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ADAPTIVE SYMPATRIC SPECIATION OF POLYCHROMATIC "ROUNDFIN" SAILFIN SILVERSIDE FISH IN LAKE MATANO (SULAWESI)

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The significance of sympatric speciation is one of the most controversial topics in evolutionary biology. Theory suggests that different factors can lead to speciation in full geographical contact, including selection and nonrandom mating. Strict criteria have been established for assessing sympatric speciation, which have been met in only a very few cases. Here, we investigate differentiation among sympatric morphospecies and color morphs of "roundfin" sailfin silversides (Telmatherinidae), small freshwater fish endemic to ancient Lake Matano in Central Sulawesi (Indonesia). Morphospecies are distinct according to body shape (geometric morphometrics), population structure (population-level amplified fragment length polymorphism [AFLP] markers), ecology, and mating behavior (habitat transects, stomach contents). Explorative genome scans based on AFLPs indicate that divergent selection affects only 1.3–4.2% of the analyzed loci, suggesting an early stage of speciation. Transect data demonstrate strong assortative mating and adaptive niche differentiation. However, we find no restrictions in gene flow among the conspicuous male color morphs. In summary, our data are consistent with a sympatric mode of divergence among three morphospecies under conditions effectively ruling out allopatric scenarios. Substantial, but incomplete, reproductive isolation suggests an early stage of speciation, most likely due to ecological selection pressure.

KEY WORDS: **Adaptive radiation, amplified fragment length polymorphism (AFLP), Malili Lakes, natural selection, sexual selection, *Telmatherina* sp. (Telmatherinidae).**

Sympatric speciation, the divergence of one population into two or more species without geographical isolation, remains a controversial topic in evolutionary biology (Gavrilets 2004; Coyne 2007). For decades, the idea of universal allopatric speciation, resulting from genetic drift and selection after geographic isolation, has dominated speciation research (Mayr 1963; Mallet 2001). Nevertheless, theory predicts that under certain conditions speciation is possible without complete physical isolation of diverging populations (Dieckmann and Doebeli 1999; Higashi et al. 1999; Takimoto et al. 2000; Doebeli and Dieckmann 2003; Gavrilets 2003, 2004; Gavrilets and Vose 2005; Kawata et al. 2007). Strong disruptive selection for alternative "fitness peaks" in association with evolution of mate discrimination ranks among the most common scenarios (Gavrilets 2004; Coyne 2007), besides

resource-specific assortative mating or hybrid speciation (Arnold 1997; Feder 1998; Seehausen 2004).

Empirical case studies suggest that several species pairs and radiations of plants and animals most likely evolved in sympatry (Schliewen et al. 1994, 2001; Berlocher and Feder 2002; Schliewen and Klee 2004; Feder et al. 2005; Savolainen et al. 2006; Friesen et al. 2007; Rolán-Alvarez 2007), but the total number of unambiguous cases remains low (Coyne 2007). The discussion currently focuses on the frequency of sympatric speciation in nature and, ultimately, on the mechanisms promoting it (Mallet 2001; Turelli et al. 2001; Via 2001; Coyne 2007). Recently, the idea of the mosaic nature of animal genomes (Templeton 1981; Mallet 1995, 2005; Feder 1998) has gained new momentum, suggesting that gene flow among recently evolved sympatric species is common and even generates adaptive variation (Wu 2001; Seehausen 2004; Mallet 2007).

Coyne and Orr (2004) suggested four criteria to reject the null hypothesis of allopatric speciation: (1) sympatry, (2) substantial reproductive isolation, and (3) sister group relationship of the diverged species with (4) implausibility of allopatric scenarios. Sister species or species flocks endemic to habitat islands, such as oceanic islands or isolated freshwater lakes, appear to be promising systems to test for these criteria because such isolated habitats allow ruling out phases of physical isolation (Via 2001; Coyne and Orr 2004). "Roundfin" sailfin silversides, endemic to isolated Lake Matano in Central Sulawesi (Indonesia), might represent an additional example of sympatric speciation, and are also a promising model to test for mechanisms promoting divergence. These small, atheriniform (Teleostei: Atheriniformes) freshwater fish are characterized by having rounded second dorsal and anal fins, distinguishing them from sympatric "sharpfin" sailfin silversides (Kottelat 1991). Three morphospecies are characterized according to the shape and size of adult, spawning individuals: high-bodied, large *Telmatherina antoniae* "large," slender and small *T. antoniae* "small," and slender, large *T. prognatha* (Kottelat 1991; Herder et al. 2006a). In contrast to the dusky-gray females, males of all three morphospecies occur in bright yellow, blue, or blue-yellow color morphs (Herder et al. 2006a) (Fig. 1). These fish are mobile and nonterritorial promiscuous substrate spawners (Gray and McKinnon 2006). Lake Matano is a comparably small (approx. 32 by 6 km; ≈164 km^2) but extremely deep (> 590 m) graben-lake, with steep walls and without major intralake barriers down to more than 400 m depth (Haffner et al. 2001, Fig. 1). It is the uppermost of "Wallace's dreamponds" (Herder et al. 2006b), that is the Malili Lakes, a hotspot of freshwater diversity (von Rintelen et al. 2007). The lakes harbor endemic radiations of snails, crustaceans, and fish (Kottelat 1990a,b, 1991; von Rintelen et al. 2007). Age estimates of 1–2 Myr are provided in the literature, but have never been tested critically (von Rintelen et al. 2004).

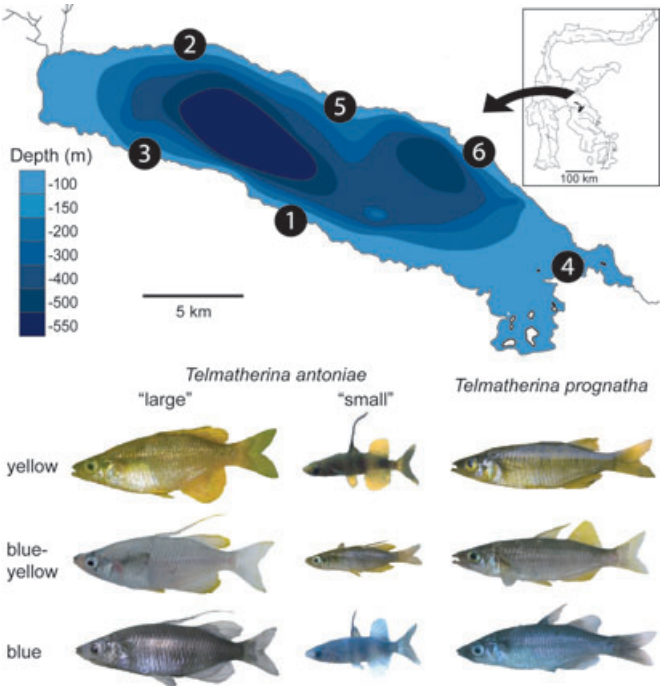

Figure 1. Lake Matano and its three polychromatic roundfin morphospecies, collected from six sampling locations distributed around the lake. Illustrations display adult, reproducing males; relative picture size corresponds to natural size. © Map by T. & K. von Rintelen, modified (with permission).

The radiation of sailfin silversides has recently attracted attention as a model system for speciation processes. Roy et al. (2004, 2007a,b) identified three mitochondrial clades in Lake Matano's *Telmatherina* and based analyses of speciation processes on those three mtDNA haplotype groups. However, nuclear data only support two monophyletic groups (Herder et al. 2006b), corresponding to morphologically well-defined roundfins and sharpfins (Kottelat 1991). The additional mitochondrial clade results from introgression of haplotypes common in stream populations, into sharpfins (Herder et al. 2006b). Hence, sharpfins carry both "native" and introgressed "stream" mtDNA haplotypes, whereas roundfins are clearly monophyletic according to both marker systems.

The present study applies Coyne and Orr's (2004) criteria for sympatric speciation to Lake Matano's roundfins. Based on data derived from nuclear population-level genetic markers, ecological field observations, and analysis of body shape and stomach contents, we test for morphological, ecological, and genetic divergence within the well defined, compact area of an ancient lake. This approach allows incorporation of hypotheses on mechanisms discussed to drive speciation processes, namely ecological selection with respect to body shape, trophic ecology, and habitat use and sexual selection with respect to male coloration.

