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Laboratory mating trials indicate incipient speciation by sexual selection among populations of the cichlid fish *Pseudotropheus zebra* from Lake Malawi

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It has been suggested that sexual selection may have played a major role in the rapid evolution of hundreds of species of cichlid fishes in Lake Malawi. We report the results of a laboratory test of assortative mating among Lake Malawi cichlid fishes from five closely related geographical populations differing in male courtship colour. Paternity of clutches was tested using microsatellite DNA typing of offspring. Out of 1955 offspring typed, 1296 (66.3%) were sired by the male from the same population as the female, which is more than three times the rate expected if females do not differentiate among males of the different populations (20%). This result indicates that mate preferences of geographical races are strongly differentiated, consistent with the races representing incipient geographical species diverging under sexual selection exerted by female preferences for different male courtship colours.

Keywords: geographical speciation; cichlid fishes; Lake Malawi; assortative mating; sexual selection

1. INTRODUCTION

Recently, considerable interest has focused on the role of sexual selection in promoting reproductive isolation and speciation (Panhuis *et al.* 2001; Boughman 2002), although the evidence from comparative studies is not universally convincing (Gage *et al.* 2002; Morrow *et al.* 2003). Models indicate that sexual selection may lead to sympatric speciation (Turner & Burrows 1995; Higashi *et al.* 1999), but it has been suggested that divergence in allopatry or parapatry is more likely (Turrelli *et al.* 2001). Geographical variation in male courtship traits may not be caused by sexual selection, but by genetic drift or natural selection. Sexual selection may lead to variation in male traits without leading to speciation, for example where signal transmission evolves to maximize detectability in different environments (Price 1998). Furthermore, variation in phenotypic traits such as male colour pattern may be independent of mate choice, even where selection has been implicated (e.g. Llopart *et al.* 2002). However, where sexual selection by female choice has exerted a dominant influence on the evolution of male courtship signals, we should expect to find an interpopulation correlation between female preferences and male courtship traits under standardized environmental conditions in the laboratory.

The existence of many recently evolved cichlid fish species in Lake Malawi (Meyer 1993; Turner 1999) provides an excellent opportunity to investigate the early stages of speciation. It has been suggested that sexual selection may have played an important role in Malawi cichlid diversification (Dominey 1984; McKaye 1991; Turner 1994, 1999). Sympatric rocky-shore 'mbuna' taxa differing largely in male colour appear to be biological species (Holzberg 1978; McKaye *et al.* 1982, 1984; van Oppen *et al.* 1998; Knight *et al.* 1998; Jordan *et al.* 2003). However, there are also many allopatric forms that, compared with the sympatric species studied to date, are less ecologically differentiated (Ribbink *et al.* 1983), are more genetically similar (Allender *et al.* 2003) and differ more subtly in male courtship colours (e.g. Konings 2001). These may represent incipient species diverging under sexual selection acting on male colour. In this case, we would predict that geographically isolated colour forms should show a high degree of assortative mating when tested in the laboratory. We test this prediction using five populations of the *Pseudotropheus zebra* complex, one of the most species-rich groups of the Lake Malawi mbuna cichlids.

2. MATERIAL AND METHODS

(a) *Experimental animals*

The study animals belong to the mbuna complex endemic to Lake Malawi. Males defend territories to which they attempt to attract females. Females are mouth brooders, carrying the eggs, which may be fertilized by up to six males (Kellogg *et al.* 1995; Parker & Kornfield 1997), out of the male territory immediately after spawning. Females retreat to quiet shelters for up to three weeks to brood the developing young.

