Body shape vs. colour associated initial divergence in the *Telmatherina* radiation in Lake Matano, Sulawesi, Indonesia

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Abstract

Highly polymorphic colouration patterns are often associated with sexual selection in fish and can be the initial cause of divergence among closely related taxa. Here we use genetic, body colour and geometric morphometric data collected on 118 fish from Lake Matano, Sulawesi, Indonesia to test if colouration is the initial cause of divergence in the radiating *Telmatherina* genus. Results reveal that all *Telmatherina* previously described in this system can be categorized into three mitochondrial lineages and that colouration is only weakly associated with early divergence. Clade-specific body shapes, however, likely adapted to microenvironments are key to the initial divergence in this system. Data also show that although colourations were not likely instrumental in seeding divergence in these fish, they appear to have developed in parallel within each clade. Our results are consistent with an emerging pattern repeated in many vertebrate radiations, whereby divergence by colouration or other display traits is preceded by specialization to environmental adaptive peaks.

Introduction

Sexual selection is a powerful mechanism driving divergence, and its ability to initiate speciation has received considerable attention (Panhuis *et al.*, 2001; Arnegard & Kondrashov, 2004; Gourbiere, 2004). Investigations of sexual selection based on assortative mating among brightly coloured fishes have been conducted both *in situ* (Seehausen *et al.*, 1997; Rico *et al.*, 2003) and under laboratory settings (Seehausen & van Alphen, 1998; Knight & Turner, 2004). Such studies often attribute divergence among taxa to differentiating colouration among closely related species. This process is thought to be due to female preference for male colouration and is especially prevalent in polygynous systems (Seehausen & van Alphen, 1999; Allender *et al.*, 2003; Knight & Turner, 2004). Sexual selection and assortative mating based on colouration is an enticing hypothesis explaining sympatric speciation as resulting selection is thought to be strong and disruptive even among closely related groups (Lande, 1981; Panhuis *et al.*, 2001; Salzburger & Meyer, 2004).

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Sexual selection among brightly coloured fishes is especially prevalent in systems characterized by high water clarity allowing mate recognition, and large amounts of predator-free space applying little cost to the development of conspicuousness (Lande, 1981; Seehausen *et al.*, 1997; Panhuis *et al.*, 2001). Under such conditions, sexual selection can generate many polychromatic sibling species relatively quickly from few common ancestral lineages in a Fisherian runaway mode (Lande, 1981; Panhuis *et al.*, 2001; Rundle & Nosil, 2005).

Recent work, however, argues that sexual selection in general, and more specifically among brightly coloured closely related species, is probably insufficient in itself to cause sympatric divergence (Arnegard & Kondrashov, 2004; Gourbiere, 2004). Rather, colouration segregation likely arises subsequent to differentiation brought on initially by ecological factors such as resource partitioning or niche specialization (Arnegard & Kondrashov, 2004; Salzburger & Meyer, 2004; Rundle & Nosil, 2005) and reinforces divergence among even closely related populations (Danley & Kocher, 2001; Streelman & Danley, 2003; Kocher, 2004).

Lake Matano, located in the south central portion of Sulawesi Island, Indonesia (Fig. 1), is an ancient tropical freshwater lake estimated to be 2–4 million years old (Brooks, 1950; Hamilton, 1979; Haffner *et al.*, 2001). It

Fig. 1 Location of Lake Matano on the continental island of Sulawesi in the Indonesian Archipelago. Beach locations where fish were collected are indicated with dark circles and alphanumeric values. Beaches with stars indicate sites used in previous studies (Kottelat, 1991) (N = North, S = South).
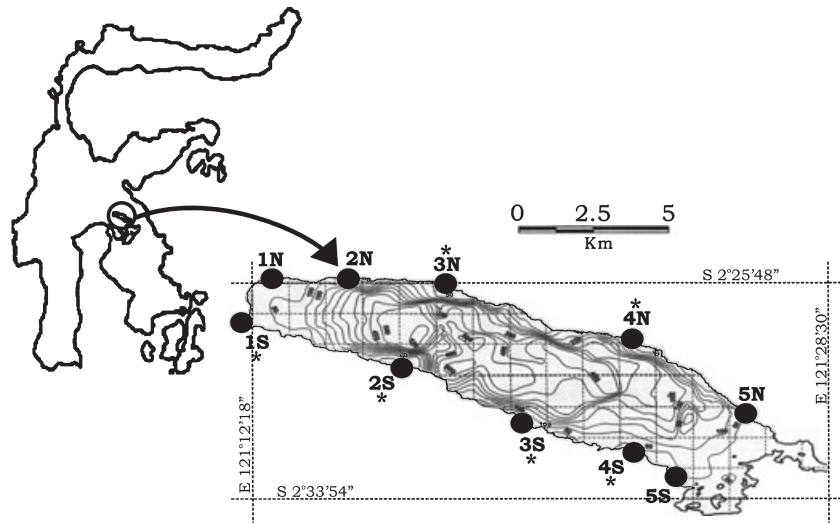
occurs along an active fault, forms the hydrological head of the Malili lakes system, and is isolated from downstream lakes by a 72-m elevation change including cascading rapids (Brooks, 1950; Hamilton, 1979; Kottelat, 1990a). Lake Matano is relatively small ($\sim$164 km^2), has two very deep basins (610 & 590 m) separated by a deep saddle (509 m), and the entire lake is surrounded by limited littoral areas (Fig. 1). The fish community in this lake is largely restricted to these shallower littoral areas with few fish reported beyond the 30–40 m contours (D. Roy, unpublished data). This graben lake is also highly oligotrophic, evidenced by its high Secchi depth (c. 23–25 m), low phytoplankton and zooplankton abundances, and by its rare top predators (Haffner *et al.*, 2001; Roy *et al.*, 2006a,b; Sabo, 2006). Four fish genera, all of which likely invaded the lake at similar times (Sparks & Smith, 2004), have endemic species in Lake Matano (Brooks, 1950; Kottelat, 1990a, 1991; Larson & Kottelat, 1992; Aarn *et al.*, 1998; Roy *et al.*, 2004). Of these, *Telmatherina* is the most genetically divergent, is characterized by a significantly greater number of colouration types and has greater variation in feeding traits than all other genera present in this system (Roy *et al.*, 2004). Roy *et al.* (2004) used criteria established by Schluter (2000) to show evidence supporting the adaptive radiation of *Telmatherina* in this system due to either sexual selection by assortative mating among the varied colourtypes, by trophic specialization in this resource limited environment, or a combination of both.