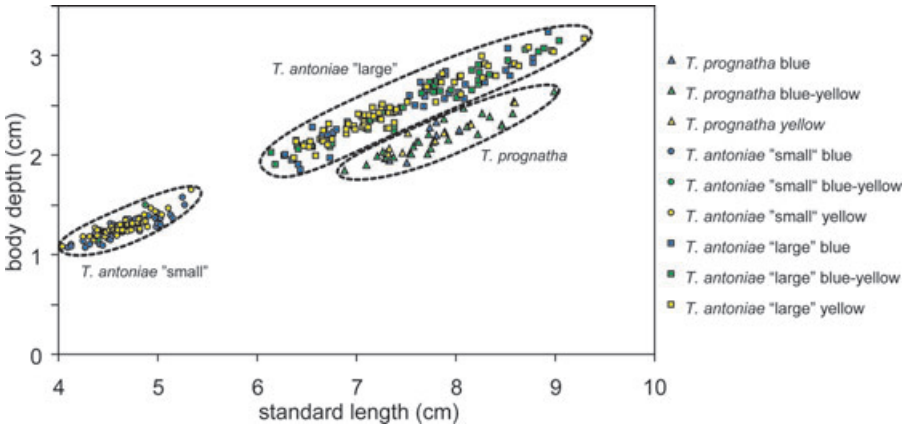

Figure 2. Body depth relative to standard length of 309 adult roundfin males, subdivided into morphospecies and color morphs. *Telmatherina antoniae* "small" is distinguished from *T. antoniae* "large" and *T. prognatha* by a gap in adult size. *Telmatherina prognatha* and *T. antoniae* "large" differ in body depth, with *T. prognatha* being more slender than *T. antoniae* "large."

Materials and Methods

SAMPLING AND ROUNDFIN MORPHOSPECIES

Sampling and transect observations were conducted in 2002 and 2004 at six areas distributed roughly equally around the shoreline of Lake Matano (Fig. 1; see online Supplementary Appendix S1). Species determination follows Kottelat (1991) and Herder et al. (2006a). "Small" and "large" morphs of *T. antoniae* are distinguished by the bimodal distribution of adult body sizes (< 5.5 cm in *T. antoniae* "small," > 6 cm in *T. antoniae* "large," and the associated abrupt increase in body depth (Fig. 2). Individual fish were obtained from different kinds of habitats within the upper 10 m of the water column using SCUBA- or snorkel-guided gillnetting. Individual coloration of body and fins was recorded using photographs and verbal, qualitative descriptions. Specimens were marked individually. A fin-clip was stored in 99% ethanol, and the fish was subsequently preserved in 4% formalin and later transferred to 70% ethanol. Exploratory sampling was conducted in the offshore area of Lake Matano (see online Supplementary Appendix S1). Voucher specimens are stored in fish collections of Zoologische Staatssammlung München (ZSM) and of Zoologisches Forschungsmuseum Alexander Koenig Bonn (ZFMK). Due to the rarity of *T. prognatha* (see transect data), the number of specimens analyzed is lower than in both other roundfin morphospecies. Because of limited availability of sufficient samples of high-quality DNA, the "blue" color morph of *T. prognatha* was excluded from the genetic analysis.

MORPHOMETRIC ANALYSIS OF BODY SHAPE

Landmark-based geometric morphometric techniques (Zelditch et al. 2004) based on 20 homologous landmarks (see online Supplementary Appendix S1) were used to quantify the body shape of 309 individuals. Principal components (PCs) and canonical variates (CVs) were calculated based on a covariance matrix derived from partial warp scores. Two methods were applied to test for significance in differentiation of PCs bearing at least 5% of variance. First, a multivariate analysis of variance (MANOVA, with Hotelling's pairwise test) was used to explore differentiation among morphospecies and color morphs based on all PCs (> 5%), followed by analysis of variances (ANOVAs) with Tukey's post hoc tests focusing on single PCs. To evaluate the integrity of morphospecies groups based on CVA scores, a jackknife test of assignment was performed (1000 resampling trials, leaving out and reassigning 10, 20, 30, 40, 50, and 80% of the specimens) (Nolte and Sheets 2005). Finally, body length and body depth were inferred from landmark data to directly compare relative body depth. For details, see online Supplementary Appendix S1.

STOMACH CONTENT ANALYSIS

Gastrointestinal tracts of 40 specimens per morphospecies were dissected. Food items present between the esophagus and pylorus were embedded in Gelvatol (polyvinylalcohol) and the relative surface area of different food items was estimated for every individual fish (see Herder and Freyhof 2006). Based on the obtained data matrix, dietary overlap (Schoener 1970) between morphospecies and the relative importance of food items (Pinkas et al. 1971) were calculated (for details, see online Supplementary Appendix S1). An ANOSIM (analysis of similarities, 10,000 replicates) was calculated from Euclidian distances to test the null hypothesis of equal content composition among morphospecies.

SCUBA TRANSECTS

SCUBA-guided transect diving was used to determine roundfin distribution with respect to habitat, season, and time of day. Observation areas were positioned around the six locations used for sampling (Fig. 1). Transects of 100 m length, subdivided in stretches of 10 m, were positioned in 1.5, 5, 10 (all in 2002 and

2004), and 20 m (only in 2002) depth, respectively. Sailfin silversides were recorded in a corridor of 4 m while diving, relating each individual to macro-, meso-, and microhabitat parameters. Each roundfin was classified according to morphospecies and mating status (courting vs. noncourting); color morphs were recorded as "yellow," "blue-yellow," or "blue." In total, 428 transect stretches covering 81,920 m^2 were included in the analysis, corresponding to a 20.48 km transect length. See online Supplementary Appendix S1 for details.

ANALYSIS OF TRANSECT DATA

To test for ecological (habitat) segregation, roundfin occurrence with respect to environmental variables was analyzed for (1) morphospecies (*T. antoniae* "large," *T. a.* "small," and *T. prognatha*), (2) courtship activity (courting vs. noncourting *T. antoniae* "small" and *T. a.* "large"; no *T. prognatha* were observed courting), and (3) color morphs (yellow and blue in *T. antoniae* "small," yellow, blue, and blue-yellow in *T. a.* "large"; not for *T. prognatha* due to its low abundance). First, canonical correspondence analysis (CCA) was used to identify the relative contribution of environmental parameters to roundfin occurrence, using the forward selection procedure implemented in CANOCO 4.0 (ter Braak and Smilauer 1998) with 9999 full model Monte Carlo permutations. The inclusion of transect length as a covariable corrected for differing transect surfaces, which are a consequence of habitat heterogeneity. In a second step, parameter effects not related to permanent habitat characteristics (season, weather, light, waves, daytime) were removed from the analysis using partial CCA (pCCA), applied to the same datasets as CCA. To test for correlation between fish abundance per transect m^2 (428 transect stretches) and single habitat parameters, Spearman rank correlation coefficients were calculated for habitat variables identified as significant by CCA or pCCA. Finally, two-sample χ^2-tests based on categories of habitat data (see online Supplementary Appendix S1) were applied to test for significant deviation from the null hypothesis of equal distribution to habitat parameters.

AMPLIFIED FRAGMENT LENGTH POLYMORPHISM (AFLP)

Following a slightly modified protocol (see online Supplementary Appendix S1) of the AFLP method (Vos et al. 1995), 330 individuals were typed with six selective amplifications, resulting in 573 polymorphic AFLP fragments (see online Supplementary Appendix S1). To allow inclusion of the factor "coloration" into the analysis, sampling was mainly confined to male specimens. For analysis of morphospecies a few female specimens (two of *T. antoniae* "small," eight of *T. prognatha*) were included, if the number of suitable males was critically low; see online Supplementary Appendix S1). Fluorescence-labeled fragments were separated on a CEQ 8000 capillary sequencer (Beckman Coulter, Inc., Fullerton, California), with an internal size standard.

