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SEXUAL ISOLATION EVOLVES FASTER THAN HYBRID INVIABILITY IN A DIVERSE AND SEXUALLY DIMORPHIC GENUS OF FISH (PERCIDAE: *ETHEOSTOMA*)

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Abstract.—Theory predicts that sexual (or behavioral) isolation will be the first form of reproductive isolation to evolve in lineages characterized by sexual selection. Here I directly compare the rate of evolution of sexual isolation with that of hybrid inviability in a diverse and sexually dimorphic genus of freshwater fish. The magnitude of both sexual isolation and hybrid inviability were quantified for multiple pairs of allopatric species. Rates of evolution were inferred by comparing genetic distances of these species pairs with the magnitude of each form of reproductive isolation: the slope of the regression of genetic distance on the magnitude of reproductive isolation represents the rate of evolution. Of the two forms of isolation, the magnitude of sexual isolation exhibited the steeper slope of regression, indicating that sexual isolation will tend to evolve to completion earlier than hybrid inviability, strictly as a by-product of evolution in geographically isolated populations. Additional evidence from the literature is used to qualitatively compare rates of evolution of sexual isolation with that of other forms of reproductive isolation. Preliminary comparisons support the prediction that sexual isolation will evolve more rapidly than other forms. Because *Etheostoma* is characterized by striking sexual dimorphism, these results are consistent with the hypothesis that sexual selection for exaggerated mate-recognition characters causes the relatively rapid evolution of sexual isolation.

Key words.—*Etheostoma*, hybrid inviability, postmating isolation, premating isolation, reproductive isolation, sexual isolation, sexual selection, speciation.

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Reproductive isolation, the reduction of gene flow between populations due to intrinsic features of organisms (Dobzhansky 1937; Mayr 1963), plays a primary role in maintaining biological diversity. At least some degree of reproductive isolation is necessary if evolutionary lineages are to remain phenotypically and genetically distinct while coexisting in nature. It is now understood that gene flow between lineages may be restricted in a variety of ways, including various forms of both premating and postmating isolation (Dobzhansky 1937; Mayr 1963). A fundamental question that remains open, however, is the chronological order in which these different forms evolve (e.g., Coyne and Orr 1998; Gleason and Ritchie 1998; Bordenstein et al. 2001). In particular, it is unclear whether one form tends to evolve before others, thereby representing the earliest possibility for diverging lineages to coexist and interact as distinct entities. Relatively few empirical data are available to indicate whether certain forms of reproductive isolation tend to evolve first, and if so, under what conditions.

Sexual (i.e., behavioral) isolation is a form of premating isolation whereby gene flow between populations is restricted due to differences in courtship behavior. Theory predicts sexual isolation will be the first form of reproductive isolation to evolve in taxa characterized by strong sexual selection (Fisher 1930; Lande 1981; West-Eberhard 1983; Carson 1986; Lande and Kirkpatrick 1988; McEvey 1993; Andersson 1994; Butlin and Ritchie 1994; Panhuis et al. 2001). Sexual selection is thought to cause the rapid evolution of mating signals and responses (Fisher 1930; Lande 1981; West-Eberhard 1983), such that individuals from geographically isolated sister populations rapidly cease to recognize each other as suitable mates, and thus fail to interbreed upon secondary contact. Sexual isolation is thought to be the first form of reproductive isolation to evolve in major lineages such as birds (Prager and Wilson 1975; Grant and Grant 1996; Grant 2001) and frogs (Blair 1964), two groups noted for widespread sexual selection of mate-recognition characters. Yet, studies demonstrating a faster rate of evolution of sexual isolation throughout major lineages characterized by sexual selection are rare (Panhuis et al. 2001).

