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Introgressive Hybridization between Color Morphs in a Population of Cichlid Fishes Twelve Years after Human-Induced Secondary Admixis

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Abstract

In the extremely species-rich haplochromine cichlid fishes of the East African Great Lakes, prezygotic isolation between closely related species is often maintained by color-assortative mating. In 1998, local fisherman working for the ornamental fish trade released different color morphs of the cichlid genus *Tropheus* into a small harbor basin in the southern part of Lake Tanganyika. This artificial amalgamation of color morphs provides a unique possibility to study mating patterns in cichlids in a natural environment over time. In a precursor study, we analyzed genotypes and phenotypes of almost 500 individuals sampled between 1999 and 2001 and uncovered a marked degree of color-assortative mating, which depended on the level of color pattern dissimilarity between morphs. Twelve years after introduction of nonindigenous morphs, we again sampled *Tropheus* individuals from the harbor basin and an adjacent, originally pure population and analyzed phenotypes (coloration) and genotypes (mitochondrial control region and 9 microsatellite loci) to assess the current status of the admixed population. Principal component analyses of color score data and population assignment tests demonstrate an increasing level of introgressive hybridization between morphs but also some ongoing color-assortative mating within morphs. The observed mating pattern might have been influenced by fluctuating environmental conditions such as periodic algal blooms or increased sedimentation causing turbid conditions in an otherwise clear lake.

Key words: *cichlid species flock,* Tropheus moorii, *faunal translocation, assortative mating, population assignment*

The formation of reproductive isolation constitutes a crucial step in organismal diversification. Reproductive isolation can evolve under a variety of mechanisms, which are broadly classified into prezygotic and postzygotic isolating barriers, both reducing gene flow between species (Coyne and Orr 2004). Several species have been shown to exhibit strong assortative mating preferences in the absence of postzygotic isolation (e.g., McMillan et al. 1997; Seehausen et al. 1997; Jiggins et al. 2004), corroborating the notion that barriers to fertilization and therein premating isolation due to courtship traits and associated preferences are likely to be common causes of reproductive isolation (for a review, see Ritchie 2007).

The extremely species-rich haplochromine cichlid fishes of the East African Great Lakes are one prominent example where prezygotic isolation by direct behavioral mating preferences has been demonstrated to be the main reproductive isolating barrier among closely related species (see, e.g., Salzburger 2009; Seehausen 2009). Although the role and relative importance of visual, olfactory, and acoustic cues used in haplochromine mate choice is still unclear, there is strong evidence for the dominant role of visual cues in a sympatric species pair from Lake Victoria (Maan et al. 2004; Stelkens et al. 2008). Intra- and interspecific variation in male nuptial coloration and corresponding female preferences are widespread in haplochromine cichlids (Seehausen 2000). The most impressive example for intraspecific color pattern variation is the genus *Tropheus* from Lake Tanganyika, with currently over 100 described color morphs distributed mostly allopatrically in the shallow, rocky habitat of the lake (Schupke 2003). Sexual selection was proposed to have contributed to the evolution of the numerous color morphs, although *Tropheus* lacks some of the features that are generally associated with sexual selection such as sexual dimorphism and polygamy (Egger et al. 2006). Phylogeographic studies

revealed a rather complex evolutionary history of the genus: recurrent major and minor lake level fluctuations were the likely cause of population displacement, secondary contact, and introgression between differentiated morphs (Baric et al. 2003; Sturmbauer et al. 2005; Egger et al. 2007). Under such a scenario, the level of introgression between morphs is probably influenced by the degree of reproductive isolation during phases of secondary contact because the presence and absence of assortative mating preferences underlie reproductive isolation and random mixing, respectively (Bateson 1983). Disassortative preferences, on the other hand, may even accelerate the fusion of gene pools (Rosenfield and Kodric-Brown 2003).