Lake Matano's clear water and brightly coloured *Telmatherina* make it an ideal system to test for putative sexual selection based on colourtype. In this study, we determine phylogenetic relationships among the various *Telmatherina* types using mitochondrial DNA sequences, and determine if colouration, or body shape, better predicts the genetic relationship by comparing both features with the derived phylogenies. The expectations are that, if genetic divergence within *Telmatherina* was

initiated by assortative mating among colourtypes, then colouration ought to match genetic structure. If body shape, however, was a more important factor driving initial divergence, then body shape should be a better predictor of genetic structure. Determining the relative importance of different selective pressures seeding divergence in this system adds to our understanding of factors capable of initiating adaptive radiations and the creation of biodiversity in natural systems.

Methods

Genetic analysis

Acoustic surveys determined that most of the fish in Lake Matano were observed within the first 40 m of the water column and relegated to the surrounding littoral areas (D. Roy, unpublished data). Consequently, approximately 50–100 *Telmatherina* were collected by seining from 10 beaches around the periphery of the lake (Fig. 1). Fish were initially separated into five morphotypes based on previous species descriptions (Kottelat, 1990b, 1991; Kottelat *et al.*, 1993) and four new morphotypes based on colouration and other phenotypic characters described by Kottelat (1990b, 1991; Kottelat *et al.*, 1993). Because of highly overlapping characters among described types making species identification ambiguous (see Kottelat, 1991; Kottelat *et al.*, 1993), *Telmatherina* were ultimately identified simply by a combination of both body shape and colouration (Fig. 2). No system-wide spatial bias was observed in the distribution of different body shape or colouration types collected from the different beaches (Roy *et al.*, 2006a).

In the field, collected specimens were anatomically sexed and only male fish were used in subsequent analyses. Collected males were anaesthetized using clove oil and pinned to a gridded cork board to carefully extend all fins in a standardized manner and photographed using
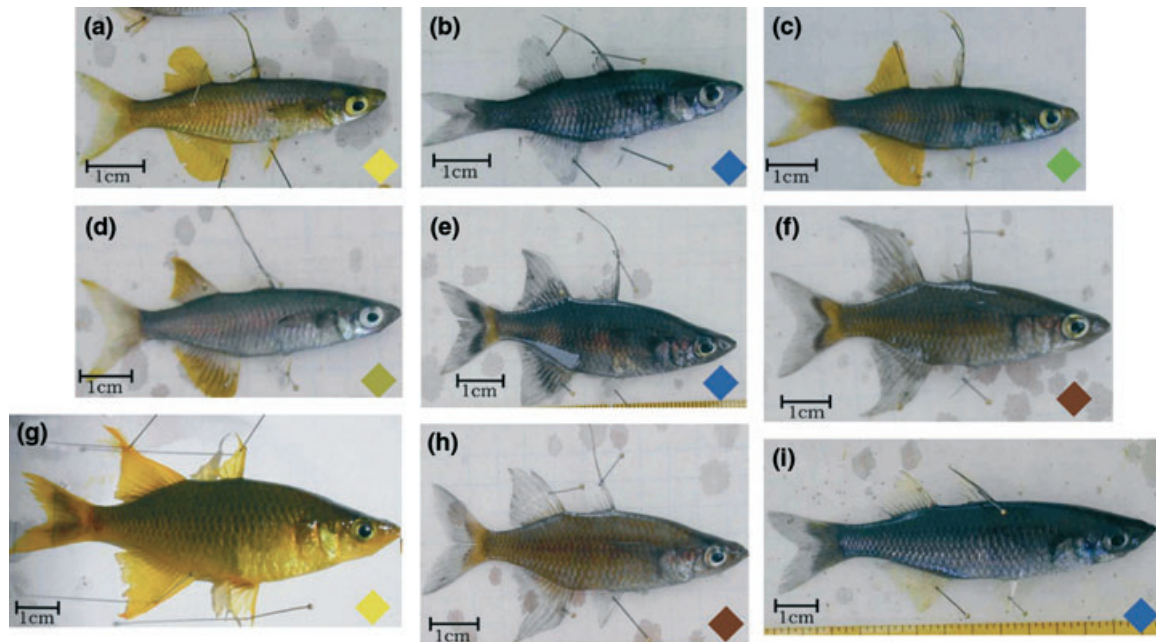
Fig. 2 Nine different *Telmatherina* morphotypes identified from Lake Matano using colouration and shape. There are five distinct colouration types; yellow (a & g), blue (b, e & i), yellow blue (c) yellow green (d) and brown (f & h). Shapes were determined using geometric morphometrics (see text). Colourations codes for DFA analysis include 1 = blue, 2 = yellow, 3 = brown, 4 = yellow blue & 5 = yellow green.

a high resolution digital camera. Fin clips were taken from each individual and stored in 95% ethanol for subsequent DNA extraction. Specimens were allowed to recover in oxygenated water before being released back to the lake. Fin clips were also collected from five *Marosatherina lagdesi*, a closely related, commonly available atheriniform fish from Sulawesi used as the outgroup (Aarn *et al.*, 1998).