Here I compare the rate of evolution of sexual isolation with that of hybrid inviability, a main form of postmating isolation, in a diverse and sexually dimorphic genus of freshwater fish. *Etheostoma* is one of three genera commonly known as darters and constitutes the largest genus of North American freshwater fish. Although taxonomic distinctions remain in flux, the genus is currently described as containing 16 subgenera, roughly 120 species, and numerous additional subspecies and geographic races (Page 1983). *Etheostoma* is also characterized by striking sexual dimorphism, a classic indicator of intense sexual selection (Andersson 1994). During the breeding season, males exhibit bright, species-specific color patterns and/or conspicuous fleshy knobs at the tips of their fin rays, thought to mimic eggs. Females, in contrast, are spotted brown and relatively cryptic. Prolific speciation and exaggerated sexual dimorphism make *Etheostoma* a classic group in which sexual isolation is expected to evolve before other forms of reproductive isolation as a result of sexual selection on mate-recognition characters.

To measure rates of evolution of reproductive isolation, I used methods similar to those developed by Coyne and Orr (1989, 1997) in their studies of *Drosophila*. For multiple pairs of species, genetic distance was compared to the magnitude of reproductive isolation. Genetic distance, plotted on the *x*-axis, represents the amount of time two lineages have been evolving independently (i.e., divergence time; see Avise 1994). On the *y*-axis is the strength of reproductive isolation, which indicates the degree to which gene flow is predicted

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to occur between the two lineages. In general, a positive correlation between genetic distance and reproductive isolation is expected: the longer two lineages have been evolving independently, the greater the strength of reproductive isolation predicted. The relationship between genetic distance and various forms of reproductive isolation has been shown to be positive in several taxa (*Drosophila*: Coyne and Orr 1989, 1997; snapping shrimp: Knowlton et al. 1993; sea stars: Foltz 1997; frogs: Sasa et al. 1998; but see Poeciliid fishes: Rosen 1979; salamanders: Tilley et al. 1990).

The rate of evolution is then represented by the slope of the regression of genetic distance on the strength of reproductive isolation, with a steeper slope indicating a faster rate of evolution. Thus, to compare the rates of evolution of different forms of reproductive isolation, each form can be measured independently and compared to genetic distance. A statistical comparison will indicate if one form of reproductive isolation exhibits a steeper slope of regression. The form that exhibits the steepest slope of regression will tend evolve to completion earliest and will most likely represent the first opportunity for diverging lineages to remain distinct in sympatry.

Only one study to date has explicitly quantified and compared the rates of evolution of different forms of reproductive isolation across a major taxon. Coyne and Orr (1989, 1997) compared rates of evolution of premating and postmating isolation in *Drosophila*. Results of these studies indicate that premating isolation evolves faster than postmating isolation, but only between sympatric species. For geographically isolated species of *Drosophila*, premating and postmating isolation appear to evolve at similar rates. These results have been interpreted as suggesting that reinforcement—selection for increased sexual isolation when there is a cost to hybridization (Dobzhansky 1937; Butlin 1989)—has accelerated the rate of evolution of sexual isolation between sympatric species (Andersson 1994). However, the rapid evolution of sexual isolation due to sexual selection is not generally thought to require the accelerating effects of reinforcement (Lande 1981; West-Eberhard 1983; Carson 1986; Lande and Kirkpatrick 1988; McEvey 1993; Andersson 1994; Butlin and Ritchie 1994). Therefore this study examines the evolution of reproductive isolation in allopatric pairs of species to test the prediction that sexual isolation will evolve to completion earlier than hybrid inviability strictly as a by-product of evolution in geographically isolated populations.

Materials and Methods

Choosing Species Pairs

Only pairs of species whose geographic ranges do not currently overlap (Lee et al. 1981; Page 1983; Etnier and Starnes 1993) were examined. If a pair consisted of two geographically isolated populations of the same species, these populations were collected from different major river drainages (e.g., Cumberland vs. Tennessee River). All individuals of a given species (or population) were collected from within a 1-km stretch of stream or river.

Pairs of species used in regression analyses were chosen to be statistically independent. Although many pairs of species were investigated (data presented below), only pairs that were both unique (i.e., no species was used in more than one pair) and phylogenetically independent were used to assess rates of evolution. To assess phylogenetic independence, a phylogeny was estimated based on nucleotide sequence data from the mitochondrial gene cytochrome *b* (see below). This phylogeny was used to indicate which pairs of species share overlapping evolutionary branches (Fig. 1); only pairs with nonoverlapping evolutionary branches were used in regression analyses (see Felsenstein 1985).