In southern Lake Tanganyika, an artificial amalgamation of several differently colored *Tropheus* morphs created a situation that is similar to secondary contact among allopatric populations after a lake level drop. The admixture event dates back to 1998, when local fishermen collected about 300 adult *Tropheus* from several sites in the southern part of the lake (the exact locations are not known) in order to export the fishes for the aquarium trade. The fishermen were refused export permits, however. But instead of returning the fishes to their original habitats, as instructed by the local authorities, the catch was released in a small harbor basin of approximately 200 m^2 in size in front of the Fisheries Department in Mpulungu, Zambia. In our precursor study (Salzburger et al. 2006), we collected samples of the admixed population in 3 consecutive years following the release of nonindigenous morphs (1999–2001) and, using both molecular and morphological techniques, examined the phenotypic and genetic structure of the population in order to assess mating patterns between morphs. Principal component analysis (PCA) based on color score data unraveled 5 distinct phenotype classes, namely the indigenous morph ("light olive") and the nonindigenous morphs "dark olive" (translocated from the Zambian east coast), "red," "red striped," and "orange" (all 3 translocated from the shoreline northwest of the Lufubu estuary; see Figure 1). Paternity analysis and a population assignment test of juveniles born after the admixis event revealed a high degree of color-assortative mating, with approximately 70% of the offspring being derived from within-color morph matings. Moreover, reproductive isolation was the strongest between the most distinct morphs (olive and reddish morphs), which also represent different mitochondrial haplotype lineages (Sturmbauer et al. 2005), whereas introgression between phenotypically and genetically more similar morphs, that is, between light and dark olive or within the reddish morphs occurred more frequently. In line with this, laboratory female mate choice experiments using several *Tropheus* morphs revealed that the level of reproductive isolation increased with increasing color pattern dissimilarity of morphs (Egger et al. 2008, 2010).

More than a decade after the translocation of nonindigenous morphs, we again sampled *Tropheus* individuals from the harbor basin to assess the current status of the admixed population and to obtain a more long-term perspective on secondary admixis in *Tropheus*. Analysis of phenotypic and genotypic data was used to uncover if color-assortative mating was maintained or broke down over time.

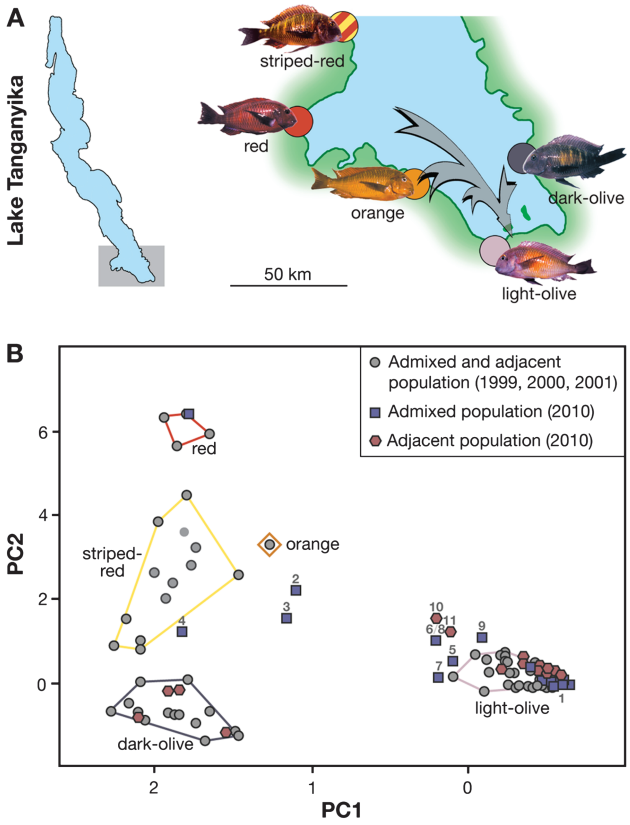

Figure 1. Morphological classification of the studied specimens of *Tropheus moorii* in the small harbor bay in Mpulungu. (**A**) Map of the southern part of Lake Tanganyika, East Africa, illustrating the human-induced secondary admixis of several nonindigenous color morphs of *T. moorii*. (**B**) PCA based on 12 landmarks related to coloration (see Salzburger et al. 2006). Several individuals (1–11; see also Figure 3) fall outside the main morphological clusters light olive, dark olive, orange, striped red, and red or show a discrepancy between genotype and phenotype and are thus likely to represent hybrids.