DNA was extracted from a random sub-sample of 118 fish while making certain to include individuals from each of the nine groups identified from Fig. 2. Extractions were performed using the Promega WIZARD® DNA extraction kit following the standard 'isolation from animal tissue' protocol (Promega, Madison, WI, USA). Amplification of 490 bp of the 16S rDNA and 600 bp of the cytochrome *b* gene was performed using published primers [16Sar-L (5'-CGCCTGTTTATCAAAAACAT-3') & 16Sbr (5'-CCGGTCTGAACTCAGATCACG-3'); Palumbi, 1996], and [GLUDG-5(5'-TGACTTGAARAACCACCG-TTG-3'); Palumbi, 1996 & CBtelm–R (GTGGAGGAG-GGGTACGACTA-3'); Roy *et al.*, 2006a]. Individual PCR reactions were performed as described in Roy *et al.* (2006a), and products were sequenced using the DTCS Quick Start cycle sequencing kit (Beckman Coulter, Fullerton, CA, USA) and CEQ8000 automated sequencer following manufacturer's instructions.

Derived sequences from both genes were aligned separately using the clustal W algorithm with a gap open penalty of 10, a gap extension of 5 and a DNA transition weight of 0.5 (Omiga 1.2; Oxford Molecular Ltd, Oxford, UK). Resulting alignments were verified by eye and tested for transition saturation using MEGA version 3.1 (Kumar *et al.*, 2001). Insertions and deletions (indels) were treated equally as complete deletions (Kocher & Carleton, 1997), and because both 16S and cytochrome *b* sequences exhibited no saturation and similar divergence rates, both fragments were combined into a single 1090-bp fragment for each individual (see Roy *et al.*, 2006a). Phylogenetic reconstruction among derived sequences was carried out by Neighbour-Joining (N-J) using the HKY85 model for base pair substitution characterized by equal base frequencies, a proportion of invariant sites (0.6005) and a gamma shape parameter (0.7095) implemented in PAUP version 4.0b10 (Swofford, 2001). This substitution model was specified by the Akaike Information Criterion resulting from a maximum likelihood survey of 56 different base substitution models implemented by MODELTEST version 3.7 (Posada & Crandall, 1998). As an additional test of phylogenetic structure and topology, Maximum Parsimony (MP) heuristic searches were performed with a Close Neighbour Interchange of 1 and an initial tree chosen by Random Addition Trees of 10 replicates. A 50% majority consensus tree was constructed from precalculated distances by 10 000 bootstrap iterations assessing nodal confidence, whereas the MP character based estimate was reiterated 1000 times.

Geometric morphometrics

Digital pictures from all genetically analysed specimens were used for geometric morphometrics (hereafter GM),

a landmark based analysis of body shape variation among specimen groups (Caldecutt & Adams, 1998; Rüber & Adams, 2001; Zelditch et al., 2004). Eighteen biologically homologous landmarks, selected based on standard landmark descriptions (Bookstein, 1991; Zelditch et al., 2004), were identified on digitized Telmatherina images using the TPSDIG program (Rohlf, 2004a). Landmarks included fin shapes as these features are diagnostic in this genus (Kottelat, 1990b, 1991; Gray & McKinnon, 2006). Fin positions were standardized by holding the fins in full extension using entomological pins. The first dorsal fin, however, was standardized using methods described by Adams (1999) because of its length and centre of articulation at its base.

Specimen landmark configurations were re-scaled to unit centroid size, translated to a common position and superimposed to minimize the least squared Procrustes distance among landmarks for all specimens (Rohlf, 1999; Zelditch et al., 2004). This generalized procrustes analysis produced a consensus configuration of landmarks based on all superimposed specimens (Rohlf, 1999; Zelditch et al., 2004). A thin-plate spline then generated interpolation functions among landmarks describing each by geometrically orthogonal components termed 'principal warps'. Thin-plate spline was also used to superimpose each specimen's landmark configuration onto the consensus configuration and calculate the amount of deviation needed along each principal warp to fit each landmark in the specimen to that in the consensus configuration. This procedure generated a set of orthogonal shape variables (partial warps) for each specimen, subsequently used in multivariate analyses (Caldecutt & Adams, 1998; Rüber & Adams, 2001; Zelditch et al., 2004). A discriminant function analysis (DFA) was performed on partial warps from all fish to determine if there were significant differences in body shapes among the genetically determined clusters. Post hoc pairwise comparisons among groups were performed using derived F-values from the generalized Mahalanobis distances to determine which groups were characterized by significantly different shapes. α-Values were adjusted to 0.0166 using Bonferroni correction keeping experiment-wise error at 0.05 (Kassam et al., 2004). Canonical variates analysis (CVA) was performed to discriminate among groups along various ordination axes minimizing variation within, but maximizing differences among, groups (Rüber & Adams, 2001; Klingenberg et al., 2003; Zelditch et al., 2004). Relative warp analysis (similar to a principal components analysis) was performed on partial warps to generate deformation grids outlining the biologically important relative shape changes for each genetically determined group. Deformation grids were constructed using mean shapes for each genetic group defined along the most important relative warps (principal component axes) and exaggerated three times to emphasize differences (Caldecutt & Adams, 1998; Rüber & Adams, 2001; Klingenberg et al., 2003; Zelditch et al.,

2004). Consensus configuration, GPA, the thin-plate spline generation of partial warps, relative warps and deformation grids were accomplished using the TPSRELW program (Rohlf, 2004b).

Colouration tests

Colouration for each specimen was scored from the digital pictures and three dominant colours (blue, yellow and brown), plus two intermediate colourtypes (yellow-blue and yellow-green) were assigned (Fig. 2). Significant relationships between colouration and genetic clade membership was assessed using an Exact test for population differentiation, with significance determined by an unbiased Markov chain approach considering colouration as an allele inherited in a haplotypic manner and clades as populations. The Exact tests were permutated 10 000 times using 10 000 de-memorization steps per batch of 100 performed in TFPGA program version 1.3 (Miller, 1997). Colouration among fish was also assigned discrete values (1–5; see Fig. 2) and added to the list of shape variable (i.e. partial warps) for DFA. DFA and CVA were repeated with the colour code included to determine whether colouration provided additional discriminating information among the Telmatherina genetic clades. All DFA and CVA were performed using STATISTICA (StatSoft, Inc. 2001, version 6, Tulsa, OK, U.S.A.).

