

Premating, Not Postmating, Barriers Drive Genetic Dynamics in Experimental Hybrid Populations of the Endangered Sonoran Topminnow

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ABSTRACT

The timing and pattern of reproductive barrier formation in allopatric populations has received much less attention than the accumulation of reproductive barriers in sympatry. The theory of allopatric speciation suggests that reproductive barriers evolve simply as by-products of overall genetic divergence. However, observations of enhanced premating barriers in allopatric populations suggest that sexual selection driven by intraspecific competition for mates may enhance species-specific signals and accelerate the speciation process. In a previous series of laboratory trials, we examined the strength of premating and postmating barriers in an allopatric species pair of the endangered Sonoran topminnow, *Poeciliopsis occidentalis* and *P. sonoriensis*. Behavioral observations provided evidence of asymmetrical assortative mating, while reduced brood sizes and male-biased F₁ sex ratios suggest postmating incompatibilities. Here we examine the combined effects of premating and postmating barriers on the genetic makeup of mixed populations, using cytonuclear genotype frequencies of first- and second-generation offspring. Observed genotype frequencies strongly reflect the directional assortative mating observed in behavioral trials, illustrating how isolating barriers that act earlier in the reproductive cycle will have a greater effect on total reproductive isolation and may be more important to speciation than subsequent postmating reproductive barriers.

THE relative contributions of premating and postmating reproductive isolation are fundamental to understanding speciation and are primary forces responsible for generating and maintaining biodiversity (MENDELSON 2003; COYNE and ORR 2004). Theory predicts that premating barriers will be “reinforced” in species pairs that come into secondary contact as a result of selection against the production of less-fit hybrid offspring (DOBZHANSKY 1951). This theory has received empirical support in the form of enhanced premating barriers in sympatric populations compared with postmating barriers (WASSERMAN and KOEPFER 1977; WAAGE 1979; OTTE 1989; RUNDLE and SCHLUTER 1998). Studies of reproductive barriers in taxa that have remained geographically isolated throughout their respective ranges are more limited and have produced mixed results. The most comprehensive analysis comes from the comparative examination of premating and postmating barriers in 171 species pairs of *Drosophila* by COYNE and ORR (1989, 1997). Their results showed no difference in the genetic distances associated with the premating behavioral and intrinsic postmating barriers in allopatric species pairs. However, using a similar experimental design, MENDELSON (2003) found that premating behavioral barriers evolved earlier than

postmating barriers in species pairs of the freshwater fish genus *Etheostoma* and claimed that sexual selection had promoted the rapid evolution of premating barriers in the absence of reinforcement (WEST-EBERHARD 1983).

In a series of laboratory trials, we examined premating behavioral barriers (HURT *et al.* 2004) and postmating reproductive barriers (HURT and HEDRICK 2003) in two allopatric species of the endangered Sonoran topminnow, *Poeciliopsis occidentalis* and *P. sonoriensis*, to assess their contributions to overall reproductive isolation. *P. occidentalis* was once the most common fish in the Gila river drainage in Arizona (Figure 1), but natural populations are now limited to four isolated watersheds. *P. sonoriensis* is found in the upper Yaqui river drainage in southeast Arizona and throughout the Yaqui drainage in Sonora, Mexico. Results from no-choice mating observations showed that the duration and sequence of mating behaviors differ in the two species, and directional assortative mating was detected in multiple-choice trials, where *P. sonoriensis* males attempted more copulations toward heterospecific females than observed in the reciprocal interspecific cross.

Laboratory crosses also provided evidence of intrinsic postmating reproductive barriers. Sex ratios from one direction of interspecific cross (*P. sonoriensis* female × *P. occidentalis* male) were highly male biased, averaging 8.3% female offspring, consistent with genic incompatibilities involving cytoplasmic organelles or maternal

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FIGURE 1.—U.S. distribution of *Poeciliopsis occidentalis* and *P. sonoriensis*. Map shows historical distribution of *P. occidentalis* (hatched gray area) and *P. sonoriensis* (solid gray area) in the United States. Source populations used in mating trials are shown as a circle and a square for *P. occidentalis* and *P. sonoriensis*, respectively.

effects (COYNE and ORR 2004). Second, brood sizes from backcrosses to F_1 females were significantly smaller than brood sizes produced from intraspecific crosses. However, postmating reproductive barriers were far from complete as all categories of F_1 and backcrosses produced offspring and crosses between *P. occidentalis* males and *P. sonoriensis* females had a larger average brood size than that of either intraspecific cross.