Materials and Methods

Sampling was carried out in March 2010 in the small harbor bay in front of the Fisheries Department, Mpulungu, Zambia. Additional samples were collected from an adjacent population ("St Georges"; ~50 m west of the admixed population), which was already used as an originally undisturbed adjacent population by Salzburger et al. (2006). Fish were collected by local divers using gill nets. Each specimen was measured, weighted, and photographed in a standardized way. Finally, a fin clip was taken and stored in ethanol for later DNA extraction before fishes were released back into their habitat.

In order to be able to compare the results between the different sampling years, we used the exactly same color score as Salzburger et al. (2006) to quantify phenotypic differences between individuals/morphs. Only adult individuals (larger

than 60 mm in total length) were included in this analysis as juveniles often have distinct color patterns. Twelve features related to coloration were used for the color score: overall body color (red/light olive/dark olive/orange), central body color (red/yellow/dark/orange), color of eyelid (red/light/dark/yellow), eye ring (blue/dark/light), operculum (red/dark/light/blue), operculum edge (red/light/dark/yellow/orange/blue), dorsal fin (dark red/light/dark/light blue/light red), base of dorsal fin (dark red/light/dark/orange/blue/light red), base of anal fin (dark red/light/dark/orange/blue/light red), base of pectoral fin (dark red/light/dark/orange/blue/light red), stripe/dot pattern (uniform/stripe/dot), and dorsal fin pattern (uniform/striped). The color score data available from adult individuals collected from 1999 to 2001 were, together with the new samples from 2010, translated into a binary data matrix and subjected to a PCA with R (v. 2.8.1, R Development Core Team 2008).

Total DNA was extracted from fin clips preserved in ethanol applying a proteinase K digestion followed by sodium chloride extraction and ethanol precipitation (Bruford et al. 1998). The 106 individuals sampled in 2010 (44 individuals from the admixed and 62 from the adjacent population) were genotyped at 9 microsatellite loci: Ppun5, Ppun7, Ppun21 (Taylor et al. 2002), UNH130 (Lee and Kocher 1996), Pzeb3 (van Oppen et al. 1997), HchiST06, HchiST38, HchiST68, and HchiST94 (Maeda et al. 2009). Because Salzburger et al. (2006) had only analyzed 5 microsatellite markers, we also re-genotyped individuals sampled in 2001 plus a set of genetically and morphologically distinct individuals for the STRUCTURE analysis (see below). Sample sizes differed in the PCA on color traits and in the microsatellite analyses and were as follows: PCA analysis (admixed/adjacent): 1999, $N = 78/23$; 2000, $N = 106/23$; 2001, $N = 66/32$; 2010, $N = 38/57$; microsatellite analysis (admixed/adjacent): 2001, $N = 73/39$; 2010, $N = 44/62$; reference: 1999, $N = 35$, 2000, $N = 9$, 2001, $N = 24$.

Fragment size calling was carried out on an ABI 3130*xl* genetic analyzer (Applied Biosystems) in comparison to the LIZ 500(−250) (Applied Biosystems) internal size standard. Genotypes were determined manually using Peak Scanner (v. 1.0; Applied Biosystems). As in Salzburger et al. (2006), we also determined the DNA sequence of a 363-bp segment of the mitochondrial control region for the samples from 2010 using published primers (Kocher et al. 1989; Salzburger et al. 2002). The PCR fragments of the control region were purified using ExoSAP-IT (USB), directly sequenced with the BigDye sequencing chemistry (Applied Biosystems), and analyzed on an ABI 3130*xl* genetic analyzer (Applied Biosystems). The DNA sequences are available at GenBank under the accession numbers JQ736031-JQ736134.