AFLP FRAGMENT DATA: CANONICAL CORRESPONDENCE ANALYSIS

The conceptual framework of CCA was used to determine the relative contribution of morphological differentiation, male coloration, and spatial distribution to the variation in the multilocus AFLP dataset (Angers et al. 1999; Giannini 2003; Schliewen and Klee 2004; Herder et al. 2006b; see online Supplementary Appendix S1 for details). An AFLP data matrix consisting of presence data for all 573 polymorphic loci for all 330 specimens code for the dependent variables in this analysis, whereas binary coded hypotheses of morphology (*T. antoniae* "small," *T. a.* "large," *T. prognatha*), overall coloration (yellow, blue-yellow, blue), and distribution (locations 1–6) are the independent variables. Variables contributing most to the explanation of the variation in the AFLP matrix were identified using the forward selection procedure as implemented in CANOCO 4.0 (ter Braak and Smilauer 1998). The analysis was repeated in a second step using a partial CCA correcting for spatial factors.

AFLP FRAGMENT DATA: ASSIGNMENT METHODS

A reassignment procedure implemented in AFLPOP (Duchesne and Bernatchez 2002) was applied to test for population differentiation among morphospecies and color morphs (Campbell et al. 2003). Allocation success was estimated for each of the candidate populations by withdrawing each specimen and recalculating allelic frequency of the populations. Specimens were then reallocated on the basis of log-likelihoods computed for each population. Resulting allocation matrices were subsequently used to test for deviations from uniform distribution under the null hypothesis that, without population structure, assignment should proceed by chance.

AFLP FRAGMENT DATA: POPULATION STRUCTURE

Analysis of molecular variance (AMOVA, Excoffier et al. 1992), as implemented in Arlequin 3.01 (Excoffier et al. 2005), was used to investigate the structure of the genetic variation with regards to (1) morphospecies, (2) coloration, and (3) sampling location. F_{ST} analogues were calculated at each level of analysis. The coloration "blue-yellow" was extremely rare within *T. antoniae* "small" and the only two specimens in the analysis were consequently also excluded for analysis of male coloration. In contrast, "blue-yellow" *T. antoniae* "large" could be included in the analysis in three ways: as a separate population, combined with "yellow," or combined with "blue" specimens. This allowed testing of whether the "blue–yellow" morph possesses distinct population integrity or might belong to one of the uniformly colored groups. The methods of Lynch and Milligan (1994) as implemented in AFLP-SURV (Vekemans 2002), were used to estimate population allele

frequencies and to calculate Nei's (1978) unbiased gene diversity (H) on different levels.

AFLP FRAGMENT DATA: OUTLIER TEST FOR SELECTION

To evaluate the proportion of AFLP loci possibly subject to selection, an explorative genome scan was conducted under the hypothesis that genetic differentiation between populations is higher for loci under divergent selection than for the rest of the genome (Campbell and Bernatchez 2004; Beaumont 2005; Bonin et al. 2006). Using Beaumont and Nichols's (1996) program package fdist2 (modified by Beaumont and Balding 2004), F_{ST} and heterozygosity of each AFLP locus were calculated for each combination of the three roundfin morphospecies and compared to simulated null distributions with $P = 0.99$ and $P = 0.95$ quantiles. To identify individual loci for which the neutral model could be rejected, F_{ST}s of AFLP loci and simulated quantiles were plotted against heterozygosity. Loci outside of the neutral distribution were detected as outliers (Beaumont and Nichols 1996; Beaumont and Balding 2004; Bonin et al. 2006; see online Supplementary Appendix S1).

AFLP FRAGMENT DATA: ISOLATION BY DISTANCE

Geographic population structure detected in *T. antoniae* "large" (see below) was analyzed using Mantel tests under the model of "isolation by distance" (Wright 1943, 1946) (see online Supplementary Appendix S1). Three distances were tested for correlation with F_{ST}s between sites: (1) direct distance, that is the shortest route (without crossing land) connecting sampling locations under the hypothesis that individuals cross open waters; (2) distance along the shoreline, that is along the contour of the lakes shore under the hypothesis that migration strictly proceeds within the few uppermost meters of the lake; (3) distance along the 50 m isobath, that is under the hypothesis that migration takes place above areas not deeper than 50 m. Differentiation in (2) and (3) was chosen due to significant discrepancies among shoreline and 50 m profile, especially in the eastern corner of Lake Matano (compare Fig. 1).

Results

SYMPATRIC DISTRIBUTION OF THREE DISTINCT PHENOTYPES

Endemic roundfin sailfin silversides were obtained in sympatry from the same sample sites throughout Lake Matano, without any indications of local endemism with respect to either morphology or coloration (see online Supplementary Appendix S2). Explorative pelagic gillnetting detected *T. antoniae* "small" offshore in the center of Lake Matano. Morphometric analyses clearly distinguished all three morphospecies, based on size and shapes of head and body. In contrast, color morphs were not distinct.

"Small" and "large" *T. antoniae* differed in adult size and relative body depth, that is by a size gap of mature specimens between 5.34 and 6.15 cm SL (Fig. 2). *Telmatherina prognatha* had a more slender body than *T. antoniae* "large" and grew larger than *T. antoniae* "small" (Fig. 2). Mean relative body depth was significantly different among all three morphospecies (Tukey HSD post hoc test: $P = 0.006$ for *T. antoniae* "small" vs. *T. prognatha*, $P < 0.001$ for *T. antoniae* "small" vs. *T. antoniae* "large," $P < 0.001$ for *T. prognatha* vs. *T. antoniae* "large"). Geometric morphometric analyses likewise supported three distinct morphospecies. PCA revealed four axes explaining more than 5% of shape variance. MANOVA indicated highly significant differentiation (global and all pairwise tests $P < 0.001$). ANOVAs of PCs 1–4 strongly supported this pattern (Table 1). CVA distinguished morphospecies along two main canonical axes, explaining together 95.15% of morphometric variance among the three groups (Fig. 3A,B). Independent of body depth (mainly CV1), head characters (projecting premaxilla, size of eye) were also dominant (CV1, CV2; Fig. 3C,D). Reassignment tests strongly supported distinct shapes of morphospecies, remaining robust even for partitions of the dataset (> 96% correct reassigned individuals for up to 50% specimens left out; see online Supplementary Appendix S2).

In contrast to morphospecies, analyses focusing on body shape among color morphs within morphospecies did not indicate any differentiation (MANOVAs based on PCs of (1) blue vs. yellow or (2) blue vs. blue-yellow vs. yellow *T. antoniae* "large" n.s., (3) blue vs. yellow *T. antoniae* "small" n.s., (4) blue vs. blue-yellow vs. yellow *T. prognatha* n.s.; ANOVAs based on single PCs are likewise all n.s.).

TROPHIC ECOLOGY

Stomach contents were analyzed to determine foraging preferences among roundfin morphospecies, and suggested trophic niche partitioning. Trophic niche differentiation was significant ($P < 0.001$; pairwise comparisons *T. antoniae* "large" vs. *T. antoniae* "small" $P < 0.001$, *T. antoniae* "large" vs. *T. prognatha* $P < 0.05$, *T. antoniae* "small" vs. *T. prognatha* $P < 0.001$; Fig. 4). Little dietary overlap was indicated between "small" and "large" *T. antoniae* ($o = 0.251$) and between *T. antoniae*

Table 1. ANOVAs with pairwise post hoc tests of roundfin morphospecies for the first four principal components (PCs)

	PC1	PC2	PC3	PC4
ANOVA (global)	**	**	**	**
T. a. "large" vs. "small"	**	**	n.s.	n.s.
T. prognatha vs. *T. a.* "large"	**	n.s.	**	**
T. a. "small" vs. *T. prognatha*	n.s.	**	**	**