Phylogenetic Analysis

Individuals from every species (or population) used in the analysis, plus an additional 11 species, were sampled to estimate a phylogeny. *Percina caprodes*, a member of the sister genus to *Etheostoma*, was used as the outgroup. The number of individuals sampled per species (population) ranged from one to four, indicated in parentheses in Figure 1.

DNA was extracted using QIAmp Tissue Kit (Qiagen, Santa Clarita, CA). Cytochrome *b* (1140 bp) was amplified using standard polymerase chain reaction (PCR) technique under the following conditions: 35 cycles at 94°C (30 sec), 55°C (30 sec), and 72°C (90 sec). Primers are published in Song et al. (1998). Additional primers were developed from sequence data and are: forward: 5′-GATTGAAGAACCACCGTTGTT-3′, reverse: 5′-CCGACATTCGGTTTACAAGACCG-3′. PCR product was purified using the WizardPrep DNA purification kit (Promega, Madison, WI). Sequencing reactions were conducted using BigDye Terminator RR mix (PE Applied Biosystems, Foster City, CA) under standard conditions. Sequencing product was purified with Sephadex (Pharmacia Biotech AB, Piscataway, NJ). Sequences were run on ABIPrism 377 and ABIPrism 3700 DNA Analyzers (PE Applied Biosystems). The sequence of cytochrome *b* for the outgroup, *P. caprodes*, was obtained from GenBank (Song et al. 1998).

Sequences were aligned in Sequencher version 3.1.1 (Gene Codes Corp., Ann Arbor, MI). Modeltest version 3.0 (Posada and Crandall 1998) was used to identify, based on Akaike information criterion, the best-fitting model of evolution for cytochrome *b*. A general time reversible (GTR) + I + Γ model of evolution (I = 0.5828, Γ = 1.302) was determined best fitting. Unusual transition/transversion rates were noted: A-C = 1.1997, A-G = 30.8976, A-T = 0.7338; C-G = 2.3347, C-T = 8.498. A heuristic search using the optimality criterion of likelihood was conducted in PAUP* version 4 (Swofford 1998). All positions of the 1140 bp of cytochrome *b* were considered in the analysis. TBR branch swapping was in effect; starting trees were obtained via stepwise addition.

Bootstrap values based on 1000 replicates were calculated using the maximum parsimony criterion, because likelihood would have proven computationally prohibitive. Characters were unordered and of equal weight. Starting trees were obtained via stepwise addition, using the simple addition sequence. TBR branch swapping was in effect.

Measuring Sexual Isolation

Sexual isolation was measured for 13 pairs of species in artificial streams at Highlands Biological Station, Macon County North Carolina (1998–2000) and Lake Texoma Bi-