To determine whether colouration or shape best predicted genetic structure, individual clade assignments based on colour and shape were compared. Because the distance (N-J) and character based (MP) phylogenies revealed identical topologies (see Results below), we used the character based topology to generate a minimum spanning haplotype network (MSN) using the TCS program (Clement et al., 2000). Possible colouration and shape-specific segregation among genetic clades was assessed by mapping individual colouration(s) and shape(s) onto the MSN. Colouration assignment to individuals was determined from digital images (described above) whereas shape was assigned by plotting 95% confidence ellipses around the genetic clades outlined in the CVA (see Fig. 4). Individuals occurring inside their genetic clade's 95% confidence ellipse were assigned that clade's respective shape whereas those falling outside the ellipse were assigned the shape of the clade in closest proximity.

Results

Genetic analysis

Sequences used in this study are available on Genbank (accession numbers DQ023624–DQ023667, DQ002506–DQ002547, DQ054795–DQ054796 and AY545822–AY545875). Aligned sequences were characterized by 204 variable sites, 182 of which were parsimoniously informative. Overall sequence differences were

characterized by three indels relative to the outgroup, and a transition to transversion ratio of 3.36. Generated phylogenies separated the 118 *Telmatherina* specimens into three distinct and well-supported clades (Fig. 3). All clades, with the exception of clade II, were supported by
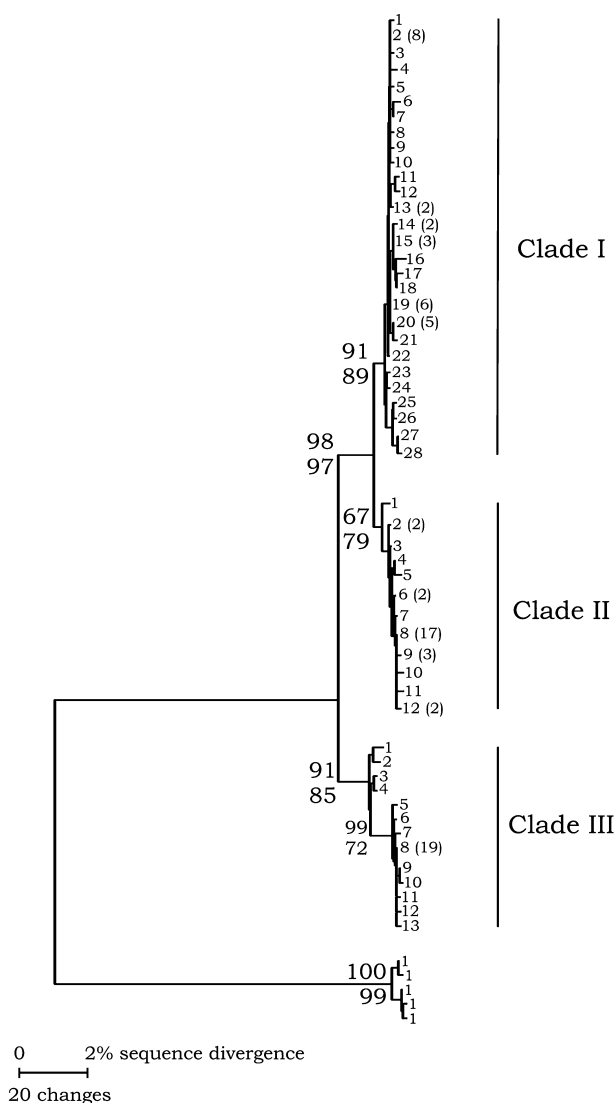

Fig. 3 Phylogenetic relationships derived from 1090 bp of the mitochondrial genome from 118 *Telmatherina* from Lake Matano, Sulawesi, Indonesia using a 50% majority-rule consensus tree determined from Neighbour-Joining algorithm with genetic distances calculated using HKY + I + G (I = 0.6055; G = 0.7095) model of base substitution implemented in PAUP version 4.0b10. Maximum parsimony (MP) recovered similar topology using Closest Neighbour Interchange of 1 with the addition of 10 replicates. Bootstrap nodal support for both distance (above) and character based (below) topologies is indicated at root nodes (10 000 for NJ and 1000 for MP). Numbers at terminal nodes indicate haplotype number and those in brackets indicate number of individuals sharing the same haplotype.

> 85% of bootstrap permutations. The observed trifurcation of the *Telmatherina* specimens into three distinct clades did not correspond to the seven different species previously reported (Kottelat, 1991), but did match previously reported phylogenies (Roy *et al.*, 2004, 2006a). Genetic variation within clades was highest amongst clade I followed by clade III and then clade II. All clades were approximately equidistant from the outgroup, but showed variable inter-clade divergence (Fig. 3). Clades I and II were more closely related but were nevertheless clearly divergent at the species level (Avise & Walker, 1999; Hebert *et al.*, 2003), and clades I and II exhibited large and similar distances from clade III (Fig. 3). The distances exhibited among clades are consistent with the presence of three distinct *Telmatherina* lineages in Lake Matano with possible internal substructures.

Geometric morphometrics

No significant differences were found in overall morphometric-based centroid sizes among *Telmatherina* clades (Kruskal–Wallis$_{116}$ = 3.84, $P > 0.05$) indicating that size had no significant influence on shape differences among them (Cavalcanti, 2004). Significant body shape differences, however, were observed among the three clades (Wilk's $\Lambda = 0.11219$, approx. $F_{64,164} = 5.088$, $P \ll 0.00001$). Pairwise, *post hoc* comparison revealed that all clades significantly differed in body shape from each other (clades I vs. II $F_{32,82} = 8.48$, $P \ll 0.00001$; clades I vs. III $F_{32,82} = 7.29$, $P \ll 0.00001$; clades II vs. III $F_{32,82} = 1.93$, $P = 0.009$). Although CVA clearly segregated all three clades from one another, some overlap was evident from the 95% confidence ellipses (Fig. 4a). Clades II and III were much more similar in body shape to each other than either was to clade I (Fig. 4a). Adding colouration as an additional variable in the DFA and CVA gave almost identical results (Wilk's $\Lambda = 0.11159$, approx. $F_{66,122} = 4.879$, $P \ll 0.00001$) and did not enhance the segregation among clades any further (Fig. 4b).