**P significant at $\alpha = 0.001$.

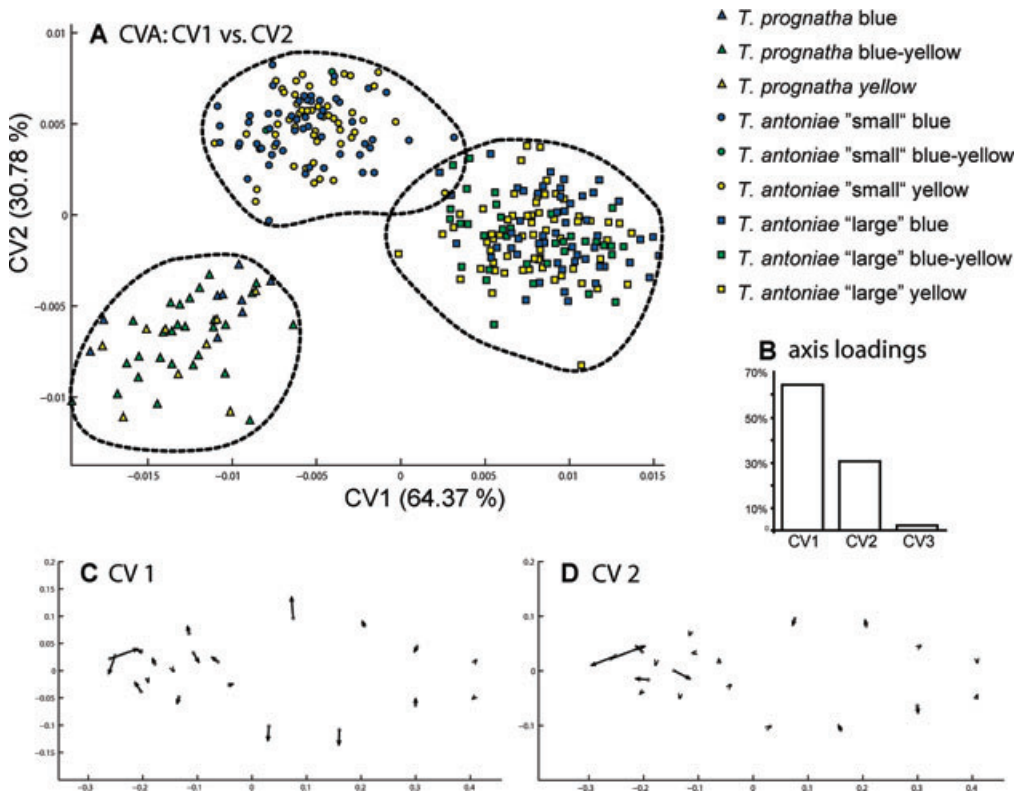

Figure 3. Canonical variates analysis (CVA) based on 20 landmarks, distinguishing roundfin morphospecies along two canonical axes. (A) CVA-plot; (B) axis loadings, with 95.15% of variance among morphospecies in the first two axes; (C) and (D) deformation vectors, indicating the changes in the relative position of landmarks for each canonical axis. CV1 separates *T. antoniae* "large" from *T. antoniae* "small" and *T. prognatha* according to body depth and differences in head shape. CV2 distinguishes *T. prognatha* from both *T. antoniae* morphospecies, mainly according to shape of head and snout.

"small" and *T. prognatha* ($o = 0.248$). Contents were less differentiated between *T. antoniae* "large" and *T. prognatha* ($o = 0.508$). Copepods were the dominant diet of *T. antoniae* "small" ($I = 84.74$), a food item almost absent in both other morphospecies. Terrestrial arthropods were of major importance for *T. antoniae* "large" ($I = 78.33$) and of relevance for *T. prognatha* ($I = 55.96$). Mollusks were only found in *T. antoniae* "large" ($I = 18.03$), whereas fish was a major source of nutrition for

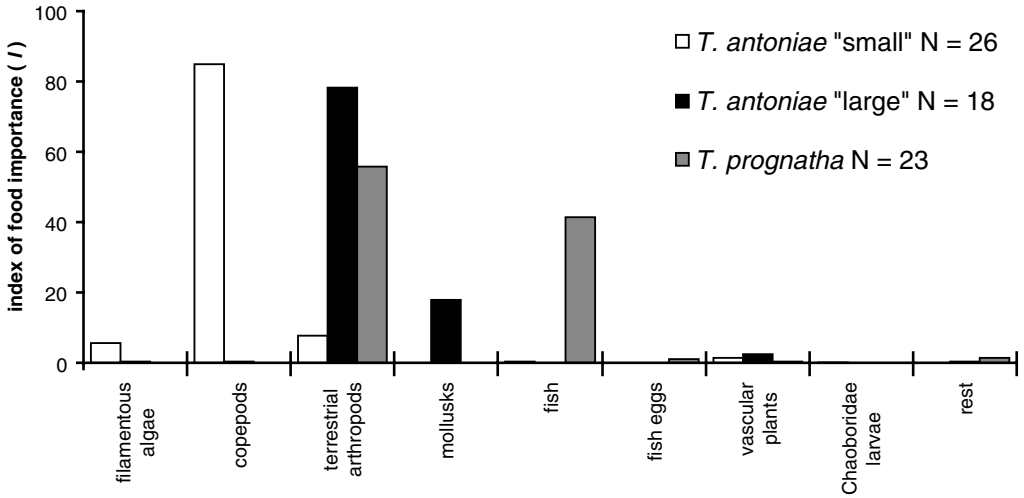

Figure 4. Dietary importance of food items identified in roundfin morphospecies. *Telmatherina prognatha* mainly feed on fish and terrestrial arthropods, *T. antoniae* predominantly on copepods (*T. a.* "small") or terrestrial arthropods and mollusks (*T. a.* "large").

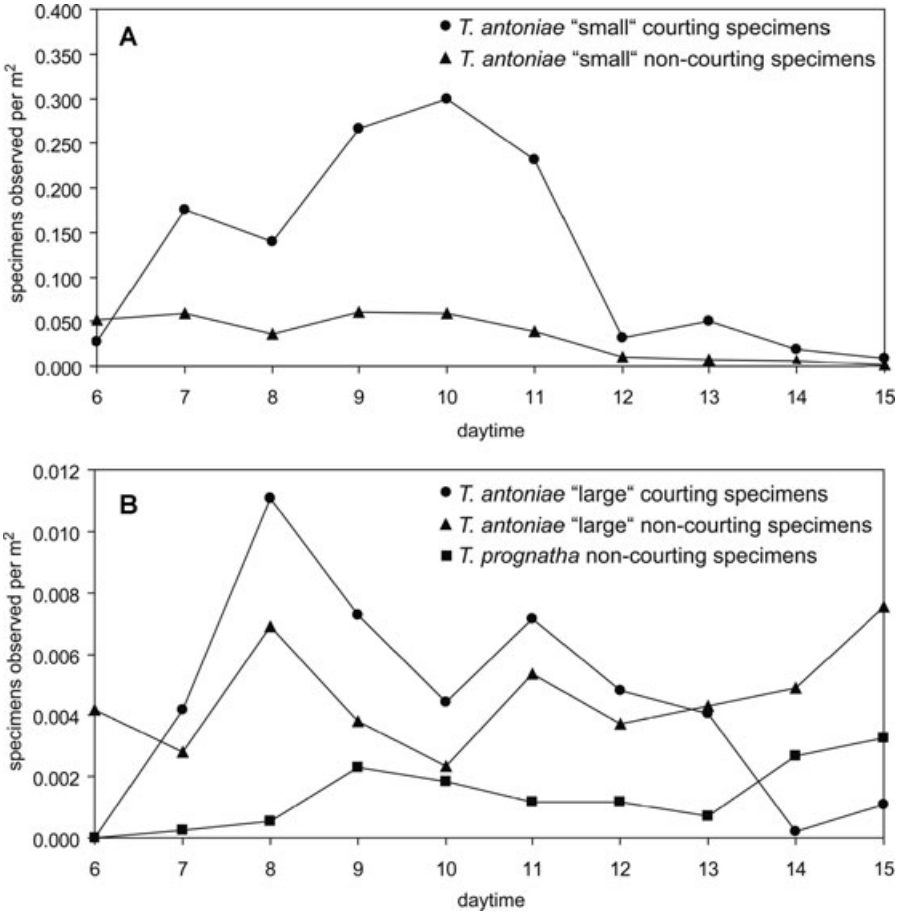

Figure 5. Abundances of roundfins at benthic transects throughout the day. (A): *T. antoniae* "small" show a conspicuous peak density in the morning, most conspicuous in courting specimens, and are nearly absent after noon. (B): In contrast, the total abundance of *T. a.* "large" is rather constant, with fluctuations between courting and noncourting behavior. *Telmatherina prognatha* is rare, but present at least between 0900h and 1500h.

T. prognatha ($I = 41.23$). The remaining food categories were of minor importance.

FLUCTUATION IN ABUNDANCE AND ASSORTATIVE MATING

Abundances of morphospecies were unevenly distributed, with a strong dominance of *T. antoniae* "small" and *T. prognatha* being rare. In contrast to both other morphospecies, the abundance of *T. antoniae* "small" sharply decreased in the sampling areas after noon. Assortative mating by size was strong.