Mitochondrial DNA sequences were aligned using CODONCODE ALIGNER (version 3.5; CodonCode Corporation) and combined with the sequences of Salzburger et al. (2006) resulting in a total of 561 sequences from 4 sampling years (1999 [$N = 113$], 2000 [$N = 194$], 2001 [$N = 150$], and 2010 [$N = 104$]). These sequences were collapsed into haplotypes using the software COLLAPSE (v. 1.2, Posada 2006). Based on the resulting 59 haplotypes, a maximum likelihood analysis was carried out in PAUP*4.0b10 (Swofford 2002) to construct an unrooted mitochondrial haplotype genealogy according to the strategy described in Salzburger et al. (2011).

Microsatellite scoring data were rounded to valid integers using the software TANDEM (Matschiner and Salzburger 2009). A population assignment test was carried out with Structure 2.1 (Pritchard et al. 2000). As reference for "pure" individuals, we included samples from the years 1999–2001 that grouped in one of the genotype classes A, B, C, or D based on the population assignment test and to the corresponding phenotype classes light olive, dark olive, and red/striped red based on the PCA in Salzburger et al. (2006). These samples were then used to test the genetic assignment of individuals collected in 2001 and 2010. We ran Markov chain Monte Carlo simulations with 500 000 replications (burn in = 50 000; admixture model with prior population information; correlated allele frequencies) for K (number of genetic clusters) = 4 (based on the samples light-olive, dark-olive, red/striped-red, and the light-olive morphs from the adjacent population). The simulations were also run with $K = 2$ (based on the samples red/striped-red and all dark-olive and light-olive individuals) and $K = 3$ (one time based on the samples light olive, dark olive, red/striped-red, without the light-olive morphs from the adjacent population and one time based on the samples light olive, including the light-olive individuals from the adjacent population, dark olive, and red/striped red) to check for stability of the genotypic assignment.

Results

In the PCA, specimens from the admixed population from sampling years 1999–2001 were, just as in our precursor study, split into 5 distinct phenotype groups (light olive, dark olive, orange, red, and striped red, see Figure 1B). All individuals from the adjacent population sampled between 1999 and 2001 were placed in the light-olive phenotype group (see Salzburger et al. 2006). The majority of specimens collected from the admixed population in 2010 also grouped within these discrete phenotype groups. Several individuals of the 2010 sampling were placed outside these clusters, though (Figure 1B). The same was the case for specimens sampled from the adjacent population in 2010 (see Figure 1B). Importantly, most "outliers" displayed color patterns intermediate between red and olive phenotypes, suggesting a hybrid origin. The changes in morph frequency over the sampling years in both the admixed and the adjacent population are shown in Figure 2. Intermediate phenotypes were not detected in 1999 but showed up in low numbers in 2000 (0.9%) and increased dramatically in abundance (from 1.5% to 19%) between 2001 and 2010. In the adjacent population, no intermediate phenotypes were identified from 1999 to 2001 but were present in 2010 with a frequency of 3.5%.

Sequencing of the mitochondrial control region revealed the presence of 59 haplotypes. Of these, 34 were found exclusively in specimens collected between 1999 and 2001, 16 were shared between individuals collected in 2010 and in

