

No barriers to gene flow among sympatric polychromatic ‘small’ *Telmatherina antoniae* from Lake Matano, Indonesia

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Genetic divergence, assortative courtship and intermale aggression were assessed between sympatric colour morphs of the sailfin silverside *Telmatherina antoniae*, endemic to Lake Matano, Indonesia. Genetic analysis using microsatellite markers showed no barriers to gene flow among *T. antoniae* primary colour morphs (blue and yellow) within sampling sites, sympatric populations or at the lake-wide level. Low but significant genetic differentiation was found between yellow morphs and mixed (blue–yellow) morphs. Behavioural surveys indicated assortative courtship does occur along primary colour lines; however, intermale aggression among paired and intruding male morphs appeared equal with respect to male colour. These observations support the hypothesis that males view other males as threats to their courtship regardless of their colour. This study supports recent work suggesting that assortative mating is present in *T. antoniae* despite a lack of reproductive isolation among colour morphs.

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Key words: colour; gene flow; intermale aggression; Lake Matano; microsatellites; *Telmatherina*.

INTRODUCTION

Since the formulation of the theory of natural selection, the maintenance of phenotypic polymorphisms within a population has been a subject of great interest to biologists. The presence of colour polymorphisms within a species offers an opportunity for crypsis, predation avoidance, assortative mating and the potential for sexual selection for a particular colour morph. Sexual selection, through female mating preferences or male competitive behaviour, has been implicated as a means of maintaining colour polymorphisms in natural populations (Kingston *et al.*, 2003; Gray & McKinnon, 2007). Because many species occupy variable habitats throughout their lifetimes, their relative conspicuousness to both potential mates and predators also varies (Gamble *et al.*, 2003; Chunco *et al.*, 2007). Frequency and density-dependent selection for particular colour morphs have been shown to maintain colour polymorphisms in differing habitats and social

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environments (Sinervo *et al.*, 2001; Svensson & Sinervo, 2004), even despite weak female preferences (Chunco *et al.*, 2007). Thus, colour polymorphisms are rarely maintained by one mechanism and are probably sustained by a variety of ecological and behavioural factors (Kingston *et al.*, 2003; Gray & McKinnon, 2007).

Among diverging, but sympatric, fishes there is mixed evidence that colour polymorphisms play a role in the speciation process (Seehausen, 2000; Boughman, 2001; Danley & Kocher, 2001; Roy *et al.*, 2007a). Assortative mating, where individuals select mates of similar colour or phenotype, however, has the potential to initiate genetic divergence *via* random genetic drift if gene flow is impeded between individuals of dissimilar colours. Such a process has been suggested for a number of fish species; notably the poeciliids (Endler, 1983; Kingston *et al.*, 2003) and African cichlids (Seehausen, 1997; Pauers *et al.*, 2004; Salzburger *et al.*, 2006). Within the latter group, there are striking examples of incipient speciation *via* sexual selection, including the *in situ* isolation of Lake Victoria's sympatric *Pundamilia nyererei* (Witte-Maas & Witte) and *Pundamilia pundamilia* Seehausen & Bouton, two species with minimal morphological differentiation with the exception of colour (Seehausen, 1997). Other species complexes show more regional variation in isolation among morphs, but have been suggested as cases of incipient speciation. For example, in hamlets (*Hypoplectrus* sp., Serranidae), colour polymorphisms have been associated with assortative mating and sexual selection (Fischer, 1980; Graves & Rosenblatt, 1980; Michiels, 1998); however, reproductive isolation is not complete, as the production of fertile hybrids is still possible (Whiteman & Gage, 2007). Furthermore, localized ecological processes appear to influence genetic structure among certain colour morphs (Puebla *et al.*, 2008).