The purpose of this study was to examine the combined effects of premating and postmating reproductive barriers on the genetic makeup of mixed *P. occidentalis*/*P. sonoriensis* populations. Twenty-two replicate laboratory populations were established, each initially consisting of equal numbers of males and females of the two species. The parental types of first- and second-generation offspring were determined by genotyping diagnostic nuclear and mitochondrial loci. Cytonuclear genotype frequencies were compared to expectations under random mating as well as to expectations based on behavioral observations and fecundity of interspecific crosses in laboratory trials. Results were used to assess the relative contributions of premating and postmating barriers to reproductive isolation and to predict the long-term genetic effect of population admixture.

MATERIALS AND METHODS

Mating trials: Captive stocks of *P. occidentalis* and *P. sonoriensis* were initiated from 20 gravid females in 1994 and 1998, respectively. Since that time, these stocks have been held at the Animal Resource Center (ARC) at Arizona State University in 1500-liter raceways averaging 500–1000 individuals per stock. Fish were maintained at constant temperature (26°) and under a 14 hr:10 hr light:dark photoperiod. Fish were fed commercial flake food one time daily at ~13:00 hr and were given weekly supplements of brine shrimp. Juvenile *P. occidentalis* and *P. sonoriensis* were randomly selected from raceway populations and raised in separate 40-liter aquaria until they reach sexual maturity, which occurs at ~4 months of age (SCHOENHERR 1974). Gonopodial formation, consisting of modification of anterior anal fin rays 3–5, signals the initiation of male sexual development (CONSTANTZ 1989). Males, upon

identification by differential elongation of the anal fin, were moved to separate aquaria to ensure that all fish were virgins upon initiation of breeding trials.

From January to March, 2003, 22 replicate populations were established in 40-liter aquaria. Each replicated population contained three adult virgin males and females of both *P. occidentalis* and *P. sonoriensis*, for a total of 12 initial fish per aquarium. We monitored regularly for the production of offspring and began sampling offspring as soon as >10 fry were observed in an aquarium. Between 7 and 8 fry were randomly selected from each population at three different times separated by 4-week intervals, to maximize the probability that offspring were sampled from more than one brood.

The initial 12 parental fish were removed from all aquaria 4 weeks after the third samples were obtained to avoid potentially complicating factors such as mate preference based on size or sexual maturity. First-generation offspring remained in the established aquaria until the production of second-generation progeny. Parents were removed when F_1 's were <12 weeks of age to prevent progeny from mating with parents. Second-generation progeny were first observed in March, 2004 and all offspring produced until August 2004 were collected.

Laboratory techniques: First-generation offspring were genotyped for a nuclear major histocompatibility complex (MHC) class II gene (HEDRICK and PARKER 1998) and for the mitochondrial gene NaDH (HEDRICK *et al.* 2005), using analysis of single-strand conformation polymorphism (SSCP) as described by GLAVAC and DEAN (1993). SSCP is a highly efficient and sensitive technique frequently used for rapid genotyping. Second-generation progeny were genotyped for the above MHC gene, the mitochondrial gene NaDH, and for two diagnostic microsatellite markers, LL53 and C15, described by PARKER *et al.* (1998, 1999). Previous molecular analyses have shown that *P. sonoriensis* and *P. occidentalis* possess nonoverlapping sets of alleles at all four loci and there is no evidence of linkage between nuclear genes (PARKER *et al.* 1998, 1999; HEDRICK *et al.* 2001). Genotyping of these markers was diagnostic in differentiating heterospecific genotypes from homospecific genotypes and in identification of the mtDNA type of the maternal parent.

Statistical analysis: A replicated goodness-of-fit test (G -test) was used to test for heterogeneity of cytonuclear genotype frequencies among replicated populations and to detect significant deviations from expected values under random mating (SOKAL and ROHLF 1981). A χ^2 -test was used to test for deviations from expectations in specific genotype categories and identify populations whose genotype frequencies differed from the overall mean (SOKAL and ROHLF 1981). Second-generation genotypes were placed in 20 different categories, depending on the number of o/o , o/s , and s/s nuclear genotypes, where s and o indicate *P. sonoriensis* and *P. occidentalis* and the mtDNA type is (o) or (s).