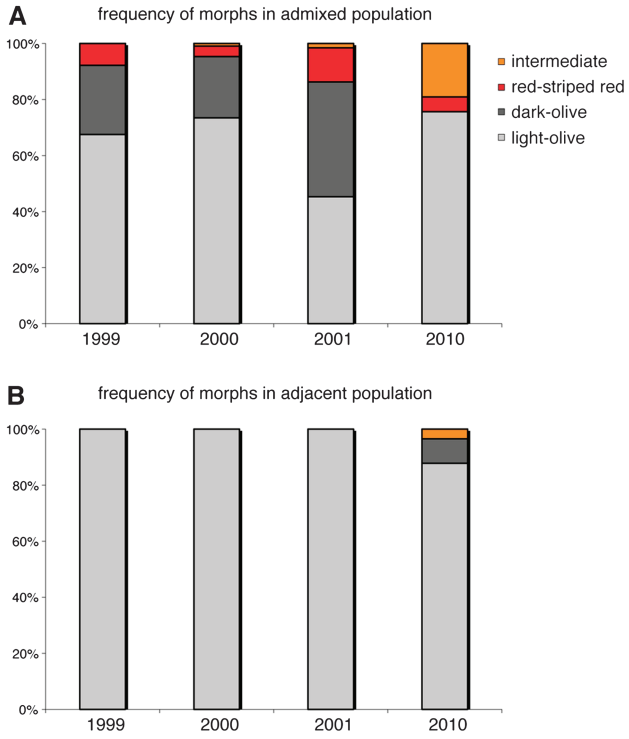

Figure 2. Bar plots representing the frequency of morphs (based on the PCA) and intermediate phenotypes in the admixed (**A**) and in the adjacent (**B**) population for the sampling years 1999, 2000, 2001, and 2010.

the earlier years and 9 haplotypes were exclusively found in specimen sampled in 2010. Six out of these haplotypes were singletons, that is, they were found in a single specimen only. In the haplotype genealogy based on a maximum likelihood analysis (data not shown) all red, red-striped, and orange specimens from 1999 were placed in one and all light- and dark-olive individuals from 1999 in the other clade. As of 2000, introgression occurred in both clades (year 2000: one light-olive and one dark-olive specimen grouped in the "red clade," one striped-red individual grouped in the "olive clade"; year 2001: one light-olive specimen grouped in the red clade, one striped-red individual grouped in the olive clade; year 2010: one light-olive and two phenotypically intermediate individual grouped in the red clade, seven phenotypically intermediate individuals grouped in the olive clade).

The microsatellite-based population assignment test (Pritchard et al. 2000) for individuals from the years 2001 and 2010 is shown in Figure 3 and Supplementary Figure 1. For both the admixed and the adjacent population sampled in 2001 and in 2010, only few individuals could be assigned to a particular phenotype group and to the "corresponding" genotype class with a probability P_a of more than 0.75. This clearly indicates a high frequency of hybridization between morphs. The genetic assignment of individuals was consistent between iterations of structure runs and between runs using different values for K ($K = 2$ and $K = 3$, data not shown). Moreover, the correct assignment of the red and striped-red morphotypes to the red/striped-red genotype class demonstrates the power of the assignment test (see Figure 3 and Supplementary Figure 1).

Discussion

Our morphological and genetic analyses of an admixed population of *Tropheus moorii* 12 years after the translocation of nonindigenous morphs not only provide evidence for extensive hybridization but also for ongoing color-assortative mating between different *Tropheus* morphs, both in the admixed and in the adjacent population. The PCA uncovered several phenotypically distinct individuals in the 2010 sample, which displayed color patterns intermediate between the red and olive morphs (Figure 1). Based on photographs of fish sampled in 2010, it seems that many more individuals (at least 11) displayed phenotypes deviating from the originally released color morphs, that is, they did not resemble naturally occurring color morphs of *T. moorii*. However, our relatively conservative color scoring, which is restricted to certain body areas only, cannot discriminate these obvious hybrids from the naturally occurring morphs. Intermediate phenotypes were not detected in the population samples from 1999 to 2001 (Salzburger et al. 2006; Figure 2), although a reevaluation of the mitochondrial DNA data suggests rare hybridization events between red and olive morphs already soon after admixis. Moreover, the new population assignment test based on microsatellite data showed extensive hybridization already in 2001 with only very few individuals being assigned to both a particular genotype class and the corresponding phenotype group (Supplementary Figure 1). Thus, our new and more refined analyses contradict our precursor study (Salzburger et al. 2006), in that hybridization among morphs happened more frequently than previously concluded. Note, however, that our new analysis is based on more microsatellite markers and a more robust and extensive set of reference specimens for the pure color morphs, which increased the sensitivity.