Within the telmatherinid species complex of Lake Matano, there are iridescent individuals of yellow, blue and mixed colours found in sympatry. Males are primarily described as polychromatic (Kottelat *et al.*, 1993; Herder *et al.* 2006a); however, females, whose body colour is primarily bluish grey (Kottelat, 1991), also exhibit blue or yellow colouration on their second dorsal and anal fins (Walter, 2009). No breeding experiments have been performed to determine whether colour is genetically or environmentally controlled, or if colour is related to age class or reproductive status. Initial divergence in the *Telmatherina* species complex was shown to be more related to body shape than primary body colour (Roy *et al.*, 2007a) with the group consisting of two major morphotypes on the basis of fin shape, 'sharpfins' and 'roundfins' (Herder *et al.*, 2006a, b, 2008). *Telmatherina antoniae* Kottelat is a 'roundfin' species that occurs in two genetically distinct morphospecies: large and small (Herder *et al.*, 2008), both endemic to Lake Matano. Population genetic analysis using microsatellites in *T. antoniae* showed genetic structure consisting of at least four, and possibly as many as six, sympatric genetic clusters (or 'populations') within Lake Matano (Walter *et al.*, 2009). The small size of Lake Matano (c.164 km², Herder *et al.*, 2008) coupled with the mobile and non-territorial tendencies of these fish (Gray & McKinnon, 2006; Herder *et al.*, 2008) probably provides the opportunity for considerable gene flow among geographic regions of the lake. Thus, variation in visible genetic polymorphisms is expected to decrease due to genetic drift and inbreeding (Hayashi & Chiba, 2004), unless individuals mate assortatively with respect to those polymorphisms.

TABLE I. Summary of sampling and genetic data from eight microsatellite loci in *Telmatherina antoniae*

Site	Colour	<i>n</i>	<i>n_A</i>	<i>A_P</i>	<i>H_O</i>	<i>H_E</i>
Lawa	Blue	19	10.1	5	0.62	0.76
	Mixed	3	4.3	0	0.75	0.69
	Yellow	28	12.1	2	0.65	0.77
Indah	Blue	17	9.6	2	0.71	0.73
	Mixed	1	1.8	0	0.75	0.38
	Yellow	37	12.4	1	0.66	0.73
Owesu	Blue	25	10.6	1	0.65	0.75
	Mixed	10	7.6	0	0.60	0.72
	Yellow	17	9.3	1	0.70	0.75
L Ondau	Blue	10	8.1	0	0.58	0.75
	Mixed	6	5.5	1	0.54	0.63
	Yellow	35	13.3	4	0.60	0.79
P Rio	Blue	32	11.9	1	0.64	0.75
	Yellow	10	7.5	0	0.73	0.70
Taima	Blue	16	10.0	2	0.66	0.76
	Mixed	3	3.6	1	0.63	0.63
	Yellow	28	12.1	1	0.63	0.79
T Merah	Blue	12	7.5	0	0.60	0.69
	Mixed	18	9.9	3	0.63	0.77
	Yellow	11	7.5	0	0.58	0.71
W Lonto	Blue	18	9.6	4	0.64	0.76
	Mixed	10	7.8	0	0.59	0.71
	Yellow	20	9.5	1	0.64	0.74

n, sample size; *n_A*, mean number of alleles per locus; *A_P*, number of private alleles; *H_O*, mean observed heterozygosity; *H_E*, mean expected heterozygosity.

In this study, genetic divergence among colour morphs was assessed using polymorphic microsatellite loci to test for mating isolation along primary colour lines in the 'small' *T. antoniae* morphospecies. Given a disproportionate number of colour morphs (Table I) found among sampling sites, genetic analyses were performed at both site-by-site and lake-wide levels. Observations of courting were performed to test for assortative mating behaviour, because males have been described as persistently courting females (Kottelat, 1991). Additionally, given the occurrence of fierce intermale fighting (Kottelat, 1991; Gray & McKinnon, 2006), behavioural surveys were used to characterize the role of colour in intermale aggression among mate-paired males and intruders.