Statistical associations between nuclear and mitochondrial markers were assessed using the gametic cytonuclear disequilibrium statistic (D), which measures the association between nuclear alleles and mitochondrial haplotypes, and three genotypic cytonuclear disequilibria statistics (D_1 , D_2 , D_3), which measure association between nuclear genotypes and mitochondrial haplotypes, as defined by ASMUSSEN *et al.* (1997):

$$D = -D_1 + D_2$$

$$D_1 = u_1 - ux$$

$$D_2 = v_1 - vx$$

$$D_3 = w_1 - wx.$$

Nuclear (mitochondrial) cytonuclear genotypes $o/o(o)$, $o/s(o)$, and $s/s(o)$ are indicated by u_1 , v_1 , and w_1 , respectively; u , v , and w indicate frequencies of nuclear genotypes o/o , o/s , and s/s , respectively; and x indicates the frequency of *P. occidentalis* mitochondrial haplotype (o).

These measures have been used to detect the directionality of matings in both natural and experimental hybrid zones (ARNOLD *et al.* 1988; SCRIBNER and AVISE 1994; AVISE 2001; SCRIBNER *et al.* 2001). Under random mating, the absolute values of all four disequilibrium statistics are expected to decay monotonically to zero by one-half each generation. This rate of decay is reduced under positive assortative mating. Expected disequilibrium values are based on random mating and second-generation expectations account for observed genotype frequencies in first-generation progeny.

Recursion equations: Recursion equations for cytonuclear genotype frequencies, modified from ARNOLD *et al.* (1988), were used to compare the observed cytonuclear genotype frequencies of first- and second-generation offspring with expectations based on the estimated strength of mate preferences observed in behavioral trials (HURT *et al.* 2004) and differential reproductive success found in experimental crosses (HURT and HEDRICK 2003). These equations were then used to model the expected change in cytonuclear genotype frequencies in a mixed population over time. Three different models were generated: the premating behavioral barrier model, the postmating barrier model, and the combined premating and postmating barrier models. Probabilities of positive assortative mating for *P. occidentalis* females and *P. sonoriensis* females are represented by α and β , respectively, so that $(1 - \alpha)$ and $(1 - \beta)$ are the probabilities of random matings. It is assumed that all hybrids mate at random. Reproductive fitness coefficients were calculated as the standardized product of the proportion of crosses that produced offspring and the average brood size of that cross category. Laboratory trials included both within-species crosses, both reciprocal between-species crosses (F_1 crosses), and the four possible crosses between F_1 's and the two pure species (backcrosses). Selection coefficients for all other possible hybrid crosses were estimated as the average observed coefficient for the parents of that cross.

For example, the expected frequency of genotype $o/o(o)$ in the next generation is u'_1 . This expectation is a function of u_1 and v_1 female frequencies and u and v male frequencies in the parental generation, the reproductive success of all cross categories that produce $o/o(o)$ offspring, and the strength of assortative mating of $o/o(o)$ females with o/o males (α), weighted by the average reproductive fitness of the population and is given by

$$u'_1 = \frac{S_1(\alpha u_1 + (1 - \alpha)u_1 u) + (1/2)S_2(1 - \alpha)u_1 v + (1/2)S_7 v_1 u + (1/4)S_8 v_1 v}{\bar{S}}$$

Here S_1 , S_2 , S_7 , and S_8 indicate the reproductive success of u_1 female \times u male, u_1 female \times v male, v_1 female \times u male, and v_1 female \times v male crosses, respectively, and \bar{S} is the average population reproductive fitness, where fractions account for Mendelian segregation of gametes (see supplementary Appendix at <http://www.genetics.org/supplemental/> for all six recursion equations). For the premating barrier model, all S_i values are equal to one, and for the postmating barrier model α and β are equal to zero.