Importantly, we again identified one genetically and phenotypically pure red individual in the 2010 sample of the admixed population, confirming ongoing color-assortative mating within the red morph. At the same time, our new data show that hybridization, also between distinct allopatric color morphs, increased over time in the admixed and in the adjacent population. This means that the adjacent population, which served as reference for indigenous fish, by now is strongly affected by the dispersal of nonindigenous morphs and hybrids (Figure 3).

Changes in the frequency of morphs in the admixed and in the adjacent population can influence the rate of hybridization. Backcrossing of hybrids into one of the parental morphs provides a route for gene flow between morphs, such that the rate of homogenization between morphs will increase steadily even if F1 hybrids are produced at very low rates. The reduction in the frequency of certain morphs can have diverse consequences, either a reduction of hybridization rates because of reduced

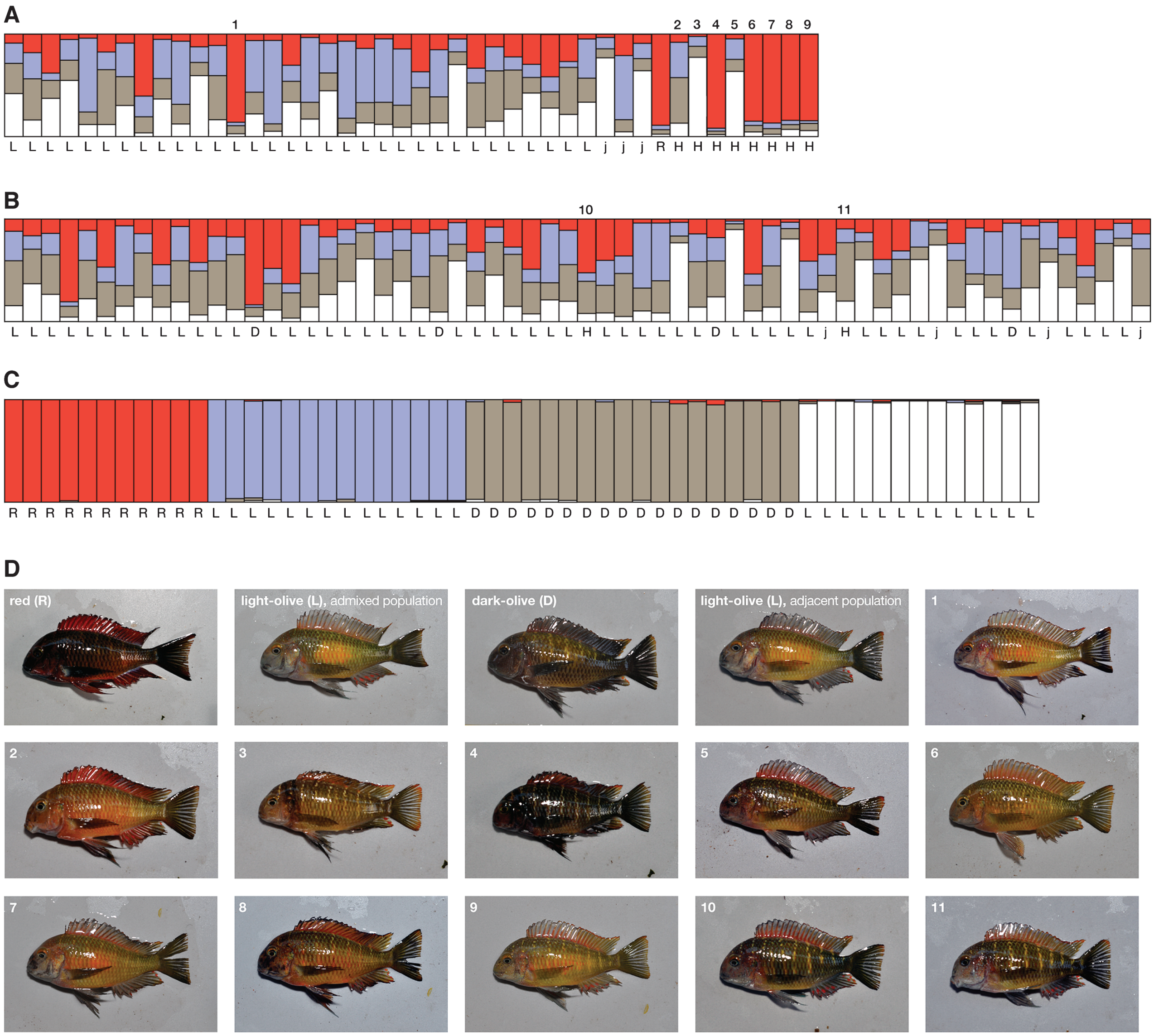

Figure 3. Results of the population assignment test based on 9 microsatellite markers. (**A–C**) Structure plot showing the individual assignment of specimens collected in 2010 in the admixed (**A**) and the adjacent population (**B**) with respect to a reference set of pure individuals (**C**) from different sampling years. The color coding refers to the genotype classes identified by Salzburger et al. (2006). (**D**) Photographs of pure specimens and 11 putative hybrid specimens. The letters indicate the phenotypic assignment to a specific phenotype class: L, light olive; D, dark olive; R, red; H, hybrid; j, juvenile.

encounters with rare morphs or an increase in hybridization rates resulting from the limited availability of homotypic partners for the rare morph.

Reproductive isolation in *Tropheus* appears to correlate with the level of color pattern dissimilarity between morphs with the red morph being the most distinct among the studied morphs (Salzburger et al. 2006; Egger et al. 2008, 2010). In previous mate choice experiments, females of some morphs discriminated against males of distinct morphs, whereas no assortative preferences were detected among similar morphs (Egger et al. 2010). Importantly, females of the resident morph did not prefer their own morph over a distinct alternative choice (Egger et al. 2010; Sefc KM, Hermann CM, Steinwender B, unpublished data), which might also have facilitated hybridization between resident and introduced morphs in the admixed harbor population.