The study populations belong to the *P. zebra* 'blue–black' complex, part of the subgenus *Maylandia* (also known by the junior synonym *Metriaclima*; e.g. Stauffer *et al.* 1997). At the majority of sites studied to date, only one 'blue–black zebra' is found. At Mphanga Rocks, two 'species' differing in male colour and in subtle details of head and jaw shape coexist (Konings 2001), but there is evidence for recent or current gene flow between them (Smith & Kornfield 2002). Although several allopatric and sympatric forms have been given formal species names (Stauffer *et al.* 1997), many of these names are not presently accepted by other authorities (Konings 2001; Smith & Kornfield 2002). Assigning species status to allopatric forms is usually arbitrary (Mayr 1982). Many *P. zebra* taxa, including those that occur sympatrically without apparently interbreeding

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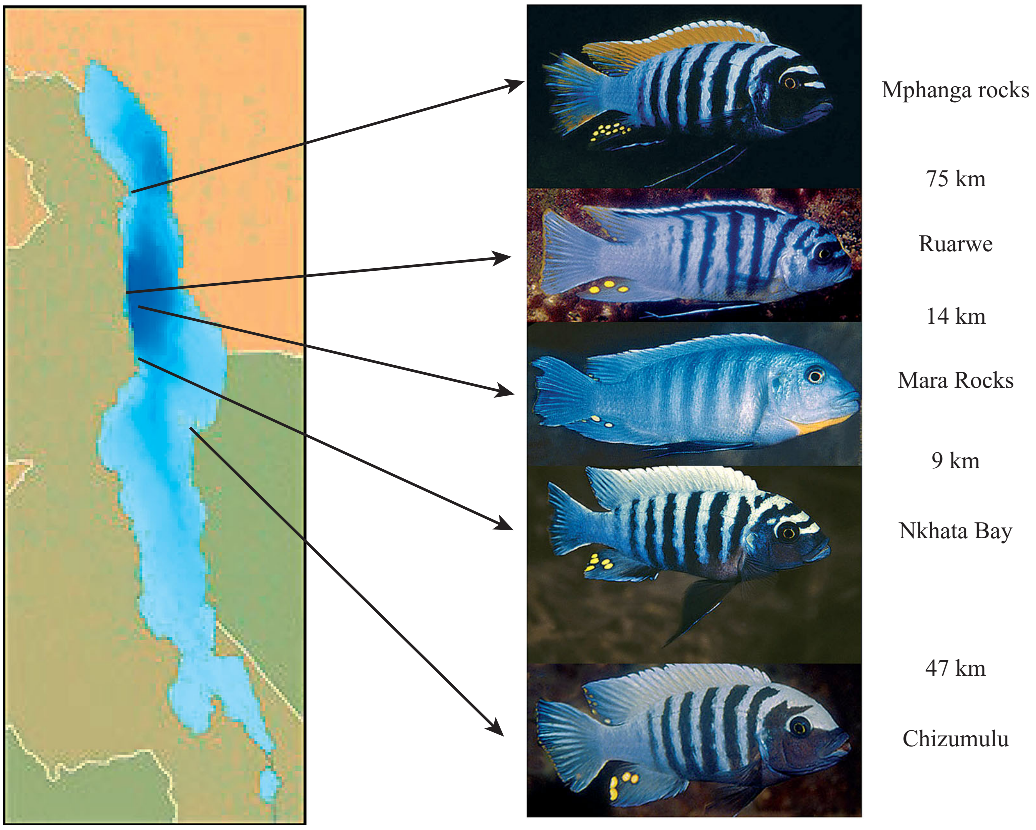

Figure 1. Experimental analysis of assortative mating was carried out using specimens collected from five populations in the northern half of Lake Malawi. Males of the populations differed in one or more elements of their breeding dress. Females (not shown) of all populations were dark brown with faint vertical bars; those from Mphanga Rocks differed in having a faintly reddish dorsal fin. Distances shown are estimates of the shortest distance between sites where populations of the fish with the colour phenotype illustrated have been found, which are not necessarily the sites where we collected the experimental fish.

(Knight *et al.* 1998), show no apparent post-mating barriers, so post-mating isolation is not a useful criterion for species status.