MATERIALS AND METHODS

A total of 428 individuals identified as 'small' *T. antoniae* on the basis of morphology (following Kottelat, 1991) were sampled non-lethally with a seine from eight sites in Lake Matano, Indonesia (2°29' S; 121°20' E; Fig. 1). *Telmatherina antoniae* are more easily identifiable *in situ* than other *Telmatherina* species and, at the time of sampling, only 'small' *T. antoniae* individuals consistently corresponded to a monophyletic mtDNA clade (Roy, 2006; Roy *et al.*, 2007b). A small section of the anal-fin tissue was removed and preserved in 95% ethanol, and all fish were quickly released to their site of capture. Confirmation of species identification was made using mtDNA restriction fragment length polymorphism (RFLP)

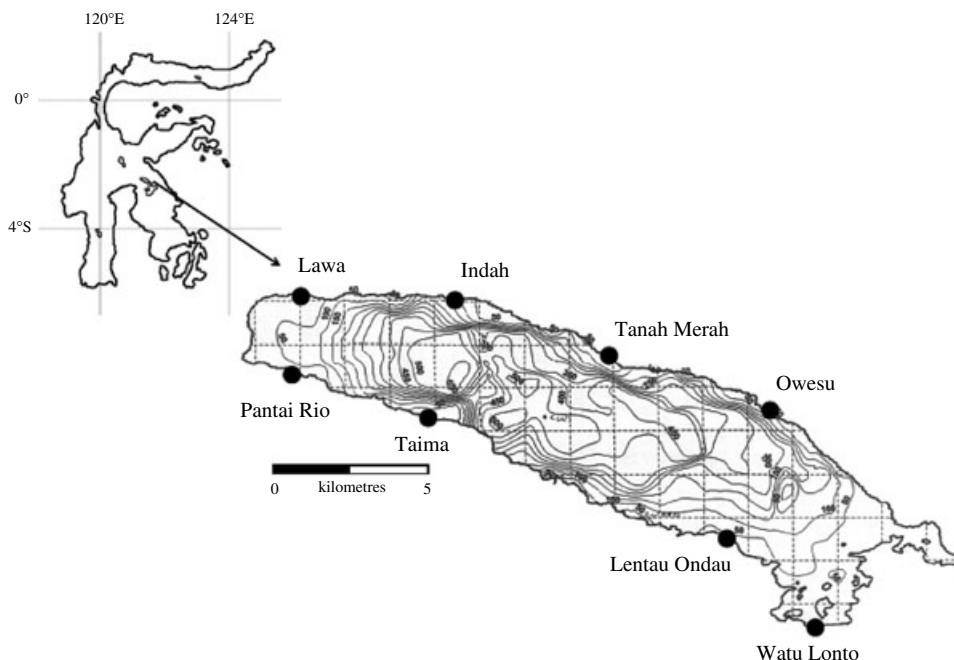


FIG. 1. Study area and sampling sites for *Telmatherina antoniae* in Lake Matano, Sulawesi, Indonesia.

analysis following Roy (2006). Samples of males and females were pooled as preliminary genetic analyses showed no genetic differentiation between males and females in the dataset. On the basis of the colour of their body (males) and second dorsal and anal fins (females), fish were sorted into three groups: blue, yellow or mixed (Fig. 2). For males, if both body and fin colour matched, fish were identified as blue or yellow; if the body was blue and fins were yellow, the male was classified as 'mixed'. No male *T. antoniae* with yellow body colouration and blue fins were observed, nor are they reported in the literature. As the female body colouration is bluish grey, females were sorted into either yellow or blue morphs on the basis of the colouration of their second dorsal and anal fins, and no females were collected that were classified as mixed.

DNA was recovered from tissue samples following the plate-based extraction method (Elphinstone *et al.*, 2003) and resuspended in 50 µl of Tris–EDTA buffer (10 mM Tris, 1.0 mM EDTA, pH 8.0). The samples were genotyped at eight microsatellite loci following Walter *et al.* (2007). A previous analysis of genetic structure in these fish showed four to six highly divergent genetic groups (sympatric populations) that were independent of the spatial distribution of the fish (Walter *et al.*, 2009). Genetic divergence among the colour morphs within the sampling sites was tested for, within the previously identified populations of Walter *et al.* (2009) and in a pooled sample of all of genotyped fish.