RESULTS

First generation: Offspring were produced in all of the 22 replicated populations and an average of $19.5 \pm$

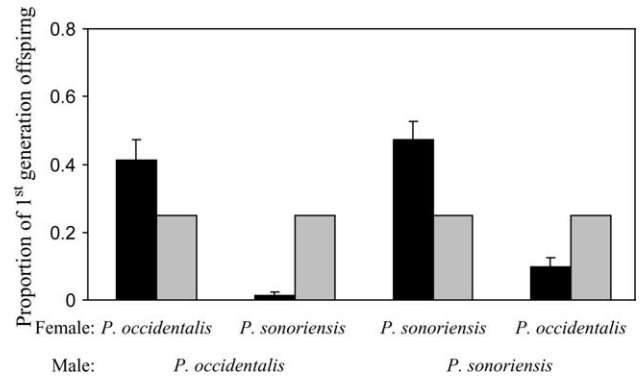


FIGURE 2.—Proportion of first-generation offspring from four cross types. Observed (solid bars) and expected (shaded bars) proportions of first-generation offspring from the four possible cross types are shown. Error bars indicate one standard error of the mean.

0.52 individuals were destructively sampled from each, totaling 430 individuals. Resulting cytonuclear genotype frequencies are shown in Figure 2. The G -test for heterogeneity of genotype frequencies among replications was significant ($G_H = 187$, $P < 0.001$). Four of the replicated populations differed significantly from the mean frequency in a χ^2 -test corrected for multiple tests using the sequential Bonferroni method. Three of these populations were fixed for one parental type, one for $o/o(o)$ and two $s/s(s)$, and the fourth replicate showed an excess of F_1 types $s/o(o)$, where o/o , o/s , and s/s indicate *P. occidentalis*, *P. sonoriensis*, and F_1 nuclear genotypes, respectively, and (o) and (s) indicate *P. occidentalis* and *P. sonoriensis* mtDNA types.

Pooled genotype frequencies were significantly different from expectations under random mating ($G_P = 286$, $P < 0.001$). This deviation is primarily due to a deficiency of hybrid offspring; that is, only 11.3% of the combined offspring genotypes were hybrids compared to the expected under random mating. Of the two hybrid categories, 87.2% were from *P. occidentalis* female \times *P. sonoriensis* male matings, significantly outnumbering offspring from the reciprocal interspecific cross ($\chi^2 = 27.94$, $P < 0.001$). Offspring from *P. sonoriensis* female \times *P. sonoriensis* male crosses made up 75.4% of the pure species progeny, significantly outnumbering offspring from *P. occidentalis* female \times *P. occidentalis* male crosses ($\chi^2 = 4.85$, $P = 0.028$).

Second generation: An average of 23.39 ± 2.79 individuals remained in each replicated population after the removal of parental individuals. Of the 22 original populations, 18 produced second-generation offspring during the sampling period. An average of 16.44 ± 9.99 progeny were sampled from each replicate, for a total of 296 genotyped individuals. Heterogeneity among replicates was not significant.

Again, the observed pooled genotype frequencies differed significantly from expectations based on random mating ($G_P = 64.58$, $P < 0.001$) (Figure 3).

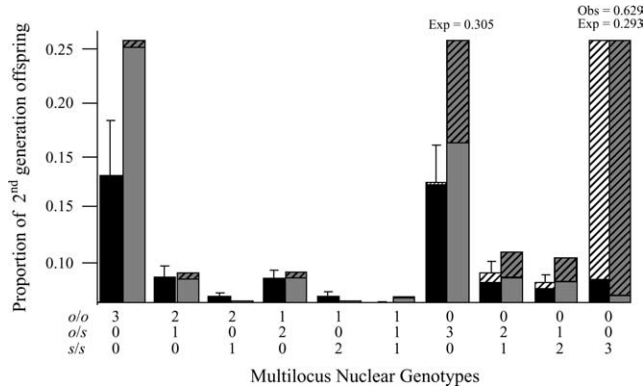


FIGURE 3.—Observed second-generation multilocus genotype frequencies (solid bars) and expected cross frequencies (shaded bars). Multilocus nuclear genotype categories are designated under each bar, where numbers indicate the number of loci of each single nuclear genotype (i.e., o/o, o/s, or s/s). Solid and hatched bars indicate the proportion of each category that possessed (o) and (s) mitochondrial types, respectively.