Altogether, 14,001 adult roundfin individuals were observed within 1.5 m above the substrate. The majority of individuals were classified as *T. antoniae* "small" (94.26%), followed by 4.79% *T. antoniae* "large," and 0.91% *T. prognatha*. Six roundfin specimens could not be assigned to any of the three groups and were excluded from subsequent analyses. In *T. antoniae* "small," 74.58% of the males and 89.25% of the females were spawning or courting, contrasted by only 41.6% active males and 61.25% females in *T. antoniae* "large." No courtship or spawning was observed in the rare *T. prognatha*. A total of 5528 roundfin pairs were observed within the area up to 1.5 m above substrate (5362 pairs of *T. antoniae* "small," 166 pairs of *T. antoniae* "large"), without recording a single mixed pair. However, one mixed pair (a large *T. antoniae* male courting with a small *T. antoniae* female) was observed above 1.5 m. Moreover, mixed pairs were observed in rare cases outside the transect areas (F. Herder, pers. obs. 2002, 2004, 2006). Abundances of roundfins within the sampling areas varied substantially with time of day (Fig. 5). In *T. antoniae* "small," courting specimens drastically outnumbered noncourting individuals between 0700 h and 1200 h; the abundance of both strongly decreased after 1200 h. The abundance of courting *T. antoniae* "large" followed the same trend, but the abundance of noncourting *T. antoniae* "large" remained largely constant and relatively high over the day. *Telmatherina prognatha* abundances weakly increased during the day.

DISTINCT PATTERNS OF HABITAT USE

Transect data were used to test for habitat-use differentiation among morphospecies and color morphs, analyzing courting and

Table 2. Results of full and partial CCAs, evaluating the significance of contribution for each single habitat parameter to roundfin distribution

Roundfin phenotypes combined in explorative CCAs											
T. a. "small"	●										
T. a. "small" courting		●		●	●						
T. a. "small" courting blue						●					
T. a. "small" courting yellow						●					
T. a. "small" noncourting		●	●		●						
T. a. "small" noncourting blue								●			
T. a. "small" noncourting yellow								●			
T. a. "large"	●										
T. a. "large" courting		●		●	●						
T. a. "large" courting blue									●		
T. a. "large" courting yellow									●		
T. a. "large" courting blue-yellow									●		
T. a. "large" noncourting		●	●		●						
T. a. "large" noncourting blue										●	
T. a. "large" noncourting yellow										●	
T. a. "large" noncourting blue-yellow										●	
T. prognatha (all noncourting)	●	●	●								
Significance of single habitat parameters											
permanent parameters											
macrohabitat	*/*	**/*		**/**	*/*						*/*
mesohabitat	*/-		**/**				**/**	*/*			
substrate							**/**	**/**			*/-
sediment											
depth			**/**			**/**		*/*			
slope	**/**	**/**	**/**	**/**			**/**		*/-		**/**
aufwuchs	*/*	**/**	*/*	**/**	*/*	**/**					*/-
wood	*/*	*/*	*/*								
vegetation	*/*	*/*	*/*		*/*		**/**	**/**			
canopy cover	**/**	**/**	**/**								
shading											
nonpermanent parameters											
light											
daytime	**	**	**			*	*	**			*
season	*	**		*	**		*	**	*		**
waves	**	**	**			*					
weather	*										*

Hierarchical analyses focus from morphospecies to courting activity to male coloration. Significant results for the total set of environmental parameters are provided ahead of, those excluding the effect of nonpermanent parameters behind the slash.

****P* **significant at** $\alpha = 0.01$, **P* **significant at** $\alpha = 0.05$.

noncourting roundfins separately. Habitat use was indicative of substantial niche differences among morphospecies, including most of the parameters analyzed (Table 2, Appendices I and II). Permanent and nonpermanent habitat factors contributed significantly to variance in morphospecies occurrence (Table 2). *Telmatherina prognatha* were most abundant in the highly structured and canopy-covered littoral zone over hard substrate (Fig. 6, Appendix I). In contrast, *T. antoniae* were most frequent in slightly deeper and less structured littoral zones, with differences in microhabitat characteristics: *T. antoniae* "large" were associated with exposed hard substrate with steep areas and submersed vegetation or aufwuchs, whereas "small" *T. antoniae* preferred soft bottom habitats lacking structuring factors like submerged wood (Fig. 6, Appendices I and II). Differences in habitat use were subtle but nevertheless significant among courting and noncourting *T. antoniae* "large" and "small" (Table 2, Appendices I and II). The same

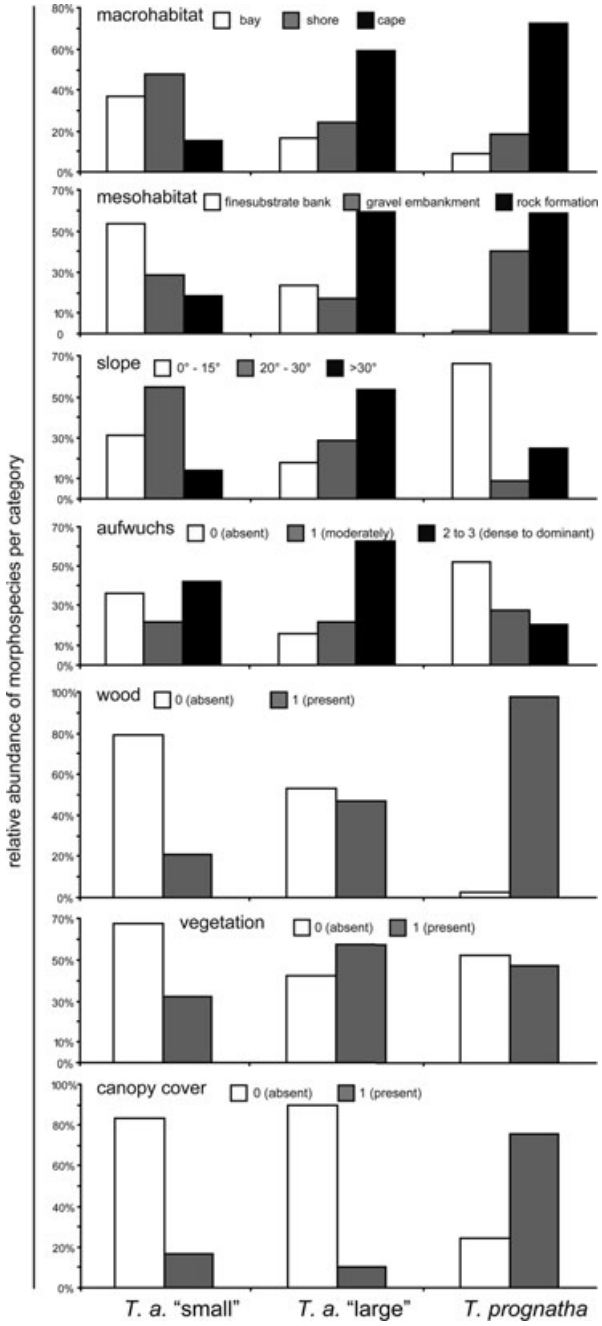

Figure 6. Habitat characteristics of roundfin morphospecies. Environmental parameters are summarized for categories and are restricted to permanent parameters explaining significant variation in morphospecies occurrence, as identified by pCCA.

applied for color morphs in both, *T. antoniae* "large" and "small" (Appendices I and II).

POPULATION STRUCTURE ACCORDING TO MORPHOLOGY, NOT TO MALE COLORATION

AFLP data were used to test for population structure according to morphospecies, male coloration, and intralake distribution. CCA, F_{ST}-based analyses, and assignment tests concomitantly demonstrated significant population structure according to morphospecies, but did not support restrictions in gene flow among color morphs. Differentiation among morphospecies was restricted to very few markers, which may be selection footprints. Local signal detected in *T. antoniae* "large" was not explained by geographical isolation. These results are based on several different analyses.