Although little is known about the relative importance of different mate choice cues in *Tropheus*, intraspecific communication is likely mediated by visual signals (at least in parts; see Wickler 1969; Nelissen 1976; Sturmbauer and Dallinger 1995). Such signals can be influenced by the physical properties of the ambient light spectra and the degree of attenuation, absorption, and scattering of the transmission medium (Lythgoe 1979; Reimchen 1989). Environmental changes altering water clarity, such as

eutrophication or sedimentation, can thus have profound effects on fish communication, sexual selection, and mating systems (Seehausen et al. 1997; Järvenpää and Lindström 2004). Excess sedimentation caused by deforestation leading to reduced light penetration has also been reported from several inshore sites of Lake Tanganyika (particularly in the North). There, high sediment loads are often correlated with low fish diversity (Cohen et al. 1993; Alin et al. 1999). Temporarily turbid water conditions may also occur naturally and on the basis of seasonal climatic cycles leading to, for example, increased sediment inflow caused by rainfall or periodic algal blooms caused by upwelling of nutrient-rich water (Plisnier et al. 1999; Langenberg et al. 2002; Bergamino et al. 2007). Such periods of higher turbidity might also lead to a temporary breakdown of reproductive barriers between *Tropheus* color morphs. It is unclear, however, whether excess sedimentation or eutrophication-induced production could have affected our study populations by increasing the spontaneous level of hybridization. Compared to the northern part of Lake Tanganyika, sites at the southern tip of the Zambian shoreline are actually regarded as low disturbance sites (Cohen et al. 1993), although (to our knowledge) no detailed data are available on eutrophication levels in the harbor basin in Mpulungu from the last 12 years. At the time of our sampling, the water in Mpulungu harbor was clear (secchi disc measurement revealed visibility to the maximum depth of the harbor basin of 2.70 m).