The study was carried out using *P. zebra* from Nkhata Bay (NB) and three other allopatric populations, from Chisumulu Island (CH), Mara Rocks (MR) and Ruarwe (RW). The fifth population used was the form with a red–orange dorsal fin from Mphanga Rocks, sometimes known as *Pseudotropheus emmiltos* (EM). A phylogeny based on amplified fragment length polymorphism (AFLP; Allender *et al.* 2003) indicated that the four populations from the western side of the lake (NB, MR, RW, EM) form a clade. At present, major habitat discontinuities prevent gene flow between populations at these sites (Rico *et al.* 2003). It is known that water levels in Lake Malawi have varied greatly (Sturmbauer *et al.* 2001), creating and eradicating barriers to dispersal. It is likely that our study populations have differentiated *in situ* since present habitat barriers were established. The fifth population, at Chisumulu Island, appears to be less closely related (Allender *et al.* 2003). This population was included in the study because of the close resemblance of the colour pattern of the males to that of males from Nkhata Bay.

All fishes used in the experiments were collected from the wild. Males of all five races share the same blue–black vertical-barred ground coloration but all differ in at least one other aspect of pattern or coloration (figure 1). Females of the most common morph in all five populations are dark brown with faint vertical bars. EM females have a slightly reddish dorsal fin, but females of the other populations could not be reliably distinguished. In our experiments, we did not use the rarer blotched or orange morph females that occur in all populations except EM (Ribbink *et al.* 1983).

(b) *Experimental protocol*

Mate-choice trials were carried out in three glass aquaria 3.5 m long, 0.8 m wide and 0.4 m deep. Water was maintained at a temperature of *ca.* 25 °C using standard aquarium thermostatic heaters, filtered using external canister filters and illuminated by fluorescent tubes on a 12 L : 12 D regime. Fishes were fed daily on a diet of flake, pellet and fresh food (pea and shrimp mix) and given regular one-third water changes.

We adopted a ‘partial partition’ design (Turner *et al.* 2001), where males were confined to 80 cm × 70 cm territories using plastic grids adjusted to allow the smaller females to pass through and mate with any preferred male. Each male territory contained a terracotta flowerpot to act as a refuge and spawning site. Three replicates were carried out concurrently. Five males, one of each race, were present in each tank, along with between three and eight females of each population. Males were randomized with respect to size and position. In every replicate, males of all races sired one or more broods indicating that all males were available as potential mates.

(c) *Paternity testing*

Before introduction into the experimental aquarium, each fish was fin-clipped. A small (*ca.* 1 mm^2) piece of tissue was cut from the soft-rayed part of the dorsal fin and placed in 100% ethanol for later DNA analysis. Females carrying clutches were removed from the aquarium after *ca.* 5 days. The clutch was removed by gently opening the female's mouth and allowing the larvae to drop into a tray of water containing an anaesthetic overdose (MS-222), before preservation in 100% ethanol. During the removal of offspring, undeveloped and presumably unfertilized eggs were occasionally found in the mouths of females, at approximately the same frequency as observed in single-population stock-breeding tanks and in forced crosses between these races, suggesting that estimates of crossing rates largely reflected mate preferences rather than differences in offspring mortality or fertilization probability.

After removal of the brood, the mother was fin-clipped and allowed to recover in an isolation tank for *ca.* 10 days before being put into a further replicate of the experiment or returned to stock.

(d) *Microsatellite analysis*

All mothers, potential fathers and offspring were typed at between one and three loci until parentage could be assigned without ambiguity. Three highly variable dinucleotide microsatellite loci were used: UNH130 (Lee & Kocher 1998), UME003 (Parker & Kornfield 1997) and Pzeb1 (van Oppen *et al.* 1997). DNA was extracted from tissue samples using a salt–chloroform protocol (Rico *et al.* 1992) and amplified using a FAM/HEX/TET-labelled forward primer for the ABI system or a Cy5-labelled forward primer for the ALFexpress system using standard PCR protocols. PCR products were resolved on either an ABI 377 (Applied Biosystems) or an ALFexpress (Amersham Pharmacia Biotech), with internal size standards. Identical sample controls were used throughout to prevent potential errors in scoring arising from hardware differences. Alleles were sized using GENESCAN and GENOTYPER software for the ABI-generated data and FRAGMENT MANAGER for the ALFexpress data.

(e) *Data analysis*

To account for multiple paternity within a clutch, we considered that each part of a clutch sired by a different male represented an independent spawning decision, and further calculations are based on these 'spawnings' rather than on whole clutches. As the numbers of eggs fertilized by different males could vary considerably within a clutch, we also report the numbers of eggs fertilized by males of different populations, as the most direct measure of mating success.