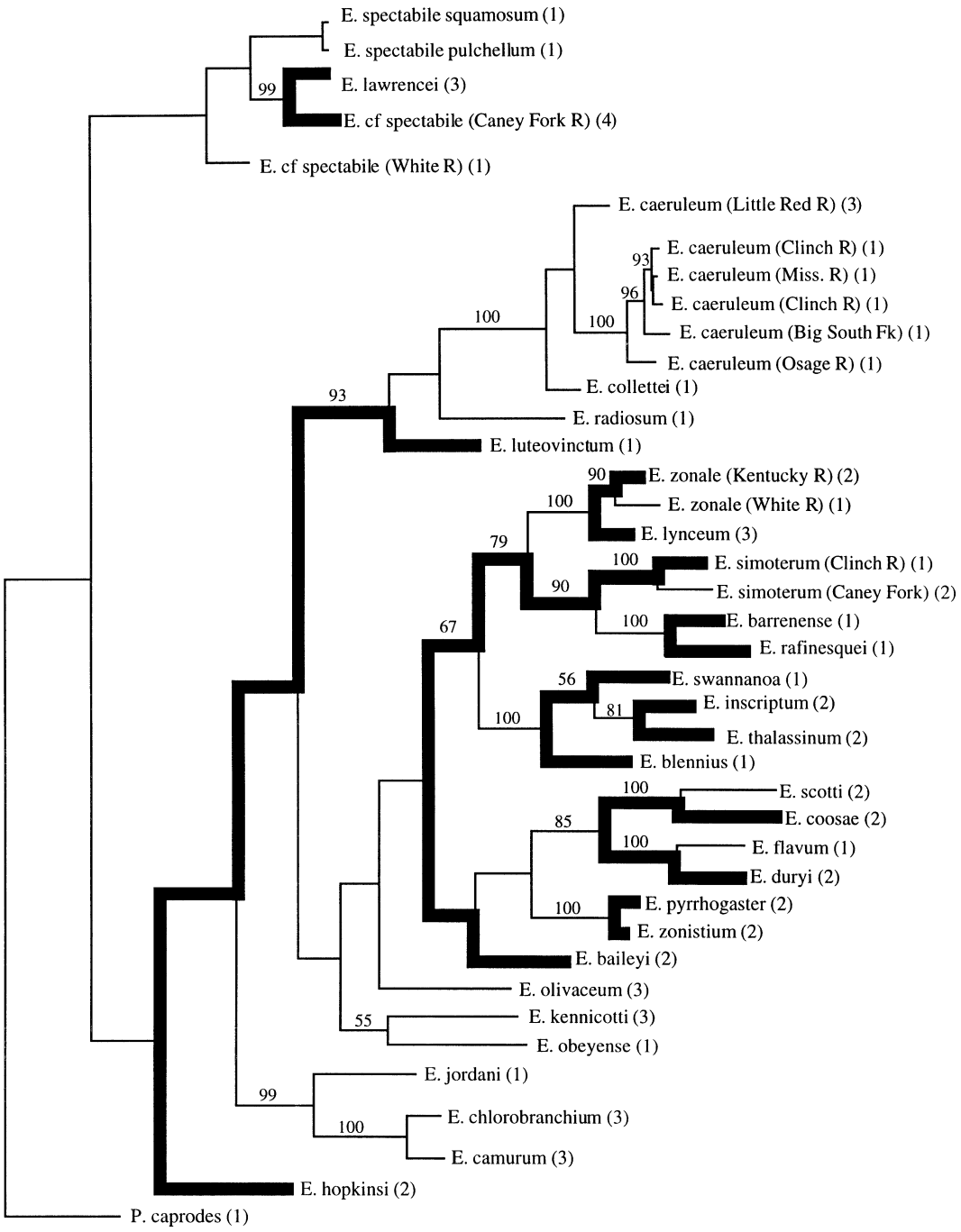

FIG. 1. A phylogenetic reconstruction of several species of *Etheostoma* based on nucleotide sequence differences in the mitochondrial gene cytochrome *b* (1140 bp). Bootstrap values greater than 50% are shown. Numbers in parentheses indicate the number of individuals sampled per population. One haplotype per monophyletic population was used here to illustrate the phylogenetic independence of species pairs used in the study. Species pairs used in the sexual isolation dataset are indicated with dark bars on the phylogeny; note that no two pairs share overlapping evolutionary branches.

ological Station, Marshall County Oklahoma (2000). Each species pair was subject to one or more multiple-mate choice trials, designed to simulate secondary contact. Each trial consisted of five males and five females of each of the two species (20 individuals) captured from the wild and transported to the station within 24 h of capture.

Trials were conducted in artificial flow tanks (Living Stream, Frigid Units, Toledo, OH; L × W × D: 84 × 24 × 22 inches); 20 individuals in this size tank falls within the range of spawning densities observed in nature (pers. obs.). Water in each trial was treated to mimic the average temperature, pH, and hardness of the two streams from which fish were collected. Temperature was controlled by a compressor built in to the Living Stream; pH and hardness were

controlled using standard aquarium buffering agents. Water flow was adjusted with submersible water pumps to mimic naturally occurring rates of flow.

To distinguish the two species in a trial (females of different species were often indistinguishable) experimental fish were lightly anesthetized and injected with a small amount of elastomer dye (Northwest Marine Technologies, Shaw Island, WA) prior to a trial. One species was marked in an upper caudal fin ray, the other in a lower ray; placement was reversed for alternate trials. Individuals were given 2–3 h recovery time before being placed in the experimental tank.