Deviations from expected values are primarily due to an excess of pure *P. sonoriensis* genotypes; 53.3% of the second-generation progeny were classified as (s/s(s)). Frequencies of pure *P. occidentalis* and F₁ [o/s(o) and o/s(s)] genotypes were less than those expected. Of the 296 genotyped progeny, 34% fell into one of the hybrid genotype categories. Among these, 90.1% had *P. occidentalis* mtDNA haplotypes, significantly greater than the proportion of hybrids with *P. sonoriensis* haplotypes ($\chi^2 = 65.9, P < 0.001$). Of the pooled nuclear alleles, 71.5% were *P. sonoriensis*, significantly outnumbering *P. occidentalis* nuclear alleles ($\chi^2 = 54.8, P < 0.001$). *P. sonoriensis* mtDNA alleles outnumbered *P. occidentalis* alleles by a smaller margin than that found for nuclear alleles at 56.7%; however, this difference was still significant ($\chi^2 = 5.4, P = 0.020$).

Disequilibrium statistics: Observed and expected gametic and genotype cytonuclear disequilibrium values are shown in Table 1. The decay of linkage disequilibrium (*D*) and genotypic disequilibrium statistics *D*₁ and *D*₃ from their initial values of 0.25, 0.25, and -0.25, respectively, was less than expected under

TABLE 1

Initial, observed, and random mating expectations of gametic disequilibrium values (*D*) and genotypic disequilibrium statistics (*D*₁, *D*₂, and *D*₃) for first- and second-generation progeny

	Initial values	First generation		Second generation	
		Observed	Expected	Observed	Expected
<i>D</i>	0.250	0.222	0.125	0.150	0.111
<i>D</i> ₁	0.250	0.201	0.125	0.088	0.101
<i>D</i> ₂	0.00	0.041	0.000	0.124	0.021
<i>D</i> ₃	-0.250	-0.243	-0.125	-0.212	-0.121

TABLE 2

Relative fitness values for reproductive success, as defined by the proportion of crosses that produced offspring and average brood size based on results from laboratory crosses

Male	Female	Proportion success	Average brood size	Relative fitness
<i>P. occidentalis</i>	<i>P. occidentalis</i>	0.85	6.19	0.66
	<i>P. sonoriensis</i>	0.54	9.28	0.63
	F ₁ (o)	0.80	2.73	0.28
	F ₁ (s)	1.00	2.67	0.34
<i>P. sonoriensis</i>	<i>P. occidentalis</i>	0.69	6.92	0.60
	<i>P. sonoriensis</i>	0.69	6.62	0.58
	F ₁ (o)	0.80	2.47	0.25
	F ₁ (s)	1.00	4.45	0.56
F ₁ (o)	<i>P. occidentalis</i>	0.80	5.44	0.55
	<i>P. sonoriensis</i>	0.80	4.57	0.46
F ₁ (s)	<i>P. occidentalis</i>	1.00	7.95	1.00
	<i>P. sonoriensis</i>	0.80	4.00	0.40

random mating. Genotype disequilibrium statistic *D*₂ increased from its initial value of 0 to 0.041 and 0.124 in the first and second generations, respectively, indicating an increasing association of o/s nuclear genotypes with (o) mitochondrial haplotypes. *D*₁ declined faster than *D*₃, due to the higher frequency of *P. sonoriensis* nuclear genotypes than of *P. occidentalis* genotypes and the rarity of o/s(o) genotypes.

Recursion equations: From earlier independent behavioral observations (HURT *et al.* 2004) we calculated the probability of assortative mating where α and β are the probabilities of positive assortative mating for *P. occidentalis* females and *P. sonoriensis*, respectively. The strength of assortative mating was found to be asymmetric, $\alpha = 0.017$ and $\beta = 0.903$; *P. sonoriensis* males performed significantly more copulation attempts toward *P. occidentalis* females than that found for the reciprocal cross. Relative fitness values based on data from reproductive trials (HURT and HEDRICK 2003) are given in Table 2. Crosses within and between pure species showed comparable reproductive success. However, fecundity differed significantly among backcross categories, with the greatest reproductive output observed in *P. occidentalis* female \times F₁(s) male crosses.