If mating is random with respect to population, the expected number of spawnings by a female of population i sired by a male of population j will be the product of the total number of spawnings in which males of population j participated and the total number of spawnings in which females of population i participated, divided by the total number of spawnings. The observed number of females of population i mating with males of population j was then divided by the expected value for this combination of mates. If this 'spawning ratio' is greater than 1.0, we conclude positive assortative mating between female i and male j relative to the other populations in the comparison. If there is a general preference for mating with individuals of the same population, we predict that the mean spawning ratio for same-population matings should be significantly greater than that of different-population matings. To test for the effects of replicate tanks, tank was included as a factor in a two-way analysis of variance using the ratio of observed to expected values for all population comparisons as the dependent variable. For this analysis, where females had produced broods in more than one replicate, the pseudoreplicate broods were deleted at random until each female was represented by a single clutch. Levene's test was used to check for homogeneity of variances, with no significant deviation from homogeneity found after $\ln(x+1)$ transformation ($F_{5,69} = 1.02$, $p = 0.412$).

3. RESULTS

A total of 1955 offspring were typed from 96 broods (mean brood size of 20.6, s.e. = 0.97, maximum of 56, minimum of 2). Eighteen clutches (18.8%) were sired by more than one male—one was sired by three males, the rest by two. Females of the five populations did not differ in their probability of multiple mating ($\chi^2_4 = 3.99$, $p > 0.10$). Most clutches ($n = 54$) were fertilized exclusively by males from the same population as the female. Among the 42 clutches wholly or partly fertilized by males from different populations from the female, 24 were entire broods sired by a single male, 17 were fertilized by two or more males—one from the same population as the female and one or two from an allopatric population—and in a single case a clutch was sired by two allopatric males.

(a) *Assortative mating*

Seventy-one (61.7%) out of 115 spawnings, comprising 1296 eggs (66.3%), were fertilized by males of the same population (table 1). Ratios of observed to expected numbers of spawnings differed significantly when pairings of males and females from the same population were compared with pairings from different populations ($F_{1,69} = 89.7$, $p < 0.001$), with neither replicate ($F_{2,69} = 0.16$, $p = 0.855$) nor interaction term ($F_{2,69} = 0.12$, $p = 0.887$) being significant. Pairings between fishes of the same population occurred between 2.04 and 6.47 times as often as expected, while the most frequent pairing between different populations was only 1.5 times more frequent than expected.

Only two combinations of matings between different populations occurred more often than expected: Chisumulu females with Nkhata Bay males (1.36) and Nkhata Bay females with Chisumulu males (1.50). Males of these two populations have the most similar breeding dress of any pair of populations (figure 1).

Mean brood sizes did not differ between clutches sired by males of the same population, those sired by males of a different population and those sired by both same-population and different-population males (ANOVA: $F_{2,93} = 1.49$, $p = 0.23$). Likewise, the proportions of undeveloped (presumably infertile) eggs in these classes of clutches were approximately the same (2.2–2.5%; G-test: $G_2 = 0.15$, $p > 0.10$). Thus, the observed levels of assortative mating seem to reflect mate choice, rather than reduced fertilization rates or reduced offspring survival in pairings between fishes from different populations.

(b) *Asymmetries of preferences*

Although some apparent asymmetries were observed in mate preferences, none of these differences was significant ($\chi^2_1 < 3.84$, $p > 0.05$; or test invalid owing to low sample sizes).