For each trial, the 20 individuals were allowed to spawn freely over the course of three consecutive days. Spawning was observed during daylight hours, from approximately 1030 h until 1730 or 1830 h, depending on natural daylength; 15-min breaks were taken at hourly intervals. All observed spawning events were recorded. Spawning events are unambiguous, consisting of 2–20 sec of concerted quivering (duration varies among species). A sexual isolation index (*SI*) was calculated for each trial by comparing the number of conspecific spawning events with the number of heterospecific spawning events (after Stalker 1942):

$$SI = [(\text{no. conspecific spawning events}) - (\text{no. heterospecific spawning events})] \div \text{total no. spawning events.} \quad (1)$$

Sexual isolation (*SI*) is expected to range from zero to one. If mating is essentially random, the number of conspecific matings will roughly equal the number of heterospecific matings, and $SI = 0$. If sexual isolation is complete, there will be no heterospecific matings, and $SI = 1$. For species pairs subject to more than one trial, the mean *SI* from replicate trials was used in the regression analysis.

Measuring Hybrid Inviability

Seven of the pairs used in the sexual isolation analysis, plus an additional two species pairs ($n = 9$), were examined for hybrid inviability. Hybrid inviability was assessed by comparing the hatching success of eggs fertilized in conspecific versus heterospecific manual crosses. For each species pair, multiple crosses of all four cross types were conducted (two conspecific and two reciprocal heterospecific). Unique pairs of parents were used for every cross, that is, no individual was used twice. For each cross, a gravid female was gently squeezed over a shallow dish of treated water to extract eggs. The number of healthy eggs extracted (i.e., yolky, with a smooth margin) was recorded. A male was then gently squeezed to release milt directly onto the eggs (see Strawn and Hubbs 1956). Eggs were placed in a controlled-environment chamber, where temperature and light cycles were adjusted to reflect the average of natural conditions. Developing embryos were monitored daily; dead embryos were removed to deter fungal growth.

The number of fry that hatched and swam vigorously and normally upon hatching was recorded for each cross. Conspecific hatching success was determined by pooling the total number of successfully hatched embryos from all conspecific crosses (A × A, B × B) and dividing by the total number of healthy eggs extracted for these two cross types. Likewise to calculate heterospecific hatching success, the total number hatched from all heterospecific crosses (A × B, B × A) was divided by the total number of eggs extracted for these two cross types. Hybrid inviability (*HI*) for each species pair was calculated as:

$$HI = [(\%\ \text{conspecific hatching success}) - (\%\ \text{heterospecific hatching success})] \div \text{total \% hatching success.} \quad (2)$$

Eight of the species pairs for which *HI* was quantified were phylogenetically independent; this subset of species pairs was used in the regression analysis.

Estimating Divergence Time

Estimates of divergence time were calculated as the genetic distance between the two species in each pair. Genetic distance was calculated based on nucleotide sequence differences in the mitochondrial gene cytochrome *b*. Sequences of cytochrome *b* were obtained and aligned according to protocols outlined above. Genetic distances were calculated in PAUP* version 4 (Swofford 1998) using maximum-likelihood distances. As determined for the phylogenetic analysis, distances were calculated based on a general time reversible (GTR) + I + Γ model of evolution (I = 0.5828, Γ = 1.302), yielding distance values that estimate the average number of nucleotide substitutions per site. To correct for intraspecific variation among haplotypes in estimating divergence time (Edwards and Beerli 2000), net interspecific genetic distance was calculated by subtracting mean intraspecific distances from mean pairwise interspecific distances (Nei 1987, eq. 10.21).