Relative warps 1 and 2 accounted for > 75% of the variation in shape differences among the three clades and deformation from the consensus configuration proved to be clade-specific (Fig. 5). Individuals in clade I were characteristically more fusiform in body shape, had rounded anal and second dorsal fins, an enlarged caudal peduncle and a terminal mouth. Based on these features we classified this fusiform body shape as 'Torpedo' (Fig. 5). The shape characteristics of members belonging to clade I seemed to correspond to previous species descriptions of *T. antoniae* (Kottelat, 1991; Kottelat *et al.*, 1993). Specimens in clades II and III were more compressiform in body shape with their bodies compressed along the posterior anterior axis (Fig. 5). Both clades – which we classified as 'Rotund' – were further distinguished from the 'Torpedo' type by longer and
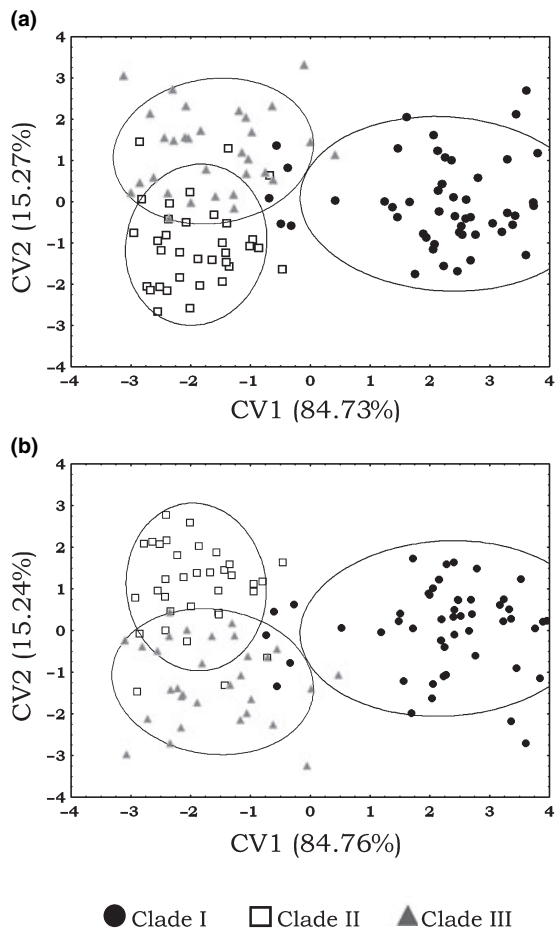
(a)

(b)

● Clade I   □ Clade II   ▲ Clade III

Fig. 4 Canonical variates ordination plots based on partial warps analysis of body shape segregating the three genetic clades identified from the phylogenetic relationships. Plots show individual *Telmatherina* scores along CV axes and 95% confidence ellipses surround each clade. In both cases CV1 and CV2 account for near 100% of the total variation among clades. Values in brackets indicate the proportion of total variation described along each axis. (a) Shape variables only. (b) Shape variables with an additional variable representing colour scored from digital images as in Fig. 2.

more pointed anal and second dorsal fins, a deeper body and a protruding mouth. Distinguishing features between clades II and III were more subtle but nevertheless apparent. Individuals in clade II were even more longitudinally compressed, had longer and more pointed fins reaching further back along the body, had a smaller and more tapered caudal peduncle and a smaller head than individuals belonging to clade III (Fig. 5). Individuals in clade III generally displayed morphological features intermediate between those in the other two clades (Fig. 5). Clade II and III specimens were subsequently classified as 'Rotund A' and 'Rotund B', respectively. Shape differences were more pronounced between both Rotund clades (clades II & III) and the Torpedo clade

(clade I), whereas the greatest genetic divergence separated Rotund B (clade III) from Rotund A and Torpedo shaped fish (clade I & II; Figs 3, 4a,b and 5).

Colouration and shape tests

Exact tests demonstrated a significant difference in colouration types assigned to the different clades ($P \ll 0.0001$). Pairwise comparisons between clades showed that colouration assignments to clade I were significantly different from those assigned to clades II and III ($P < 0.001$ and $P < 0.001$, respectively). No significant differences, however, were found between clades II and III ($P = 0.97$). Using colourtype as a grouping variable in an additional DFA for shape rather than genetic clade revealed a significant difference in the shapes assigned to colouration groups (Wilk's $\Lambda = 0.13341$, approx. $F_{136,313} = 1.848$, $P \ll 0.002$). *Post hoc* comparisons in this case did not show significant shape differences among yellow, blue or brown *Telmatherina* (blue vs. yellow $F_{32,80} = 0.80$, $P = 0.76$; blue vs. brown $F_{32,80} = 1.26$, $P = 0.20$; yellow vs. brown $F_{32,80} = 1.24$, $P = 0.22$), but did show significant differences between yellow blue vs. blue ($F_{32,80} = 2.46$, $P < 0.00001$), yellow blue vs. brown ($F_{32,80} = 2.52$, $P < 0.00001$), yellow green vs. blue ($F_{32,80} = 2.83$, $P < 0.00001$) and yellow green vs. brown ($F_{32,80} = 2.28$, $P < 0.00001$). Yellow blue and yellow green *Telmatherina*, however, were not significantly different in shape from each other ($F_{32,80} = 1.42$, $P = 0.1163$). CVA segregated both yellow blue and yellow green coloured fish from all other colourtypes but a high degree of overlap was observed among blue, brown and yellow colourtypes (Fig. 6). The first two CV axes accounted for just over 75% of the shape variation among colourtypes, substantially less than what was accounted for by clade membership (compare Figs 4 and 6).