The mean number of alleles, number of private alleles, observed and expected heterozygosities for each colour morph from each site was calculated using GENALEX 6.1 (Peakall & Smouse, 2006). Pair-wise F_{ST} (Weir & Cockerham, 1984) estimates among colour morphs were performed both site-by-site and using individuals pooled from all sampling sites in MSA 4.0 (Dieringer & Schlötterer, 2003). Fisher's exact tests were performed to test for significant differences in allele frequency distributions among blue, yellow and mixed colour morphs using pooled individuals from all sites in GENEPOP 4.0 (Raymond & Rousset, 1995). Given the sympatric population genetic structure for this species (Herder *et al.*, 2008; Walter *et al.*, 2009), hierarchical genetic differentiation was tested using AMOVA (ARLEQUIN 3.01; Excoffier *et al.*, 2005) using three approaches: (1) colours nested within sampling sites, (2) colours nested within four sympatric populations (STRUCTURE groups, Walter

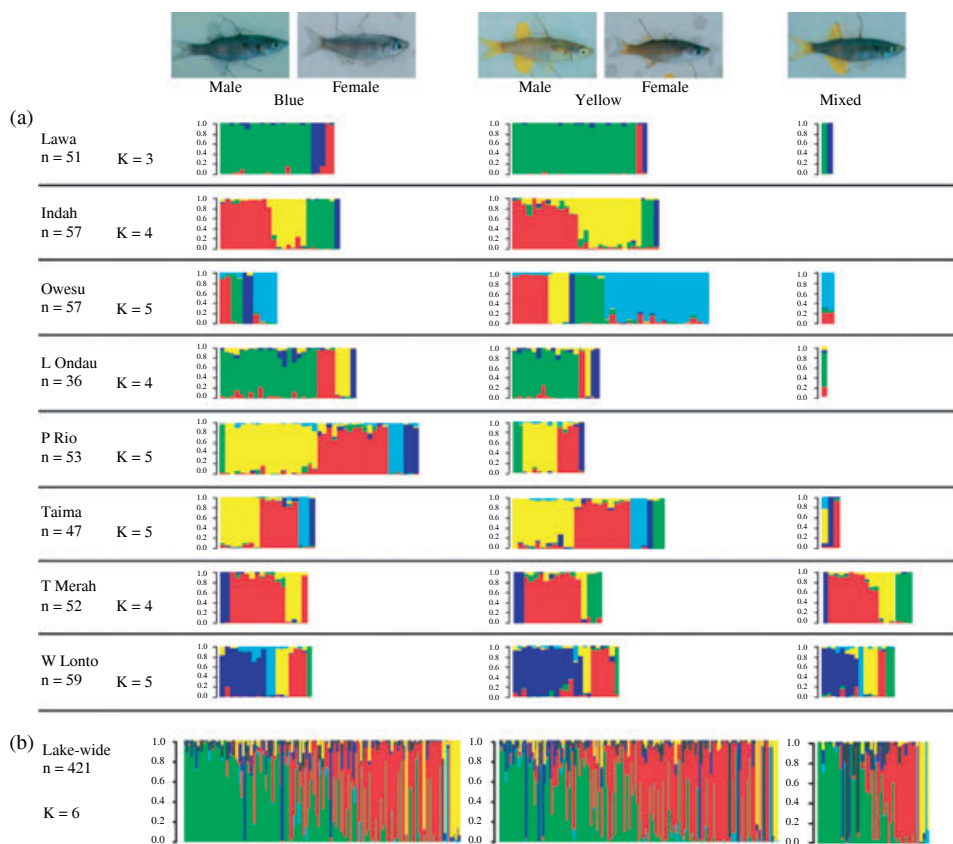


FIG. 2. Multilocus genotype clustering of *Telmatherina antoniae* colour morphs using BAPS 4.14. Each bar corresponds to an individual fish in the blue, yellow or mixed groups; the colour of the bars represents the proportion of an individual fish's genetic assignment among the genetic clusters. (a) Clustering performed by sampling site ($K = 3$ – 5). Note: the colour of the bars is not comparable across sampling sites as each site underwent a separate clustering analysis, differences in the number of individuals with those in Table I are due to removal of outliers identified by BAPS. (b) Clustering performed with pooling of all samples for a lake-wide estimate ($K = 6$).