(c) *Individual variation in female preferences*

Eight females mated in all three replicates, and a further 12 in two replicates. In 17 out of 23 cases (73.9%) where

Table 1. Spawnings (and total numbers of eggs) sired among pairwise combinations of populations of *Pseudotropheus zebra* complex (pooled over three replicates).

	male						
female	CH	MR	NB	EM	RW	total	percentage of clutches (eggs) fertilized by males of own population
Chisumulu (CH)	19 (296)	1 (27)	11 (183)	1 (1)	5 (50)	37 (557)	51.4 (53.1)
Mara Rocks (MR)	1 (12)	9 (117)	2 (37)	2 (22)	2 (63)	16 (251)	56.3 (46.6)
Nkhata Bay (NB)	8 (134)	0 (0)	9 (106)	2 (23)	1 (8)	20 (271)	45.0 (39.1)
Mphanga Rocks '*emmiltos*' (EM)	1 (10)	0 (0)	1 (1)	26 (634)	4 (44)	32 (689)	81.3 (92.0)
Ruarwe (RW)	0 (0)	0 (0)	1 (26)	1 (18)	8 (143)	10 (187)	80.0 (76.5)
total	29 (452)	10 (144)	24 (353)	32 (698)	20 (308)	115 (1955)	61.7 (66.3)
percentage of male mating that was with females of own population	65.5 (65.5)	90.0 (81.3)	37.5 (30.0)	81.3 (90.8)	40.0 (46.4)	61.7 (66.3)	

a female mated with a male of her own population, a different male of the same population sired all or part of the next clutch laid by that female. By contrast, out of the 12 spawnings where a male from a different population sired all of part of the clutch, the female was never found to mate with a male of that allopatric population again. This suggests that individual females do not have strong preferences for males of particular allopatric populations.

4. DISCUSSION

Previous studies have demonstrated reproductive isolation among sympatric species of Lake Malawi cichlid fish in laboratory trials (Knight *et al.* 1998; Jordan *et al.* 2003). One study (Couldridge & Alexander 2002) has demonstrated that females prefer to remain close to conspecific males during a choice trial among fishes from populations that may be allopatric in origin (the source of the fishes is not known) but probably represent races of species that co-occur in sympatry. Our experiment is the first published use of genetic paternity testing, to our knowledge, to demonstrate a high level of assortative mating among allopatric colour forms of Lake Malawi cichlids. Our results indicate that these populations have already developed at least partial reproductive isolation, consistent with at least incipient species status.

The Nkhata Bay and Chisumulu populations did not show any marked degree of assortative mating with respect to each other. These two populations are not likely to be among the most closely related of the five populations (Allender *et al.* 2003). It seems most likely that the lack of assortative mating between these two populations is a result of the similarity of their male breeding colours.

Our study indicates that geographically isolated populations of *P. zebra* have undergone divergent sexual selection leading to coevolution of male courtship traits and female preferences, as predicted by models of speciation based on Fisherian runaway sexual selection (e.g. Lande 1981). It is difficult to disprove the proposal that the different male traits evolved for other reasons, for example to maximize visibility in local environmental conditions (Boughman 2002). However, assortative mating in these incipient species is substantial in controlled conditions. This indicates that females of these different populations do not merely prefer the male that appears brightest in one particular environment. Evidence for clear correlations between mate preferences and male courtship phenotypes is consistent with a role for sexual selection in speciation (e.g. Naisbit *et al.* 2001; Panhuis *et al.* 2001; Masta & Maddison 2002).

Some studies, for example on *Heliconius* butterflies (McMillan *et al.* 1997) and striped mice (Pillay 2000), have also provided evidence for association between courtship signals and mate preferences in geographically isolated populations. Other species show very different patterns. For example, sympatric reproductively isolated kokanee and sockeye salmon both strongly prefer brighter red mates (Craig & Foote 2001). The parapatric butterfly fishes *Chaetodon punctatofasciatus* and *C. pelewensis* are different in colour, but do not mate assortatively (McMillan *et al.* 1999). Male *Papilio canadensis* butterflies prefer to mate with female *Papilio glaucus* rather than conspecific females (Deering & Scriber 2002). Even where divergence of mate preferences among populations occurs, and is correlated with variation in courtship traits, speciation may be prevented, for example by largely indiscriminate sneaky mating by male guppies (Magurran 2001) and Arctic charr (Jonsson & Jonsson 2001). Elucidating differences in geographical patterns of mate preferences and the factors that may modulate how these can be translated into reproductive isolation is likely to be important in clarifying the differences among taxa in the rates and mechanisms of speciation.

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