The MSN confirmed that most colouration types are spread about all clades with the exception of yellow blue and yellow green which are predominantly in clade I (Fig. 7a). In addition, brown is present but underrepresented in clade I whereas both blue and yellow have similar proportions in all three clades (Fig. 7a). Thus, although some colourations proved to be clade-specific, most were not and many colours were exhibited by the same haplotype (e.g. clade I haplotypes 2, 13, 19, 20; clade 2 haplotypes 2, 6 and 8; clade III haplotypes 8; Fig. 7a). Thus, predicting clade membership using colouration proved to be ineffective except in the case of yellow blue and yellow green *Telmatherina*. Conversely, predicting clade membership based on shape, although not perfect, was generally more successful (Fig. 7b). Most torpedo shaped fish were found in clade I (except for one) and all but one Rotund A were found in clade II. Similarly, all but five Rotund Bs were found in Clade III (Fig. 7b). Thus, shape is a better predictor of clade membership than is colour.
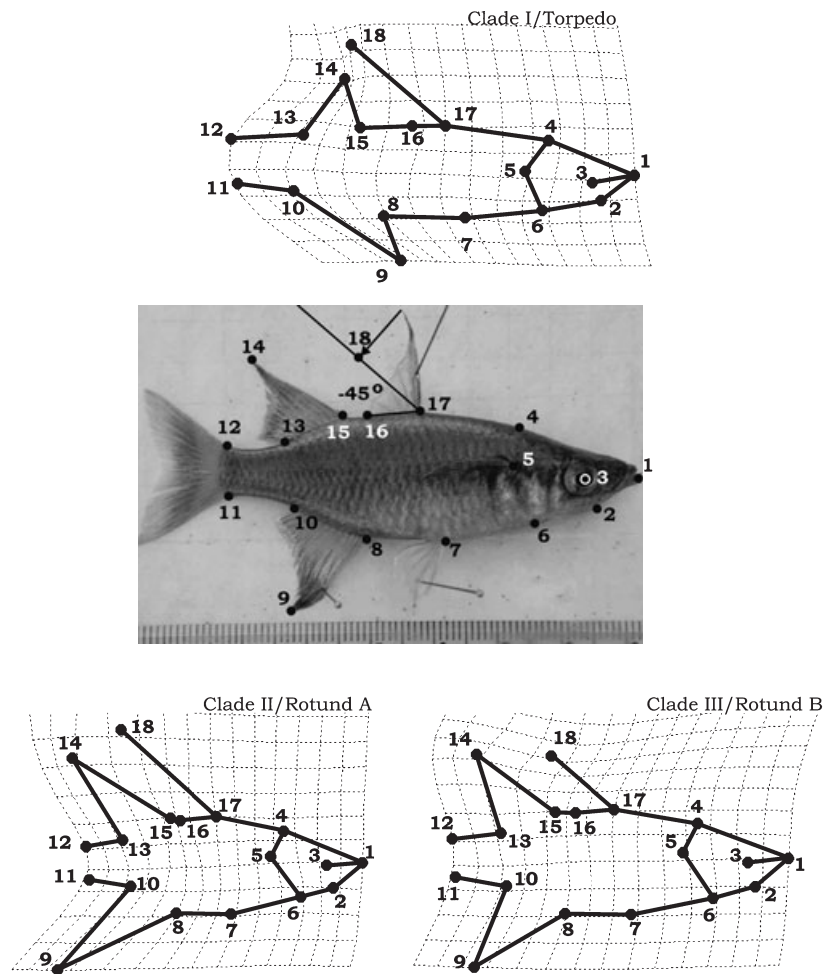
Fig. 5 Image of typical *Telmatherina* showing overlaid landmarks used in Generalized Procrustes superimposition analysis determining overall consensus configuration. Deformation grids, generated from thin-plate spline analysis of partial warps (shape variables), demonstrate how consensus configuration must be warped to fit average shapes determined for each clade and outline biologically meaningful shape differences among clades. Generated deformation grids show shape change using first two relative warps which account for > 75% of the shape differences among clades. Mean shapes for each clade was exaggerated three times to emphasize differences.

Discussion

Understanding the mechanisms regulating speciation in natural systems is critical for clarifying the evolutionary processes generating biodiversity. This is especially important in systems involved in species radiations where the number and rate of species formation is high (Schluter, 2000). A common paradigm among studies of radiation in brightly coloured polychromatic species is that sexual selection based on male colouration and female preferences is the cause of rapid divergence (Lande, 1981; Panhuis *et al.*, 2001; Knight & Turner, 2004). In this study we show that this is likely not the reason for initial divergence of *Telmatherina* in Lake Matano. Rather, the development of specific body shape adaptations to particular microenvironments is key to initial *Telmatherina* divergence within the confines of this highly oligotrophic system. Our results are consistent with the 'radiation in stages' pattern demonstrated in many other adaptive radiations, whereby species initially diverge by body shape specialization to environmental adaptive peaks, followed by communicatory cues such as

that initiated by sexual selection among colouration types (e.g. Danley & Kocher, 2001; Streelman & Danley, 2003; Kocher, 2004).

In this study, we identified three distinct *Telmatherina* mitochondrial lineages within Lake Matano matching those previously identified (Roy *et al.*, 2006a). In general, however, the three clades were not consistent with previous work describing seven different *Telmatherina* species in this system based on traditional morphometric techniques, except for clade I which may correspond to *T. antoniae* (Kottelat, 1990b, 1991; Kottelat *et al.*, 1993). It is possible that this study did not include all possible *Telmatherina* types from Matano as some may not be susceptible to our sampling techniques. Our sampling procedures, however, were consistent with those reported from previous work and included most of the same sites (see Fig. 1 & Kottelat, 1991). As such, it is unlikely that four additional types of *Telmatherina* went unnoticed. Moreover, although morphometric techniques can be a better representation of overall and more recent character evolution reflecting both nuclear and mitochondrial divergence among species, they may also
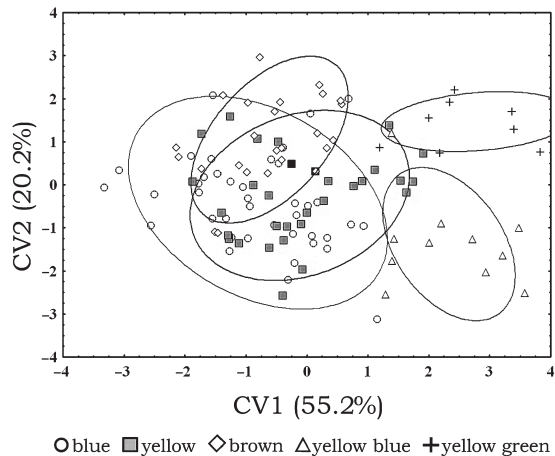
Fig. 6 Canonical variates ordination plot based on partial warps analysis of body shape segregating the *Telmatherina* based on colouration types defined in Fig. 2. Plot shows individual *Telmatherina* scores along CV axes and 95% confidence ellipses surround each colouration type. CV1 and CV2 accounting for just over 75% of the total variation among colouration types and values in brackets beside CV axes indicate the proportion of total variation described along each axis.

