164/168	3	154	168	zebra
3	24	gold <sup>d</sup>	14	150	150	144/150	8	150/150	6	-	-	-	-	144	150	gold
3	25	gold <sup>d</sup>	23	150	150	144/150	8	150/150	15	-	-	-	-	144	150	gold

<sup>a</sup>Sample lost before extraction. <sup>b</sup>Sample lost during extraction. <sup>c</sup>Suspected infertile eggs. <sup>d</sup>Three cases of pseudoreplication: different broods were found to have been spawned by the same mother. <sup>e</sup>One offspring with a maternally inherited allele of 242 bp (rather than 252 bp). Consistent at all other loci. <sup>f</sup>Multiple paternity – 170/176 and 176/210 genotypes found for two individuals in brood 2, 176/210 for one individual in brood 3. Both broods were screened at a further three loci. Three individuals match another *P. callainos* male (same male in all three offspring: genotype 166/176 *Pzeb1*) at two other loci, the third locus being nondiagnostic (both males sharing identical genotypes). <sup>g</sup>One individual with paternal 176 bp instead of 174 bp. This is a probable mutation or technique artefact and not a further example of multiple paternity (from screening the other three loci). <sup>h</sup>Two individuals where maternal allele didn't amplify, but consistent results at all other loci. <sup>i</sup>Two offspring were found with a maternal allele of 230 bp. Both show parental consistency at all other loci. <sup>j</sup>Four samples where the paternal allele amplified only but consistent across all other loci. <sup>k</sup>Maternal allele amplified only in one individual, paternal only in three individuals, all consistent at three other loci, paternal allele amplified only in one sample. <sup>l</sup>Paternal allele amplified as 208 bp. Paternal alleles consistent in all cases at further three loci. <sup>m</sup>In one individual the maternal allele amplified as 196 bp, possible mutation, PCR or ALF artefact. <sup>n</sup>One individual where the paternal allele amplified only.

Samples from 16 individuals could not be amplified at any loci. Six of these samples were suspected to be infertile eggs (discolouration of the egg noted before extraction). Loss of the remaining 10 was probably due to failure of the extraction process. For 12 offspring tested, only a single *Pzeb1* allele amplified, but consideration of the allele sizes of potential parents indicated that they should have been heterozygous at this locus. Each of these 12 samples amplified without ambiguity at a minimum of two further loci. In three cases, pairs of broods were found to have the same mother and as such cannot be considered as independent replicates (broods 1 and 3; 10 and 11; 24 and 25). Multiple paternity was found in the only situation where more than one male of a putative species was present in the tank; *P. callainos* in replicate 1. The largest and behaviourally dominant male sired all 12 offspring from brood 1, 9/11 from brood 2 and 12/13 from brood 3. The other three offspring were sired by a second male of the same putative species. None of the other four males of this species produced any offspring. Multiple paternity is already documented in *Pseudotropheus* spp. (Kellogg *et al.* 1995; Parker & Kornfield 1996), although not in aquaria. Seven cases of offspring alleles differing from the parental alleles were found at the *Pzeb1* locus. In each of these parentage was determined by screening all four loci (see broods 3, 5, 14, 18). In only one of these cases was the difference between parental and offspring alleles greater than 2 bp in length. These could be due to mutation or PCR/gel artefacts. A mutation rate of  $2.7 \times 10^{-2}$ /generation seems high, although it is within the generally accepted range of  $10^{-5}$ – $10^{-2}$  for microsatellite loci (Jarne & Lagoda 1996). It is therefore plausible that these represent true mutations; however, in the absence of sequence data this is highly speculative.

Thus, 264 offspring from 22 independent broods (seven *P. callainos*, eight *P. zebra*, seven *P. 'zebra gold'*) were the product of complete assortative mating, with two cases of multiple paternity where the offspring of one brood were sired by two different males, both of which belonged to the same putative species.

#### Spawning frequency and sequence

The spawning sequence within each tank is shown in Table 2. No significant differences were found in the

distribution of spawning over time of the three putative species (runs test,  $Z = 0$ ,  $Z = 0.407$ ,  $Z = 0.371$ , respectively,  $P > 0.5$  for all). This strongly suggests that there was no temporal variation in spawning events with respect to female type.

#### Discussion

Our finding of complete assortative mating in three independent replicate experiments lends strong support to field studies reporting reproductive isolation among these taxa (van Oppen *et al.* 1998). Our results illustrate unequivocally that assortative mating can persist in the absence of ecological or environmental cues, such as microhabitat choice or seasonality, as indicated by the lack of differences observed among taxa in the distribution of spawning events over time. Other studies on haplochromine cichlids in aquaria have suggested that behavioural dominance amongst heterospecific males can create conditions which prevent free female choice from the potential mates available (Crapon de Caprona 1986). As in all experimental tanks males belonging to each of the three putative species were able to breed, heterospecific behavioural dominance effects did not influence the spawning events discussed here. Furthermore, results showing complete reproductive isolation are considerably strengthened by the occurrence of multiple paternity in two *P. callainos* broods, indicative of either furtive mating (not as yet documented for these species) or of two males holding spawning territories within the same tank. That either of the above occurred within one tank suggests that despite multiple paternity being an 'option' open to both males and females, no cases were found of two heterospecific males siring offspring within the same brood.

Laboratory experiments indicate that hybridization among these taxa is possible when conspecific mates are absent, suggesting that there are no mechanical barriers preventing interbreeding.

We suggest that the observed reproductive isolation is maintained by direct mate choice. Given the male characteristics of bright colouration and exaggerated fins (M. Knight, unpublished data), female choice is circumstantially inferred. Female choice has been demonstrated in other maternal mouthbrooding cichlids (Hert 1991;

**Table 2** Spawning sequence through with respect to female type for each of the three experimental tanks, where C = *Pseudotropheus callainos*; B = *P. zebra*; and G = *P. 'zebra gold'*

Spawning order	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
Experiment 1	C	B	G	G	B	B	C	G	C	G	B	G	C	C	C	G										
Experiment 2	G	G	B	G	C	C	B	G	C	C	C	C	G	G	G	G	C	B	G	C	G	B	G	G	G	G
Experiment 3	B	B	C	C	C	B	C	C	C	G	C	B	G	G	B	B	C	G	B							

Seehausen *et al.* 1997; Taylor *et al.* 1998); however, whether both sexes or just one play a role in mate recognition within this species complex is as yet unknown. A study looking specifically at this issue is currently in progress.

If direct mate choice is an important isolating mechanism in these taxa one possibility is that sexual selection has in itself brought about the separation of lineages. Although still controversial, the theoretical plausibility of sexual selection playing a direct role in the process of speciation (e.g. Lande 1981, 1982; Wu 1985; Turner & Burrows 1995; Payne & Krakauer 1997) is now largely accepted. The debate has now shifted, focusing on whether such a process can have had any real significance as a mechanism of evolutionary change (see, e.g. Butlin 1987; Butlin & Tregenza 1997 for discussion). Although some empirical support exists (Kaneshiro & Boake 1987; Barraclough *et al.* 1995), many authors remain sceptical.

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