Expected frequencies of cytonuclear genotypes for first- and second-generation progeny were calculated for three different models of reproductive isolation, which included the premating behavioral barrier model, the postmating barrier model, and the combined model. Expected values for all three models as well as observed genotype frequencies are shown in Table 3. Predictions for first- and second-generation cytonuclear genotype frequencies for all three models are significantly different from observed values. However, the direction of change in cytonuclear genotype frequencies is qualitatively consistent with both the premating and the combined models. The discrepancy

TABLE 3
Observed and expected cytonuclear genotype frequencies under male and female choice models for first- and second-generation progeny

Cytonuclear genotype	First generation				Second Generation			
	Premating only	Expected		Observed	Premating only	Expected		Observed
		Postmating only	Premating and postmating			Postmating only	Premating and postmating	
<i>o/o(o)</i>	0.254	0.268	0.278	0.413	0.149	0.227	0.174	0.158
<i>o/o(s)</i>	—	—	—	—	0.005	0.053	0.004	0.003
<i>o/s(o)</i>	0.246	0.244	0.244	0.099	0.276	0.263	0.279	0.231
<i>o/s(s)</i>	0.024	0.255	0.025	0.014	0.030	0.235	0.029	0.017
<i>s/s(o)</i>	—	—	—	—	0.075	0.055	0.069	0.044
<i>s/s(s)</i>	0.476	0.233	0.452	0.474	0.465	0.167	0.446	0.547
χ^2	72.05	284.38	60.71		12.31	338.11	14.32	
<i>P</i> -value	<0.001	<0.001	<0.001		0.006	<0.001	0.003	

between predictions under the premating and combined barrier models and the observed frequencies is primarily due to a greater deficiency of observed hybrid nuclear genotypes and the theoretical underestimate of the increase in *s/s* nuclear genotypes.

Long-term expected values for cytonuclear genotypes under the three models are shown in Figure 4. The premating behavioral barrier model and combined barrier model predict similar changes in nuclear genotypes. However, the two models differ in their predictions for mtDNA haplotype frequencies. The combined

model predicts the fixation of (*s*) mtDNA haplotypes in ~25 generations, which is ~15 generations earlier than the predicted fixation of *s* nuclear alleles and consistent with the observed changes. However, the premating behavioral model predicts that both (*s*) and (*o*) haplotypes are maintained at equal frequencies. Because sexual selection favoring *P. sonoriensis* males will have no effect on mitochondrial frequencies, the premating behavioral barrier model predicts no change in the frequency of mitochondrial haplotypes. The postmating barrier model shows a very different pattern from the other two models and the observed frequencies; *o/o(o)* and *o/s* nuclear genotypes are maintained at relatively high frequencies, while *s/s* genotypes show a slow and steady decline. For mtDNA frequencies, the postmating model predicts that (*o*) haplotypes rise to fixation, which is the opposite direction of change from the observed haplotype frequencies.

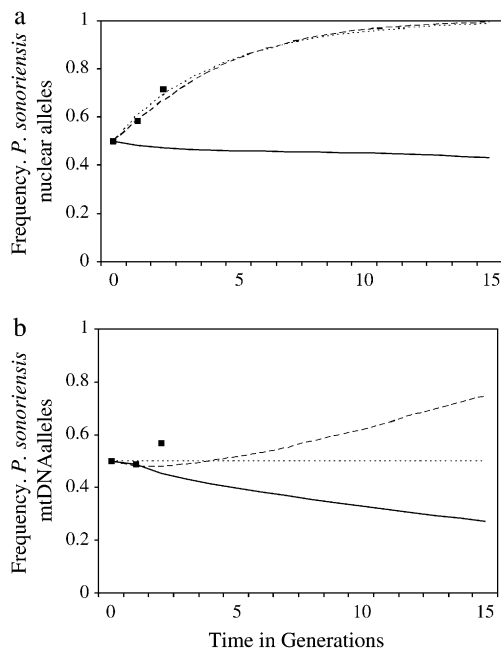


FIGURE 4.—Expected long-term (a) nuclear allele frequencies and (b) mtDNA allele frequencies under premating behavioral barrier, postmating barrier, and combined barrier models. (■) Observed allele frequencies. (···) Premating. (—) Postmating. (---) Combined.

DISCUSSION

To study reproductive barriers that contribute to speciation it is necessary to examine taxonomic pairs that are at an incipient stage in the speciation process. Studies of species pairs that are completely reproductively isolated cannot distinguish reproductive barriers that contributed to species formation from those that develop after reproductive isolation is complete (COYNE and ORR 1989). In a review of >40 pairs of allopatric fish species, MCCUNE and LOVEJOY (1998) estimated that the time required for speciation ranges from 0.8 to 2.3 million years. The estimated time of separation for *P. sonoriensis* and *P. occidentalis* falls in the center of this range. Assuming a molecular clock of ~1% per million years (BERMINGHAM *et al.* 1997; GARCIA *et al.* 2000), divergence of mitochondrial genes cytochrome B, control region, and NaDH are consistent with between

1 and 2 million years of separation (HEDRICK *et al.* 2005). Their estimated time of divergence, which places them at a stage early in the speciation process, and the ease with which we are able to breed and experimentally examine topminnows in a laboratory setting, make this an excellent system to study the evolution of reproductive barriers in allopatry.