Concerning the evolutionary relevance, the human-mediated amalgamation of distinctly colored *Tropheus* morphs resembles the natural situation during major low stands of the water level in Lake Tanganyika, leading to the admixis of formerly isolated populations (Sturmbauer 1998; Kornfield and Smith 2000; Sturmbauer et al. 2001). Clearly, if reproductive barriers (e.g., through assortative mating) are strong enough, secondarily admixed populations will show no (or very low levels) of gene flow (i.e., hybridization). This seems to be the case between the red morph and the remaining morphs, as a more or less stable assemblage of genetically and morphologically pure red specimens persisted until more than 10 years in the Mpulungu harbor basin (albeit at low frequency since the very beginning of this "experiment"). Based on the observation that assortative mating is strongest between the red morphs, which are also genetically the most distinct, and the remaining types, we had previously suggested that reproductive isolation in *Tropheus* is correlated with the time since divergence (in allopatry) (Salzburger et al. 2006). This was corroborated by more recent work demonstrating that, in some areas of Lake Tanganyika, distinct color morphs of *Tropheus* can coexist without introgression—although, in these cases, the morphs are genetically more distinct than the ones in the admixed harbor population (Egger et al. 2007; Herler et al. 2010).

On the other hand, if populations that come into secondary contact are not yet completely reproductively isolated, (introgressive) hybridization will lead to the fusion of morphs. This way, a lake level drop may result in "speciation reversal" (sensu Seehausen et al. 2008) in the admixture zones. In the Mpulungu harbor, it appears that only the red morphs persisted, whereas the other morphs are—at least genetically—largely admixed by now (Figure 3). Apparently, there are also many more intermediate phenotypes in the admixed population in 2010, although a more detailed phenotypic analysis, which probably would reveal intermediate types other than the extreme cases depicted in Figure 3, is hampered by the much lower quality of the nondigital photographs from 1999 to 2001. Besides eliciting reinforcement of reproductive isolation and the fusion of populations/species (introgressive), hybridization can influence evolution by producing new "transgressive" morphs (see Rieseberg et al. 1999). In *Tropheus*, a tree-based method for identifying hybrid taxa (Egger et al. 2007) already indicated that distinct morphs interbreed upon secondary contact and that some new morphs originated from hybridization between existing morphs.

The role of hybridization as a mechanism promoting diversification and speciation in the animal kingdom has been supported by empirical studies in recent years (see Seehausen 2004). Reproductive isolation in cichlid fish species flocks is mostly due to prezygotic isolation by direct behavioral mating preferences and hybrids are often viable and fertile (Stelkens, Young, et al. 2009). Thus, the diversity of complex species assemblages might have at least in part originated via hybridization of ancestral lineages (Salzburger et al. 2002; Joyce et al. 2011), and it has been shown that phenotypic novelty can be produced by transgressive segregation in cichlids (Seehausen 2004; Stelkens, Schmid, et al. 2009). Just as in other cichlid lineages, the evolutionary history of the genus *Tropheus* appears to have been greatly affected by environmental changes such as lake level fluctuations, enabling secondary contact between previously allopatric morphs, and possibly natural eutrophication or sedimentation. Our study shows that despite strong behavioral mating preferences, introgressive hybridization between *Tropheus* morphs can be extensive and might have contributed to the evolution of the numerous color morphs.

Supplementary Material

Supplementary Figure 1 can be found at http://www.jhered.oxfordjournals.org/.

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