et al., 2009) and (3) colour nested within six sympatric populations (BAPS groups, Walter *et al.*, 2009).

An independent heuristic approach for examining genetic structuring relying on Bayesian assignment was used to partition individuals into genetic clusters on the basis of an individual's multilocus genotype using BAPS 4.14 (Corander *et al.*, 2003). This analysis detects underlying genetic structure and determines whether this structure corresponds to the colour groups. Individual runs were performed for each sampling site, then data were pooled among all sites and the analysis re-run to examine lake-wide structuring. First, BAPS clustering of individuals at each of the sampling sites was performed independently in three replicates, followed by an admixture analysis. The admixture analysis was performed ignoring clusters with fewer than five individuals ($n = 421$, following removal of seven individuals), simulating 200 individuals in 50 iterations, to determine the number of population clusters.

Courtship and intermale aggressive behaviour was assessed by surveys of 33 courting male–female pairs, 17 of which the paired male was blue, and 16 of which the paired male was yellow, at Watu Lonto (Fig. 1) along two 10 m transects. Watu Lonto was selected

for behavioural surveys on the basis of water clarity due to minimal turbidity, and logistic constraints, which prevented behavioural surveys being performed elsewhere in the lake. Courting male–female pairs were observed for 3 min each, and when male–male aggressive displays were observed, the colour of the paired male, the colour of the extra-pair male and the retention or loss of the courted female was recorded. Contingency tables were used to test for assortative pairing along colour lines, that is, did blue male–blue female pairs occur more often than blue male–yellow female pairs along transects? The test was performed also for yellow male pairs. Contingency tables were also used to test for biases in aggressive displays of the paired male with respect to the colour of extra-pair males. Because of small sample sizes, the aggressive display analysis was performed by pooling the pairs into ‘like’ (blue–blue and yellow–yellow) and ‘unlike’ (blue–yellow and yellow–blue) categories with respect to the colour of the paired male.

RESULTS

Across pooled sites, 15 private alleles were found in the blue morphs, 11 in the yellow and three among the mixed (Table I). Site-by-site F_{ST} estimates showed only one significant comparison before Bonferroni correction; Owesu (Table II). Pooled lake-wide F_{ST} estimates showed significant genetic differentiation between yellow and mixed morphs only ($F_{ST} = 0.014$, $P < 0.05$), and no significant genetic differentiation between blue and yellow morphs ($F_{ST} = 0.003$, $P > 0.05$), or blue and mixed ($F_{ST} = 0.006$, $P > 0.05$). Despite no significant differentiation among colour morphs at sites with the highest numbers of mixed individuals, differences

TABLE II. F_{ST} estimates among colour morphs of *Telmatherina antoniae* nested within sites

Site	Color morph	
Lawa ($n = 51$)	Blue	Mix
Mix	0.014	
Yellow	–0.001	0.028
Indah ($n = 57$)	Blue	
Yellow	0.002	
Owesu ($n = 57$)	Blue	Mix
Mix	0.028	
Yellow	0.012	–0.002
L Ondau ($n = 36$)	Blue	Mix
Mix	0.070	
Yellow	0.006	0.069
P Rio ($n = 53$)	Blue	
Yellow	0.001	
Taima ($n = 47$)	Blue	Mix
Mix	0.003	
Yellow	0.006	0.007
T Merah ($n = 52$)	Blue	Mix
Mix	0.017	
Yellow	0.012	0.014
W Lonto ($n = 59$)	Blue	Mix
Mix	0.010	
Yellow	0.006	0.016