In sympatry, biological species status can be inferred from genetic data, since any genetic structure will be broken down in the absence of reproductive barriers. However, for allopatric taxa, it is usually not known to what degree genetic divergence implies reproductive isolation. Since its initial discovery, the Sonoran topminnow has undergone a series of changes to its taxonomic status. Originally describing it as different species (GIRARD 1859), several authors either synonymized or retained these classifications (QUATTRO *et al.* 1996). Recent molecular genetic evidence has shown that *P. occidentalis* and *P. sonoriensis* (known also as the Gila and Yaqui topminnow, respectively) are more different than their morphology suggests and that distinct species status is appropriate (MINCKLEY 1999; HEDRICK *et al.* 2001).

To estimate the strength of isolation required to maintain species distinctiveness should secondary contact occur, COYNE and ORR (1989) designed the "isolation index" to measure the strength of reproductive barriers in biologically isolated sympatric species pairs of *Drosophila*. The average isolation index was 0.907 with a lower-bound 95% confidence interval of 0.854. Therefore, they concluded that an isolation index of ≥ 0.85 is sufficient to prevent fusion of allopatric taxa upon secondary contact. Using an index analogous to a "total" reproductive isolation index employed by COYNE and ORR (1989), we estimated the total strength of isolation between *P. occidentalis* and *P. sonoriensis*, where $I = 1 - (\text{frequency of hybrid offspring} / \text{frequency of homospecific offspring})$. A value of 0 indicates random mating and values can range from $-\infty$ (complete disassortative mating) to 1 (complete isolation). This index differs from that of Coyne and Orr in that their "total isolation index" looked separately at pre-mating and postmating barriers and then combined them, while our index simultaneously accounts for both categories of isolating mechanisms. The isolation index for *P. occidentalis/P. sonoriensis* calculated from first-generation genotype frequencies was 0.872, falling within the range of what is required for "species status."

Observed cytonuclear genotype frequencies from first- and second-generation progeny indicate that both species preferentially mate with conspecifics. Evidence for this includes a greater proportion of parental types and a corresponding deficiency of both types of heterozygotes from what is expected under random mating. Positive assortative mating is also reflected in disequilibrium statistics for both first- and second-generation offspring; both gametic and genotypic disequilibrium

statistics, D , D_1 , and D_3 , did not decay as rapidly as expected under random mating.

Cytonuclear genotype frequencies indicated that the strength of assortative mating differed greatly in the two species. Offspring from *P. occidentalis* female \times *P. sonoriensis* male crosses significantly outnumbered offspring from the reciprocal cross. This directionality resulted in an initial increase in the genotypic disequilibrium statistic D_2 from its value of zero. Asymmetrical accumulation of reproductive barriers is thought to be a common first stage of reproductive isolation. When two males differ in sexual vigor, asymmetries in assortative mating may occur where the more vigorous male is the less discriminating species (MARKOW and HOCUTT 1998). The greater association of F_1 nuclear genotypes with (*o*) mitochondrial haplotypes was consistent with behavioral observations showing that *P. sonoriensis* males were more vigorous and less discriminating than *P. occidentalis* males. *P. sonoriensis* males spent more time courting females and attempted more copulations than did *P. occidentalis* males. Also, a higher proportion of *P. sonoriensis* copulation attempts were directed toward heterospecific females than that found for the reciprocal interspecific cross (HURT *et al.* 2004). The greater discrimination of *P. occidentalis* males may explain the faster decline of *P. occidentalis* nuclear alleles compared to *P. occidentalis* mitochondrial haplotypes. Choosy males will have fewer potential mates and therefore contribute fewer nuclear alleles to the next generation of offspring. The frequencies of maternally inherited mitochondrial haplotypes may not be affected by asymmetrical assortative mating, as female fecundity is more likely to be limited by egg production than by the availability of potential mates.