Explorative CCA analyses revealed significant support of morphospecies (*T. antoniae* "small," $P = 0.000$; *T. antoniae* "large," $P = 0.001$), "yellow male coloration" ($P = 0.034$), and sampling location (locations one, $P = 0.031$ and five, $P = 0.007$) in the dataset. Likewise, partial CCA excluding the spatial effect of sampling location supported significance of morphospecies (*T. antoniae* "small," $P = 0.001$; *T. antoniae* "large," $P = 0.001$) and "yellow male coloration" ($P = 0.041$). Hierarchical AMOVAs grouping individuals by (1) morphospecies, (2) coloration, and (3) sampling location indicated low but significant morphological and spatial structure in the dataset (morphospecies: $F_{ST} = 0.019$, $P < 0.001$; sampling locations (all three morphospecies): $F_{ST} = 0.008$, $P < 0.005$; sampling locations (*T. antoniae* "large"): $F_{ST} = 0.026$, $P < 0.001$. See online Supplementary Appendix S2 for details). Pairwise comparisons supported distinct population characteristics for all three morphospecies (pairwise F_{ST}s, all $P < 0.001$: *T. antoniae* "large" vs. "small": 0.015; *T. antoniae* "large" vs. *T. prognatha*: 0.028; *T. prognatha* vs. *T. antoniae* "small": 0.019). This result is largely maintained when comparing morphospecies from single sampling locations (see online Supplementary Appendix S2). Significant spatial structure was recovered by AMOVA among populations of *T. antoniae* "large" (2.63% variance, $F_{ST} = 0.026$; $P < 0.001$), but not in *T. antoniae* "small" (see online Supplementary Appendix S2). Populations of *T. antoniae* "large" were significantly distinguished in 11 of 15 pairwise comparisons (see online Supplementary Appendix S2). However, Mantel tests did not support significant positive correlation among local differentiation and geographic distance in *T. antoniae* "large"—neither according to direct distance between locations nor according to shoreline distances or the 50 m profile.

Reassignment tests supported population structure among morphospecies (Fig. 7). Significant deviation from uniformly distributed reassignment was detected when reassigning all three morphospecies (*T. antoniae* "large": $P < 0.001$, $\chi^2 = 59.609$; *T. antoniae* "small": $P < 0.001$, $\chi^2 = 40.707$; *T. prognatha*: $P < 0.05$, $\chi^2 = 7.635$; confirmed by subsequent pairwise tests; see online Supplementary Appendix S2). However, none of the assignments revealed unequivocally distinct populations. Low levels of differentiation were also indicated by gene diversity estimators (online Supplementary Appendix S2).

In contrast to the CCA results, male coloration was not supported as indicative of population structure by AMOVA or reassignment tests—either based on the complete dataset of

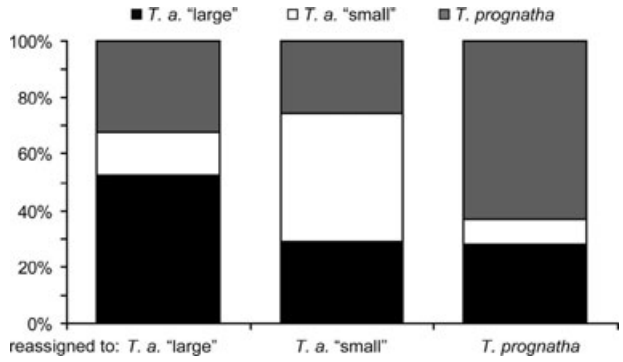

Figure 7. Reassignment of individuals according to morphospecies. Test for significance of deviation from uniform distribution under the null hypothesis that, without population structure, assignment should proceed by chance.

male roundfins or when testing explicitly within *T. antoniae* "large" or *T. antoniae* "small" (Fig. 8, see online Supplementary Appendix S2). Explorative genome scans comparing pairwise differentiation of individual AFLP loci among the three morphospecies (Fig. 9) indicated that differentiation exceeding neutral expectations is present in a low number of loci (number of loci under $P > 0.99/P > 0.95$: *T. antoniae* "large" vs. "small": 11/25; *T. antoniae* "large" vs. *T. prognatha*: 3/4; *T. prognatha* vs. *T. antoniae* "small": 4/11). In total, only 1.3–4.2 % of the loci analyzed here were identified as "outliers" under the $P > 0.99$ criterion. The remaining vast majority of genomic markers did not deviate from neutral predictions and are therefore unlikely to be directly affected by selection (Beaumont and Nichols 1996; Beaumont and Balding 2004; Beaumont 2005).

SYNTHESIS: MORPHOSPECIES IDENTITY AND CHARACTERISTICS

A high ratio of courting to noncourting specimens and a widely lacking geographic population structure strongly suggest that inshore habitats serve *T. antoniae* "small" mainly as courting- and spawning areas during early daytime. This is supported by additional evidence: (1) noncourting *T. antoniae* "small" individuals were present offshore; (2) inshore abundance strongly changed with time of day (Fig. 5A,B); (3) a feeding specialization for copepods, which can be assumed to be present in open waters; and (4) the total abundance of *T. antoniae* "small" was significantly higher than that of the other roundfin sailfin silversides, possibly based on large pelagic plankton resources. The most plausible alternative hypothesis to predominantly pelagic *T. antoniae* "small" is that these fish move to areas deeper than the 20-m transect stretches applied in the present study after spawning. However, (1) direct evidence for *T. antoniae* "small" occurring in the pelagic zone, (2) abundances of both, courting and noncourting specimens, decreasing sharply below 5 m, (3) detection of only very few specimens observed deeper than 20 m (qualitative data based on long distance visual inspection and several deep dives of areas more than 10 m below maximum diving depth for transect diving), and (4) anoxic conditions below 100 m depth in the Lake (Crowe et al. 2008) do not support this alternative.

Habitat use, feeding, and population structure of *T. antoniae* "small" widely contrasted with the "large" morph, which had lower abundance along transects, lower proportion of courting or spawning activity, mostly uniform presence during the day, and foraged on prey clearly not (mollusks) or less likely (terrestrial arthropods) available offshore. Consistent with a benthic ecology, populations of *T. antoniae* "large" were locally structured. Similarly, food items dominating the diet of *T. antoniae* "large" are indicative of inshore feeding. The nutrition of *T. prognatha* matches a priori expectations of a piscivorous fish, based on its slender and short-finned overall appearance (Kottelat 1991).

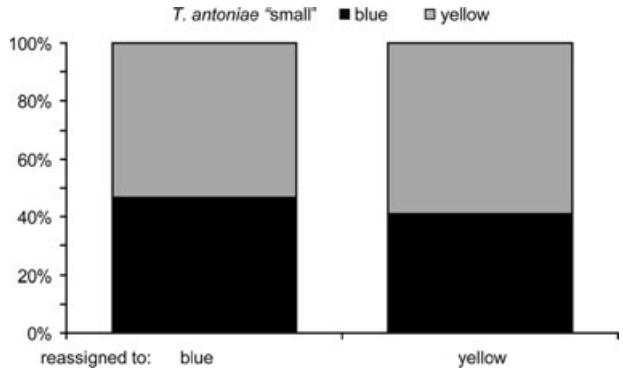

Figure 8. Reassignment of *T. antoniae* "small" individuals according to blue or yellow male coloration. Test for significance of deviation from uniform distribution under the null hypothesis that, without population structure, assignment should proceed by chance.

Discussion

Ongoing controversies have refueled the discussion about the importance of sympatric speciation in nature (Coyne 2007). As only very few unambiguous cases are known, the question remains whether sympatric speciation is indeed rare or a more widespread phenomenon hidden by difficulties in providing unambiguous evidence (Bush 1994; Via 2001; Coyne and Orr 2004; Rolán-Alvarez 2007). The present study applies Coyne and Orr's (2004) criteria for sympatric speciation to a monophyletic radiation of highly mobile fish restricted to a uniformly shaped lake.