All values are non-significant ($P < 0.05$), except for the *italicized* value before Bonferroni correction.

in relative number of colour morphs among sites have the potential to confound the estimates of F_{ST} particularly if mixed individuals are undersampled or consist of differing proportions of individuals of the sympatric populations. Exact tests showed one significant pair-wise comparison: yellow *v.* mixed ($P < 0.01$). The hierarchical AMOVA with colours nested within sampling sites showed low but significant spatial differentiation, but no significant genetic variation among colour morphs [Table III(a)]. Similarly, AMOVA also failed to show significant genetic differentiation among colours nested within the previously identified genetic clusters or populations [Table III(b)]. Results of the genotype clustering using BAPS indicated the presence of three to five genetic clusters per site, and six clusters among the pooled samples (Fig. 2); however, none of these groups exclusively corresponded to a particular colour morph.

Mate-paired *T. antoniae* included individuals of both primary colour morphs, but no mixed individuals. Surveys of 33 male–female pairs showed 17 pairs of which the males were blue and 16 pairs in which males were yellow (Table IV). Contingency table analyses showed that females were not randomly distributed with respect to the colour of the paired male, with 24 ‘like’ pairings compared with nine ‘unlike’ pairings, $\chi^2_{0.05,1} = 6.81$, $P < 0.01$. Among the male–female pairs, six of the 17 blue male pairs experienced two or more intruders during observation, whereas three of 16 yellow male pairs experienced more than one intruder. The number of intruders a male experienced did not seem to have an effect on subsequent aggressive displays, and these data were included with all samples. No significant colour biases were observed for aggressive displays toward extra-pair males, $\chi^2_{0.05,1} = 1.88$, $P > 0.05$.

DISCUSSION

The lack of genetic differentiation along primary colour lines both within the sympatric population clusters and among the sampling sites indicates considerable gene flow among primary colour morphs. Furthermore, the small but significant genetic differentiation among yellow and mixed morphs is possibly indicative of recent divergence. Examples of species exhibiting conspicuous colour polymorphisms with little or no underlying genetic differentiation at neutral loci occur as a result of very recent divergence (Köblmüller *et al.*, 2008), reproductive status or similar condition-dependent polymorphisms (Grosenick *et al.*, 2007). The basis for blue and yellow polymorphisms in *T. antoniae* are unknown but likely to have both a genetic and an environmental component as both blue and iridescence are generally produced by interference effects of structural patterning, compared with yellow colours that are produced by pigments, such as carotenoids derived from the diet (Moyle & Cech, 1988).

The remarkably clear waters of Lake Matano (secchi disc depth 20 m, Haffner *et al.*, 2001) probably facilitated the evolution of colour morphs *via* both natural and sexual selection. Individuals of a particular colour may experience negative selection in environments where they appear most conspicuous to predators (Ryan,

TABLE III. Hierarchical AMOVA with (a) colour morphs of *Telmatherina antoniae* nested within spatial sites and (b) within sympatric populations identified in Walter *et al.* (2009)

(a) Site-based analysis					
Source of variation	d.f.	S.S.	Variance components	Per cent of variance	Fixation index
Among sites	7	89.92	0.0751	2.14	0.030
Among colours within sites	14	61.67	0.0317	0.90	0.009
Within individuals	818	2782.07	3.4011	96.96	0.021
Total	839	2933.67	3.5078		
(b) Sympatric population-based analysis					
Source of variation	d.f.	S.S.	Variance components	Per cent of variance	Fixation index
Among four populations (STRUCTURE)	3	122.62	0.2249	7.46	0.075
Among colours within populations	8	23.03	0.0016	0.05	0.001
Within individuals	728	2030.13	2.7886	92.49	0.000
Total	839	2933.67	3.5078		
Among six populations (BAPS)	5	83.37	0.1490	6.55	0.068
Among colours within populations	11	26.13	0.0057	0.25	0.003
Within individuals	817	1731.17	2.1189	93.19	0.066
Total	833	1840.67	2.2737		