overestimate species numbers if some of these morphological traits are generated by phenotypic plasticity (e.g. Messmer *et al.*, 2005). Whether or not more recent (perhaps roughly < 20000 years before present) reproductive isolation within *Telmatherina* clades has occurred is impossible to determine using our mitochondrial phylogeny alone. Nevertheless, the constructed phylogeny presented here represents the evolutionary history of *Telmatherina*, and is useful in determining deep evolutionary relationships among the *Telmatherina* in Lake Matano, particularly with reference to their initial divergence (Kocher, 2004; Salzburger *et al.*, 2005).

All evidence suggests an intralacustrine origin of the three *Telmatherina* clades from a common colonizing ancestor (Brooks, 1950; Sparks & Smith, 2004). Lake Matano is isolated from the remaining Malili Lakes by an elevation change of more than 70 m (Kottelat, 1990a; Roy *et al.*, 2006b). Although such geological discontinuities can form rather quickly, evidence suggests that this topographical feature is ancient (Hamilton, 1979; Hope, 2001; Roy *et al.*, 2006b). The formation of Lake Matano predates that of other Malili systems based on its tectonic origins, its great depth and elevation, deviations along the active fault, and its level of endemism (Brooks, 1950; Hamilton, 1979; Whitten *et al.*, 2002). From this, Brooks (1950) postulated that species from Matano served as propagules for downstream colonizations. More recent studies support this hypothesis by demonstrating a paucity of downstream species present in Matano (Kottelat, 1990b, 1991; von Rintelen *et al.*, 2004; Roy *et al.*, 2006b). The apparent lack of spatial segregation among the three mitochondrial lineages further suggests

that this intralacustrine divergence occurred sympatrically. Fish used in this study were taken from various beaches where all clades were often caught together in a single seine (Roy *et al.*, 2006a). Smaller scale differences in habitat preference (e.g. rock formations or the extension of sandy reefs) were not quantified in this study, however, and may be informative in linking different *Telmatherina* types to specific microhabitats, especially as body shape differences among the three clades suggest ecological specializations (see also Gray & McKinnon, 2006).

The intralacustrine divergence of *Telmatherina* in Matano may have resulted from fluctuating lake levels as has been purported for many of the African Rift Valley cichlids (Salzburger & Meyer, 2004). This scenario seems questionable, however, considering Matano's depth and bathymetry. Matano water levels would need to drop several hundred metres (509 m) below current levels before isolated basins could be established. Moreover, although small scale spatial variation in climate conditions and temperature are possible, sedimentary cores taken in this region suggests that conditions on Sulawesi have been relatively constant over the last two glaciation (*c.* 1.6 million years) (Hope, 2001; Visser *et al.*, 2004). Therefore, the drastic lake level fluctuations necessary to postulate historic vicariance events in Matano because of changing climate patterns or temperatures are unlikely.

An increasing number of empirical studies document sympatric speciation attributable to ecological adaptation among various body shapes: Darwin's finches (Stern & Grant, 1996), Anolid lizards (Losos & Miles, 2003), Lake whitefish and Arctic charr (Skúlason *et al.*, 1993; Bernatchez, 2004; Østbye *et al.*, 2005) and Neotropical cichlids (Barluenga *et al.*, 2006) among others. In most reported cases, divergence because of microhabitat adaptation separates taxa into distinctively divergent forms (Streelman & Danley, 2003). In this study we demonstrate the occurrence of three clade-specific shapes with two extremes and an intermediate recovered from the GM analysis. Clade I's 'Torpedo' shape makes it well suited to an open-water pelagic existence where food items are chased. In particular, the slender body and broad caudal peduncle enhances swimming speed and a terminal mouth is consistent with pelagic feeding on organisms residing within the mid-water domain (Moyle & Cech, 1996; Kassam *et al.*, 2004). The 'Rotund A' shape of clade II with its larger and longer fins and protruding mouth make it better suited to more littoral/benthic areas where manoeuvrability among mangrove roots, submerged rocky outcrops, or reefs is essential (Moyle & Cech, 1996; Kassam *et al.*, 2004). A protruding mouth is also better suited for gleaning invertebrates from different substrates than a terminally set one (Sibbing & NagelKerke, 2001; Bouton *et al.*, 2002; Kassam *et al.*, 2004). The 'Rotund B' shape of clade III is also distinct but is characterized as intermediate between the other two clades and suggests an intermediate habitat choice
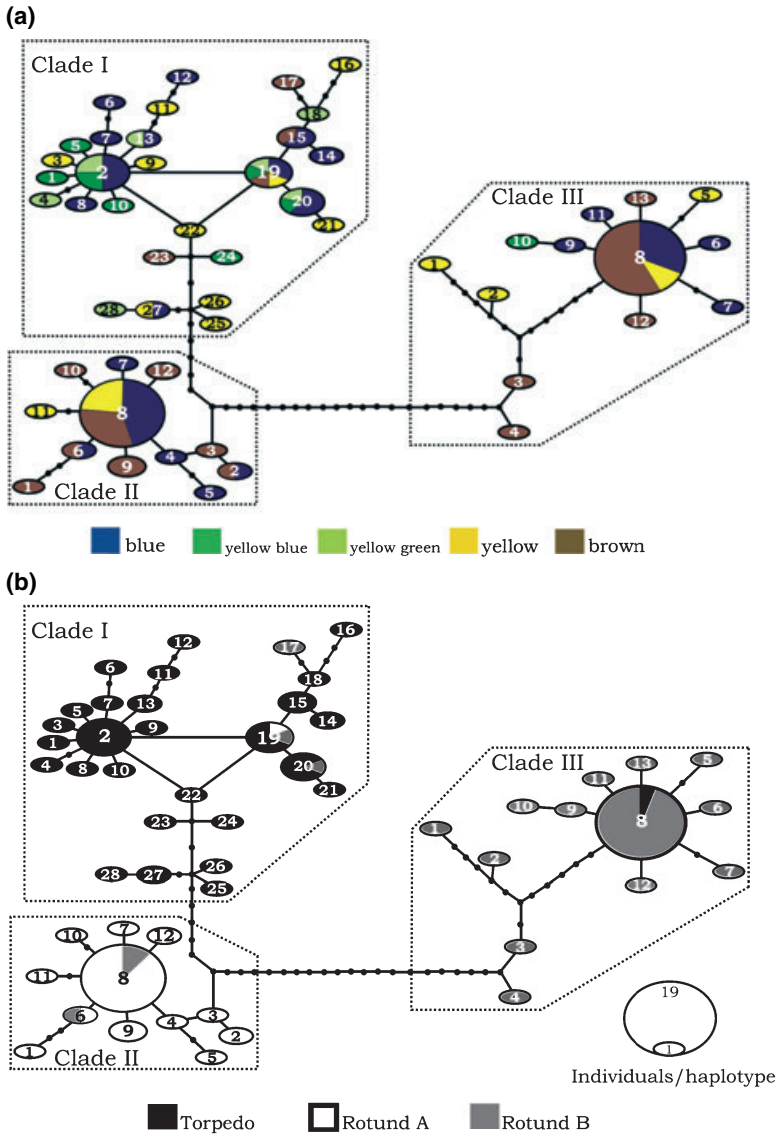
(a)