Observed cytonuclear genotype frequencies were qualitatively most similar to expectations from the combined model that included both behavioral observations and intrinsic postmating barriers. This model predicted the observed asymmetrical deficiency of hybrid genotypes as well as the rapid increase of *s/s* nuclear genotypes. The combined model also predicted the slower increase in (*s*) mitochondrial haplotypes compared with nuclear alleles, reflecting the stronger conspecific mate preference of *P. sonoriensis* females. The combined barrier model could not completely account for the excess of *o/o* (*o*) genotypes and the corresponding deficiency of F_1 's in the first generation of offspring. However, mating parameters used in the pre-mating behavioral and combined barrier models were based solely on observations of male choice. It is likely that females also exhibit mate choice; discrimination against *P. sonoriensis* males by *P. occidentalis* females would result in more *o/o(o)* and fewer F_1 (*o*) progeny, which may account for this discrepancy. In addition, nonrandom mating of hybrid progeny was not accounted for in the model and may have an influence on the genetic makeup of F_2 and later-generation progeny.

The “behavioral barrier only” model was more similar to the “combined barrier” model and the observed cytonuclear genotype frequencies than to the “postmating barrier only” model. Premating barriers are expected to have a greater impact on the total reproductive isolation of incipient species because they act earlier in the reproductive life cycle (COYNE and ORR 2004). Furthermore, the two models that include premating barriers and postmating barriers alone suggest that premating barriers are more effective at preventing gene flow between these two species. Under the premating barrier model, hybrid nuclear genotypes are nearly eliminated after 15 generations, while under the postmating barrier model they are at high frequency after 25 generations. It is likely that taken alone, postmating barriers would not be sufficient to prevent fusion of these two species if secondary contact were established.

Our results support findings in *Etheostoma* (MENDELSON 2003), which concluded that premating barriers evolved earlier than reproductive (postmating) barriers in allopatric as well as sympatric populations. One proposed explanation for this observation is that rapid divergence of sexually selected traits can result from intraspecific competition for mates. Unlike *Etheostoma*, *Poeciliopsis* males are not known to be under strong sexual selection. *Poeciliopsis* males do not perform elaborate courtship displays (REZNICK *et al.* 1996; HURT *et al.* 2004), and males do not possess obvious sexually selected traits such as brightly colored males of the related guppy, *Poecilia reticulata*. Topminnows do, however, live in dense populations where intraspecific competition for resources, including mates, is likely to occur. It is also possible that a delay in the onset of intrinsic postmating reproductive isolation is a common trend in the speciation of fishes. In a comparative study, RUSSELL (2003) found no evidence for hybrid inviability or sterility in species pairs with <7% genetic divergence at the mtDNA gene cytochrome oxidase B. Most other taxonomic groups show signs of intrinsic postmating isolation at <2% genetic divergence of mitochondrial coding sequences (COYNE and ORR 2004).

An unexpected result was the rapid increase in frequency of (s) mtDNA haplotypes that could not have been predicted from reproductive or behavioral studies alone, but was predicted from the combined barrier model. Premating barriers caused no change in mtDNA frequencies and the postmating barrier model predicted an increase in (o) mtDNA; however, the combined model showed a decrease in (o) mtDNA, which is consistent with the observed changes. The change in the allelic composition of the populations appears to be a result of the combined effects of premating barriers and differential reproductive output of different cross categories. *P. occidentalis* females had higher overall reproductive success than *P. sonoriensis* females at 0.702 and 0.517, respectively. However, F₁ females with (o) mtDNA had a very low average reproductive success at 0.265. In the combined

barrier model, premating barriers cause a reduction in the *P. occidentalis* nuclear alleles, forcing (o) mtDNA haplotypes onto a hybrid background where they decline due to the lower reproductive success of these crosses.

This study is novel in that independent estimates of premating and postmating reproductive isolation of these two endangered species were used to predict changes in genetic makeup of crossed populations. The model that encompassed both types of estimates was the most consistent with the observed results. In particular, premating isolation in these allopatric species appears to dominate changes over time in the mixed populations. In addition, these results have implications for conservation. The disturbance of physical barriers separating previously allopatric populations can have unpredictable results and can potentially lead to the loss of biological diversity. Upon reaching secondary contact incipient species undergo a race between fusion, extinction, and coexistence, with coexistence being the least likely of these three outcomes (SPENCER *et al.* 1986; COYNE and ORR 2004), and in our particular example this would predict the extinction of the Gila topminnow.

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