Individuals sampled for the distance analyses were collected from the same populations and at the same time as those used in the behavioral and hybrid inviability assays. Two exceptions are *E. simoterum* and *E. spectabile pulchellum*, for which DNA sequences were not available. The population of *E. simoterum* used to measure sexual isolation and hybrid inviability came from Sweeten Creek, a direct tributary of the Tennessee River; *E. simoterum* sampled for genetic analysis came from the Clinch River system, a different tributary of the Tennessee River. The population of *E. spectabile pulchellum* used to measure sexual isolation came from Pennington Creek of the Red River system (OK); *E. spectabile pulchellum* in the genetic analysis came from the Arkansas River system.

Although using genetic distance to estimate divergence times requires the somewhat controversial assumption of a molecular clock (Zuckerkandl and Pauling 1965), whereby genes or other segments of DNA evolve at roughly similar rates in different populations, this assumption becomes safer when comparing closely related lineages as in this study (Avise 1994). Cytochrome *b*, in particular, has been used to estimate divergence times (Johns and Avise 1998) and is generally likely to meet this assumption.

Statistical analysis comparing the slopes of the regressions of genetic distance on sexual isolation and on hybrid inviability was conducted in SAS (SAS Institute, Inc., Cary, NC).

Because the *x*-axis is not bounded in the same manner as the *y*-axis, the Student's *t*-test was modified to compare the slopes of the two datasets (D. Burdick, pers. comm.). Specifically, the standard error of the slope of regression is taken as a product of variation in both *x* (genetic distance) and *y* (sexual isolation or hybrid inviability), such that *t* was approximated as

$$t = \frac{\beta_{SI} - \beta_{HI}}{SE_{\text{diff}}}, \quad (3)$$

where

$$SE_{\text{diff}} = \left[\sqrt{\frac{SSE_{SI} + SSE_{SI}}{df_{SI} + df_{HI}}}\right]\left[\sqrt{\left(\frac{SE_{\beta_{SI}}}{s_{SI}}\right)^2 + \left(\frac{SE_{\beta_{HI}}}{s_{HI}}\right)^2}\right]; \quad (4)$$

s_i^2 is the mean squared error of *y* (sexual isolation or hybrid inviability), SSE_i is the sum of squares error (*y*), $SE_{\beta i}$ is the standard error of the slope, and df_i is the degrees of freedom in each dataset.

Results

Phylogenetic Analysis

The tree topology with the highest likelihood score is presented in Figure 1. Species pairs used in the regression analysis for sexual isolation are overlaid on the phylogeny to illustrate the phylogenetic independence of these pairs. Bootstrap values greater than 50% are presented. One node received greater than 50% bootstrap support but was not identified in the maximum-likelihood tree: bootstrap analysis indicates 66% support for a clade containing *E. radiosum* and *E. luteovinctum* as sister to the *E. caeruleum–E. collettei* clade. This inconsistency does not affect inferences of phylogenetic independence for species pairs used in the analysis.

The topology of the maximum-likelihood tree generally corresponds to currently accepted subgeneric classifications based on morphological analyses, except for the placement of the *E. spectabile* species complex and *E. hopkinsi*. Both *E. spectabile* and *E. hopkinsi* are currently included in the same subgenus, *Oligocephalus*, which also includes *E. caeruleum*, *E. collettei*, *E. radiosum*, and *E. luteovinctum* (Page 1981; Bailey and Etnier 1988).

Eight populations were characterized by more than one haplotype of cytochrome *b*. When all haplotypes were included in the analysis, seven of these eight populations were shown to be monophyletic, with bootstrap support ranging from 97% to 100%. Mean intraspecific (intrapopulation) distances for these populations were low, $d = 0.0003–0.019$. Therefore, to simplify the illustration of phylogenetic independence, one haplotype per population was used to generate the phylogeny depicted in Figure 1. *Etheostoma caeruleum* from Little Red River, Arkansas, proved an exception; thus both sampled haplotypes are included in the phylogeny.

Sexual Isolation

In total, 13 allopatric pairs of species were examined for sexual isolation. Spawning data from each mating trial are presented in Table 1. Isolation indices ranged from nearly zero to one, with the most distantly related pairs of species exhibiting complete sexual isolation. The relationship between genetic distance and the strength of sexual isolation for these pairs is presented in Figure 2a.