(b)

Fig. 7 Unrooted minimum spanning haplotype network determined (using Maximum Parsimony settings) among individual *Telmatherina* sampled in this study. Network derived from 1090 bp sequences of mtDNA (cytochrome *b* & 16S). Haplotype sizes reflect number of individuals sharing particular haplotype (scale in lower right corner). Haplotype numbers consistent with those given in Fig. 3 are indicated inside haplotype ovals. (a) Demonstrates colouration mapping onto network whereas (b) represents shape.

for this clade using a combination of littoral/benthic and pelagic environments within the lake. Microenvironment adaptation of each clade predicted from their shapes as described is consistent with studies revealing clade-specific diet and isotopic signatures reflecting items within these respective microenvironments (Roy *et al.*, 2006a) and of various forms selecting different environments to spawn (Gray & McKinnon, 2006). Thus, body shape does match initial genetic divergence within *Telmatherina* and is likely related to microenvironment adaptation.

Assortative mating and sexual selection among colouration types has been proposed as a driving mechanism responsible for the creation of many sibling cichlid species in the African Great Lakes (Seehausen & van Alphen, 1999; Allender *et al.*, 2003; Knight & Turner,

2004). It is tempting to propose that the same mechanism may be operating in Lake Matano, especially considering the high water clarity and negligible number of top predators in the system. Results from this study, however, reveal that colouration was generally not found to be clade specific, except for the prevalence of yellow blue and yellow green colouration types in clade I. All other colourations were present in substantial numbers in all clades. This recurring pattern of similar colouration types in each of the three genetic clades may be the result of parallel evolution within each clade, and suggests that colouration patterns may be important to intra-clade segregation and speciation (Rundle *et al.*, 2000; McKinnon & Rundle, 2002; but see Gray & McKinnon, 2006). In the 'radiation in stages' model of adaptive radiation described by Danley & Kocher (2001),

Streelman & Danley (2003) and Kocher (2004), colour is a potent initiator of divergence in more derived stages of radiations, subsequent to body shape. In our study, however, the MSN tends to discount this possibility, at least in as much as it is related to mtDNA lineages and thus to initial divergence as segregation of haplotypes within clades based on colouration was not observed. Whether or not colouration is related to more recently evolved segregating patterns within each clade is beyond the resolution of our genetic markers, but does not appear to be so (Gray & McKinnon, 2006). Nevertheless, our results do not indicate that colouration is in any way related to initial divergence among the three *Telmatherina* lineages.

As an alternative to the scenarios presented, colouration may be linked to body shape and both traits acted together to cause initial divergence among the *Telmatherina* clades. Several lines of evidence tend to discount this hypothesis. First, if colouration and body shape were linked, either trait should be equally diagnostic in terms of assigning clade membership for any random individual within the population. This, however, is clearly not the case (Fig. 7). Secondly, adding colouration as a variable in the clade specific shape analysis should have substantially enhanced segregation among clades by shape. This, however, was not observed and adding colour produced an identical shape segregation pattern and enhanced the explanatory power of the first CV axis only marginally (Fig. 4b). Finally, if colour was linked to shape, colouration should predict *Telmatherina* shape. Apart from yellow-blue and yellow green showing segregation, blue, yellow and brown coloured individuals showed substantial overlap and were not significantly different in shape (Fig. 6). Thus predicting body shape using colour would be highly inaccurate.

Conclusion

This study provides genetic, morphological and body colouration data demonstrating that the *Telmatherina* radiation in Lake Matano was most likely initiated by body shape adaptation to microenvironments and that colouration had little to with this initial process. Multiple colouration types within each clade, however, may indicate that colour is an important factor associated with more recent divergence and the development of reproductive isolation within individual clades, as suggested by the 'radiation in stages' model of vertebrate radiation. Continued study of speciation processes in *Telmatherina* in this relatively simple and pristine ecosystem will provide valuable insight towards refining theories of speciation and adaptive radiation in natural systems.

Acknowledgments

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