2001; Coyne & Orr, 2004) but alternatively encounter positive selection by potential mates (Andersson, 1994; Chunco *et al.*, 2007). Gray *et al.* (2008) have suggested that differential reproductive success across visual environments is responsible for the occurrence of blue and yellow polymorphisms in the closely related *Telmatherina sarasinorum* Kottelat, also from Lake Matano. According to their hypothesis, environment-contingent sexual selection occurs where males of a particular colour are favoured in habitats in which that colour appears most conspicuous (Gray *et al.*, 2008). The elaborate courtship displays of males appear to further enhance male conspicuousness, which may be highest when sexually receptive females are abundant and lowest when predators are abundant, initiating a trade-off between these countervailing forms of selection (Bertram *et al.*, 2004).

Although assortative courtship along primary colour lines was most common, male–female pairs with dissimilar colouring were also noted, supporting previous observations (Kottelat, 1991; Gray & McKinnon, 2006). Studies of sexual selection tend to place a greater emphasis on female mate choice rather than choice by males or intermale competition, when in fact the latter two modes may represent substantial selective forces. Intermale competition frequently occurs in small aquaria and spatially restricted artificial populations (Farr, 1977; Martin & Hengstebeck, 1981). For *T. antoniae*, the lack of bias in male aggression against dissimilar intruding males suggests that males view males of different colour as an equal threat as those of similar colour. Such a behavioural response to males of any colour may reflect a response to sneaking mating tactics. There is opportunity for sneaking among all telmatherinid males (Gray & McKinnon, 2006), which may override assortative mating and contribute to among-colour gene flow. Gene flow would erode neutral genetic differentiation, at least between the blue and the yellow morphs. The mechanism behind the significant genetic differentiation between yellow and mixed morphs is unknown but may reflect very recent divergence, and suggests a possibility that the mixed coloured males mate less successfully with yellow females. It has been speculated that mixed morphs in other telmatherinids may be less ‘fit’ intermediate offspring of the primary colour morphs (Gray *et al.*, 2008), which may also account for their overall lower numbers and patchy distribution. Additionally, the differential abundance, distribution and exclusive male composition of mixed category may explain the significant F_{ST} estimates among yellow and mixed individuals at the lake-wide scale, despite a lack of sex-biased differentiation (Walter, 2009).

This study shows that despite assortative courtship on the basis of similar colour in Lake Matano *T. antoniae*, gene flow is high between blue and yellow colour morphs. These findings are consistent with Herder *et al.* (2008), who found a similar lack of genetic differentiation among blue and yellow male ‘small’ morphs of *T. antoniae* using AFLP markers. Nonetheless, this result leads to the question of how the colour polymorphisms are maintained in this endemic species. Environment-contingent sexual selection (known to occur in a closely related sympatric species, Gray *et al.*, 2008) may be responsible for the maintenance of these polymorphisms in the face of substantial gene flow. Alternatively, the colour variation may reflect condition-dependent polymorphisms associated with territoriality, age or other ontogenetic factors. Finally, gene flow among the colour morphs may reflect recent disruption of a reproductive

TABLE IV. Summary of behavioural data for male–female pairs of *Telmatherina antoniae* colour morphs along 10 m swimming transects at Watu Lonto in Lake Matano

Colour pairings	<i>n</i>	Extra-pair male colour	
		Blue	Yellow
Blue male			
Blue female	13	7	8
Yellow female	4	1	2
Total	17		
Yellow male			
Yellow female	13	3	0
Blue female	3	3	1
Total	16		

Extra-pair male counts are for aggressive response by the paired male. *n*, number of individuals.

barrier possibly followed by hybridization. Clearly, *T. antoniae* of Lake Matano represent a fascinating system to study the mechanisms of phenotypic polymorphism maintenance in the face of gene flow.

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