Nine of the 13 pairs examined are phylogenetically independent, indicated in Table 1 (see also Fig. 1). These pairs were subject to regression analysis (Fig. 2b). The slope of the regression of genetic distance on the strength of sexual isolation is significant. Genetic distance appears to be a good predictor of sexual isolation in this genus ($r^2 = 0.63$, $\beta = 1.704$, $F = 11.67$, df = 1, 8, $P < .02$).

Individuals could not be distinguished within a trial; however, in no trial was mating restricted to a few individuals, as it was not uncommon to observe several pairs of individuals spawning simultaneously. Therefore, despite a small number of total mating trials per species pair, sampling within a trial was intensive, as most of the 20 individuals in a trial spawned. Total number of spawnings per trial ranged from 19 to 213. Average number of spawnings per trial was 61.6. For species pairs subject to only one trial, the average number of spawnings per trial was 91.3.

Variance in *SI* across replicate trials within a species pair was generally low. One exception is *E. rafinesquei* and *E. barrenense*, which exhibit relatively low isolation in one replicate and relatively high in another, although both replicates indicate that sexual isolation for this pair is of intermediate magnitude. The average of these two values was used in regression analysis.

Hybrid Inviability

The strength of hybrid inviability was estimated for nine pairs of species (Table 2). The average number of unique sets of parents contributing to estimates of conspecific hatching success (A × A, B × B) was 7.7, and the average number contributing to heterospecific hatching success (A × B, B × A) was 7.4. Hybrid inviability indices varied widely across species pairs, ranging from $HI = -0.21$ to 0.77. Notably, none of the pairs exhibited complete hybrid inviability, and for all but one pair, this form of postmating isolation has not evolved past $HI = 0.5$. The relationship between genetic distance and hybrid inviability for the total number of pairs examined ($n = 9$) is presented in Figure 2c.

Eight of the species pairs examined for hybrid inviability were phylogenetically independent. Regression analysis based on these eight pairs does not indicate a statistical relationship between genetic distance and the strength of hybrid inviability (Fig. 2d; $r^2 = 0.0062$, $\beta = 0.157$, ns).

Comparing the Slopes of Regression: Sexual Isolation Versus Hybrid Inviability

The slopes of the regressions of the two datasets containing statistically independent data are significantly different, with the slope of the regression of genetic distance on sexual isolation greater than that of genetic distance on hybrid inviability ($P < 0.05$, modified Student's *t*, Fig. 2).

Discussion

Results of this study indicate that sexual isolation between allopatric populations of *Etheostoma* will tend to evolve to

TABLE 1. Number of spawning events per cross type in replicate mating trials for 13 allopatric pairs of *Etheostoma* species. Sexual isolation indices (*SI*) indicate the strength of sexual isolation in each trial. $\overline{SI}$ indicates the mean sexual isolation index across replicate trials, calculated for species pairs for which more than one replicate was conducted. Genetic distance is based on cytochrome *b* and represents the average number of nucleotide substitutions per site (GTR + I + Γ).