ADAPTIVE DIFFERENTIATION OF MORPHOSPECIES

We found that three roundfin morphospecies, readily identified by body and head shape characteristics, occur fully sympatric all around the lake. "Large" and "small" *T. antoniae*, as well as *T. prognatha*, occupy distinct ecological niches, partially related to pelagic versus benthic life style. Morphospecies are differentiated

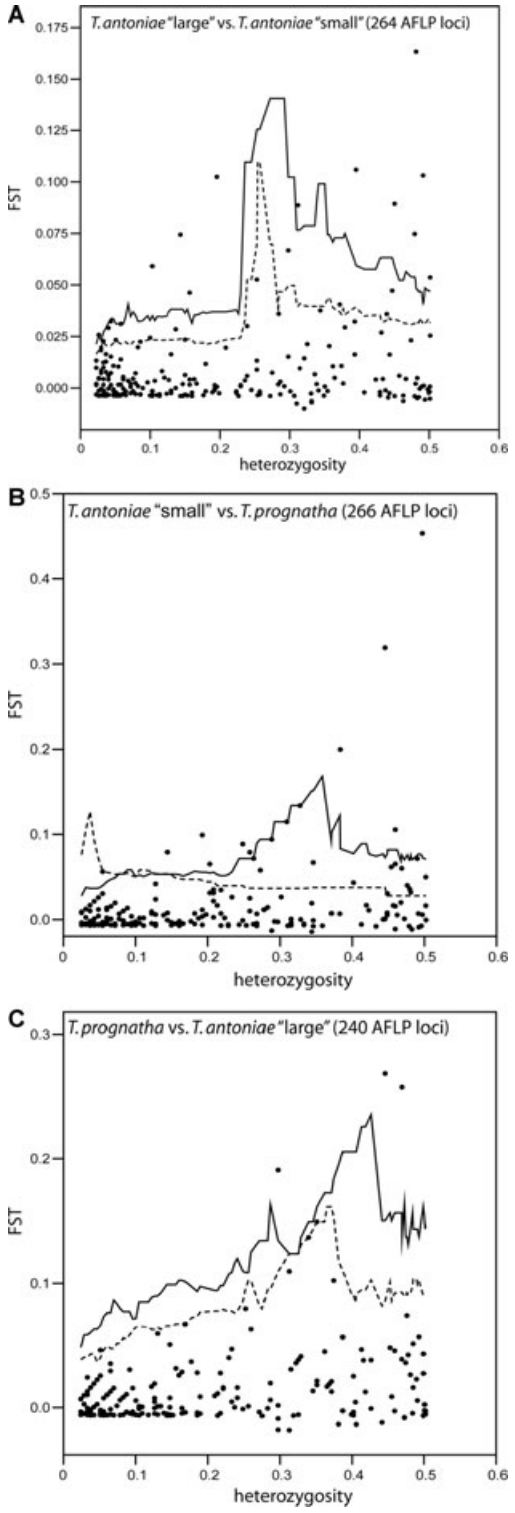

Figure 9. Explorative Genome Scans comparing differentiation of single AFLP loci to the expected neutral distribution under $P > 0.99$ (solid line)/0.95 (dashed line), with respect to heterozygosity: (A) *T. antoniae* "large" vs. *T. a.* "small"; (B) *T. a.* "small" vs. *T. prognatha*; (C) *T. prognatha* vs. *T. a.* "large." The number of AFLP loci analyzed depends on their presence in both compared groups, and the criterion of 2% diversity for each single locus. Loci exceeding expected neural F_{ST}s are potentially related to selection.

genetically, but population structure is weak and genome scans indicate that only small parts of the genome are differentiated. All of this suggests that reproductive isolation among roundfins is either of very recent origin, or speciation is incipient with incomplete reproductive isolation. Both interpretations are congruent with the lack of mitochondrial lineage sorting and the absence of a clear phylogenetic signal within roundfins (Herder et al. 2006b). Distinct body shapes associated with alternative patterns of habitat use and feeding habits suggest that adaptation according to ecological selection is most likely the causal root of roundfin divergence.

NO COLOR-SPECIFIC POPULATION DIFFERENTIATION

Evolution and maintenance of male color polymorphisms can be driven by a variety of mechanisms, and might contribute to sympatric speciation by sexual selection (Gray and McKinnon 2007). However, present results do not indicate population divergence among the strikingly different male color morphs. This suggests that divergent sexual selection on male coloration is in this case not a prime force driving speciation, as suggested by various models and (cichlid) case studies (Seehausen and van Alphen 1998; Danley and Kocher 2001; Kocher 2004; Maan et al. 2004; Kawata et al. 2007). Polychromatism present in most species of the flock (Kottelat 1990b, 1991; Herder et al. 2006a), also suggests that distinct male colorations are not maintained by the visual environment of extremely clear Lake Matano alone. Rather, color polymorphisms stabilized by factors not directly related to assortative mating seem to provide an appropriate explanation—factors that need further study. A significant signal for yellow male coloration detected in the AFLP data, despite population structure not being correlated with color, supports the idea of a heritable polymorphism not associated with population divergence. As time of day is strongly correlated with mating activity in roundfins (Fig. 5), we hypothesize that fluctuating environmental factors associated with light might shape color polymorphism in sailfin silversides, as shown for similarly polychromatic *Lucania* bluefin killifish (Fuller 2002; Fuller and Travis 2004). Alternatively, fitness correlates of male color morphs and visual environment in closely related, sympatric sharpfin sailfin silversides (Gray et al. 2008) might provide another potential explanation for maintenance of color polymorphism in roundfins. Interestingly, a recent theoretical model (Chunco et al. 2007) strongly supports both ideas by demonstrating that sexual selection acting on two distinct male color morphs in heterogeneous microhabitats can maintain color polymorphisms over a wide parameter range.

SYMPATRIC SPECIATION

Sympatric speciation is theoretically possible, if disruptive ecological (natural) selection is combined with assortative mating, or if sexual selection acts alone (Turner and Burrows 1995;

Dieckmann and Doebeli 1999; Higashi et al. 1999; Kondrashov and Kondrashov 1999; Doebeli and Dieckmann 2000; Takimoto et al. 2000; Lande et al. 2001; Gavrilets and Waxman 2002; Gavrilets 2003, 2004). Mating of Lake Matano's roundfins is highly assortative for "small" and "large" *T. antoniae*. This is associated with genetic differentiation and distinct habitat and food resource utilization in all three morphospecies. This fits exactly the major theoretical claims for sympatric speciation.

Occurrence and mating of all three morphospecies at all sampling locations around Lake Matano clearly fulfils the criterion of sympatry. Despite different peaks in occurrence along habitat parameters, mating and spawning occurs without geographical restriction. Reproductive isolation is supported by significant population structure among morphospecies. Strong assortative mating in full sympatry, as observed in roundfins, is discussed as a major prezygotic mechanism reducing recombination among sympatrically diverging populations (Coyne and Orr 2004), and serves as independent evidence supporting substantial reproductive isolation. Due to the lack of clearly distinct spawning habitats, assortative mating appears unlikely to be a byproduct of differential habitat use in roundfins, as it is known from host-specific insects (Feder 1998).

Coyne and Orr's (2004) criterion for sympatric speciation "sister group relationship of the diverged species" is problematic to test if no clear dichotomous phylogenetic structure is detectable, as in the case of roundfins (Herder et al. 2006b). Therefore, we propose incorporation of predictions derived from recent theoretical work modeling adaptive radiations into this third criterion. In line with empirical results (Schliewen and Klee 2004), mathematical and verbal models indicate that strictly dichotomous phylogenetical signals may be absent in early phases of adaptive radiations due to ongoing gene flow among diverging lineages (i.e., the "hybrid swarm phase") (Seehausen 2004, Gavrilets and Vose 2005). Hence, we suggest rephrasing this criterion as "clearly monophyletic group of closely related lineages" in the case of young species flocks. In that sense, we consider this criterion as fulfilled.

The implausibility of past allopatry is perhaps the most challenging criterion for sympatric speciation. However, roundfins exclusively inhabit a lake with a shape, depth profile, and small size hardly allowing for any geographic barriers acting as constraints on gene flow. Major, separating intralake structures or refugia like satellite lakes are neither present nor plausible (see Fig. 1 for lake bathymetry, and the topographical regional maps Peta Rupabumi Indonesia No. 2213-61, -62, -34 for details) and surrounding streams and lakes are devoid of roundfins (Herder et al. 2006a). Alternatively, separate invasions by nonsister species followed by hybridization after secondary contact could lead to a pattern erroneously assumed to be the result of sympatric speciation. In this case, parts of the genome would be shared between two nonsister species as a consequence of hybridization, mimicking monophyly by masking the allopatric phylogenetic signal. However, application of multilocus nuclear markers, in combination with explicit tests for reticulate phylogenetic signal and a well-supported mtDNA monophyly (Herder et al. 2006b), provides robust arguments against this hypothesis.

Significant spatial differentiation, as evident in *T. antoniae* "large," appears at first glance to contradict the idea of sympatric divergence. However, as differentiation is not increasing with distance among sites, structure detected most likely reflects other signals than allo- or parapatry in terms of isolation by distance. One possible explanation might be low dispersal and shoaling in benthic *T. antoniae* "large," leading to a site-specific signal. Alternatively, subtle differences in habitat composition might reduce gene flow along certain shoreline areas, whereas other stretches are extended but barrier-free. Further studies, incorporating detailed habitat mappings, are required to test this hypothesis. However, intrapopulation structure of *T. antoniae* "large" seems not to be linked to speciation processes, and therefore constitutes no argument against sympatric divergence of roundfins in toto.

The remaining, moderately plausible scenario for allopatric divergence might be that ancestral roundfins were benthic and unable to cross the pelagic zone. This could have resulted in an initial spatial split of allopatric populations that then could have given rise to multiple ecological morphospecies, similar to the recent ones. The present-day situation would imply in this case secondary sympatry of three morphospecies. Apart from not being parsimonious, genome-scan results did not identify a primary "allopatric" phylogenetic signal: a lack of genome-wide differentiation contrasts expectations for allopatric speciation, which would predict accumulation of numerous random mutations throughout the entire genome (Via 2001; Savolainen et al. 2006).

Evidence suggests that only a few loci (possibly with pleitropic effects on other genes) are responsible for characters under divergent natural or sexual selection in cichlids (Kocher 2004), sticklebacks (Raeymaekers et al. 2007), and coregonids (Rogers and Bernatchez 2005). In the context of speciation, theoretical (Mallet 1995; Wu 2001; Gavrilets and Vose 2005), as well as empirical results (Emelianov et al. 2004; Kronforst et al. 2006), indeed predict that both advantageous and neutral alleles can be shared and remain undifferentiated among diverging populations. In contrast, divergence should accumulate at certain, specific loci. The lack of strong genome-wide differentiation in sympatric roundfins accordingly suggests that only small proportions of the genome are correlated with species-specific divergence. This result renders sailfin silversides of Lake Matano a highly promising model group to test for the role of gene flow of neutral genes versus genes for adaptive speciation.

In summary, our data are consistent with a sympatric mode of divergence in the face of gene flow due to ecological selection. Conspicuously, spectacular male color polymorphisms are

not associated with population divergence. In contrast, divergent natural selection and strong assortative mating appear to explain the existence of three ecologically, morphologically, and genetically distinct morphospecies in the deepest of "Wallace's Dream-ponds."

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Appendix 1. Spearman Rank Correlations calculated for roundfin occurrence and associated habitat parameters. Parameters are restricted to those previously identified by pCCA to contribute significantly to variation. Hierarchical analyses are first based on roundfin morphospecies in toto (A), focusing then to courting activity (B), and male coloration in *T. antoniae* "small" (C) and *T. antoniae* "large" (D).

Level of rank correlation	(A) Morphospecies			(B) Courting in *Telmatherina antoniae*				(C) Color in *Telmatherina antoniae* "small"			
	T. antoniae			*T. a.* "small"		*T. a.* "large"		Courting Yellow	Noncourting Yellow	Courting Blue	Noncourting Blue
	"small"	"large"	*T. prongnatha*	Courting	Noncourting	Courting	Noncourting				
Macrohabitat	n.s.	0.135**	0.101*	n.s.	n.s.	0.104*	0.135**	n.s.	n.s.	n.s.	n.s.
Mesohabitat	n.s.	0.132**	0.195**	n.s.	n.s.	0.113*	0.113*	n.s.	−0.118*	n.s.	n.s.
Substrate	−0.125**	0.132**	0.203**	−0.11*	−0.136**	0.132**	0.112*	−0.133**	−0.159**	n.s.	n.s.
Depth	n.s.	n.s.	−0.36**	n.s.	n.s.	n.s.	−0.098*	n.s.	n.s.	n.s.	−0.158**
Slope	−0.114*	0.153**	−0.194*	n.s.	−0.143**	0.141**	0.172**	n.s.	−0.159**	0.096*	−0.165**
Aufwuchs	n.s.	0.205**	n.s.	n.s.	n.s.	0.281**	0.163**	n.s.	−0.111*	n.s.	−0.106*
Wood	−0.1*	n.s.	0.413**	−0.106*	n.s.	n.s.	n.s.	−0.097*	n.s.	n.s.	n.s.
Vegetation	n.s.	0.111*	n.s.	n.s.	n.s.	0.096*	0.099*	n.s.	n.s.	n.s.	n.s.
Canopy cover	−0.109*	n.s.	0.554**	−0.115*	n.s.	n.s.	n.s.	−0.112*	n.s.	n.s.	n.s.
Season	−0.197**	−0.143**	0.103*	−0.214**	−0.102*	−0.162**	−0.1*	−0.218**	−0.099*	−0.142**	n.s.
Daytime/light	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Weather	0.171**	0.17**	n.s.	0.148**	0.147**	0.117*	0.187**	0.132**	0.118*	0.128**	0.138**
Waves	0.188**	n.s.	0.187**	−0.173**	−0.165**	n.s.	n.s.	−0.143**	−0.139**	−0.178**	−0.138**

Level of rank correlation	(D) Color in *Telmatherina antoniae* "large"					
	Courting Yellow	Noncourting Yellow	Courting Blue	Noncourting Blue	Courting Blue-yellow	Noncourting Blue-yellow
Macrohabitat	n.s.	0.161**	n.s.	0.124*	n.s.	n.s.
Mesohabitat	n.s.	n.s.	0.134**	0.107*	n.s.	n.s.
Substrate	n.s.	n.s.	0.139**	0.13**	n.s.	n.s.
Depth	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Slope	0.13**	0.179**	0.138**	0.117*	n.s.	n.s.
Aufwuchs	0.211**	0.195**	0.26**	0.188**	n.s.	n.s.
Wood	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Vegetation	n.s.	n.s.	n.s.	0.132**	0.11*	n.s.
Canopy cover	n.s.	n.s.	n.s.	0.98*	n.s.	n.s.
Season	0.128**	0.138**	0.164**	0.119*	n.s.	n.s.
Daytime/light	n.s.	0.105*	n.s.	n.s.	n.s.	n.s.
Weather	0.107*	0.186**	0.104*	0.139**	n.s.	0.114*
Waves	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

****P** significant at $\alpha = 0.01$, ***P** significant at $\alpha = 0.05$.

Appendix II. Pairwise χ^2-tests: distribution of roundfins according to categories of single habitat parameters identified to contribute to roundfin distribution by pCCA. Analyses focus to (A) roundfin morphospecies, (B) courting activity and (C) male coloration.

Pairwise χ^2-tests	(A) Morphospecies			(B) Courting in *Telmatherina antoniae*				(C) Color in *T.a.* "small"	
	T.a. "small" *vs. T.a.* "large"	*T.a.* "small" *vs. T. prognatha*	*T.a.* "large" *vs. T. prognatha*	single vs. paired "small"	single vs. paired "large"	paired "small" vs. "large"	single "small" vs. "large"	single blue vs. yellow	paired blue vs. yellow
Macrohabitat	**	**	*	**	n.s.	**	**	**	**
Mesohabitat	**	**	**	n.s.	**	**	**	**	**
Substrate	**	**	**	**	n.s.	**	**	**	**
Depth	**	**	**	**	**	**	**	**	**
Slope	**	**	**	**	**	**	**	**	**
Aufwuchs	**	*	**	**	**	**	**	*	**
Wood	**	**	**	**	**	**	**	*	**
Vegetation	**	n.s.	*	**	**	**	**	**	**
Canopy cover	**	**	**	**	**	n.s.	**	*	**
Season	*	**	**	**	**	n.s.	n.s.	n.s.	**
Daytime	**	**	**	**	**	**	**	**	**
Weather	**	n.s.	n.s.	**	n.s.	**	**	n.s.	n.s.

**P significant at $\alpha = 0.001$, *P significant at $\alpha = 0.05$.

Supporting Information

The following supporting information is available for this article:

Appendix S1 Supplementary Material and Methods
Appendix S2 Supplementary Results

Supporting information may be found in the online version of this article.

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