Species pair	Replicate	Number of spawning events				*SI*	$\overline{SI}$	Genetic distance (cyt *b*)
		A × A	A × B	B × A	B × B			
(A) *E. zonistium* (Tennessee R, TN)[1]	1	49	12	88	64	0.06	0.16	0.02
(B) *E. pyrrhogaster* (Obion R, TN)	2	11	3	4	1	0.26		
(A) *E. lynceum* (Mississippi R, TN)[1]	1	26	20	14	10	0.03	0.03	0.05
(B) *E. zonale* (Ohio R, KY)								
(A) *E. lawrencei* (Cumberland R, TN)[1]	1	11	6	3	6	0.31	−0.004	0.05
(B) *E.* cf *spectabile* (Caney Fork R, TN)	2	7	8	5	7	0.04		
	3	5	16	3	4	−0.36		
(A) *E. rafinesquei* (Barren R, KY)[1]	1	27	15	20	12	0.05	0.38	0.10
(B) *E. barrenense* (Green R, KY)	2	19	10	0	41	0.71		
(A) *E. thalassinum* (Santee R, SC)[1]	1	8	43	1	45	0.03	−0.03	0.10
(B) *E. inscriptum* (Savannah R, SC)	2	20	14	19	4	−0.16		
(A) *E. swannanoa* (French Broad R, NC)[1]	1	9	0	0	15	1.0	1.0	0.18
(B) *E. blennius* (Buffalo R, TN)	2	0	0	0	44	1.0		
(A) *E. barrenense* (Barren R, KY)	1	52	35	10	36	0.32	0.32	0.23
(B) *E. simoterum* (Clinch R, TN)								
(A) *E. duryi* (Tennessee R, TN)[1]	1	49	0	15	27	0.67	0.64	0.34
(B) *E. coosae* (Coosa R, TN)	2	25	5	9	34	0.62		
(A) *E. simoterum* (Tennessee R, TN)[1]	1	32	0	2	20	0.93	0.82	0.40
(B) *E. baileyi* (Rockcastle R, KY)	2	14	1	3	10	0.71		
(A) *E. spectabile* (Red R, OK)[2]	1	5	5	0	10	0.5	0.61	0.46
(B) *E. radiosum* (Red R, OK)	2	22	12	0	8	0.43		
	3	18	1	1	17	0.89		
(A) *E. simoterum* (Clinch R, TN)	1	78	3	21	30	0.64	0.64	0.52
(B) *E. coosae* (Coosa R, TN)								
(A) *E. luteovinctum* (Duck R, TN)[1]	1	9	0	1	36	0.96	0.96	0.59
(B) *E. hopkinsi* (Savannah R, SC)	2	5	0	1	37	0.95		
(A) *E. collettei* (Saline R, AR)	1	18	0	0	12	1.0	1.0	0.68
(B) *E. spectabile* (Red R, OK)								

[1] Unique, phylogenetically independent pairs used in the regression analysis.

[2] *Etheostoma spectabile pulchellum* and *E. radiosum paludosum* were collected from the Washita River of southeastern Oklahoma, where their ranges do not currently overlap.

completion earlier than hybrid inviability. A statistical difference in the slopes of regression of genetic distance on sexual isolation versus hybrid inviability indicates that complete sexual isolation will evolve, on average, at a smaller genetic distance than complete hybrid inviability. This study therefore provides empirical evidence that sexual isolation throughout a major taxon will evolve faster than another form of reproductive isolation, strictly as a by-product of evolution in geographically isolated populations.

The implication that hybrid inviability has not evolved to any great extent at these levels of divergence in *Etheostoma* is corroborated by results of a more extensive study in which hybrid inviability was examined in darters (Hubbs 1967). That study reported almost no reduction in hybrid survival across numerous pairs of *Etheostoma* species, many of which were more distantly related than the pairs used here. Notably, this and other studies (Hubbs 1958; Linder 1958; Strawn 1961) have followed hybrid survival in *Etheostoma* up to and including adulthood and have found no evidence of reduced hybrid survival at any stage of development. Thus, although hybrid inviability was measured in the present study at an early stage of development, previous studies suggest hybrids will remain viable, at least into their first year of adulthood.

As in the present study, hatching success in Hubbs (1967), and in Hubbs and Strawn (1957), was generally low for both conspecific and heterospecific cross types. It is not yet clear whether these low hatching rates in the laboratory reflect naturally occurring levels. Authors of the previous studies attribute low hatching success to the stripping technique in the laboratory: eggs may be damaged as they are extruded, sperm counts may be lower in stressed males, and unnatural water hardness may exist in the laboratory. All attempts were made in the present study to replicate natural water conditions, and extruded eggs appeared healthy and viable. Nonetheless, the high mortality rate may obscure a pattern in the data, and future studies may reveal a correlation between genetic distance and the magnitude of hybrid inviability in *Etheostoma*. It is important to note, however, that no species pair, either in the present or previous studies, exhibits complete hybrid inviability, whereas several pairs exhibit complete sexual isolation.

Alternatively, hybrid inviability may not be detectable in the laboratory, if hybrids are maladapted to either parent's environment (i.e., environment-dependent postzygotic isolation; Rice and Hostert 1993). However, species used in this analysis were always paired with members of the same sub-

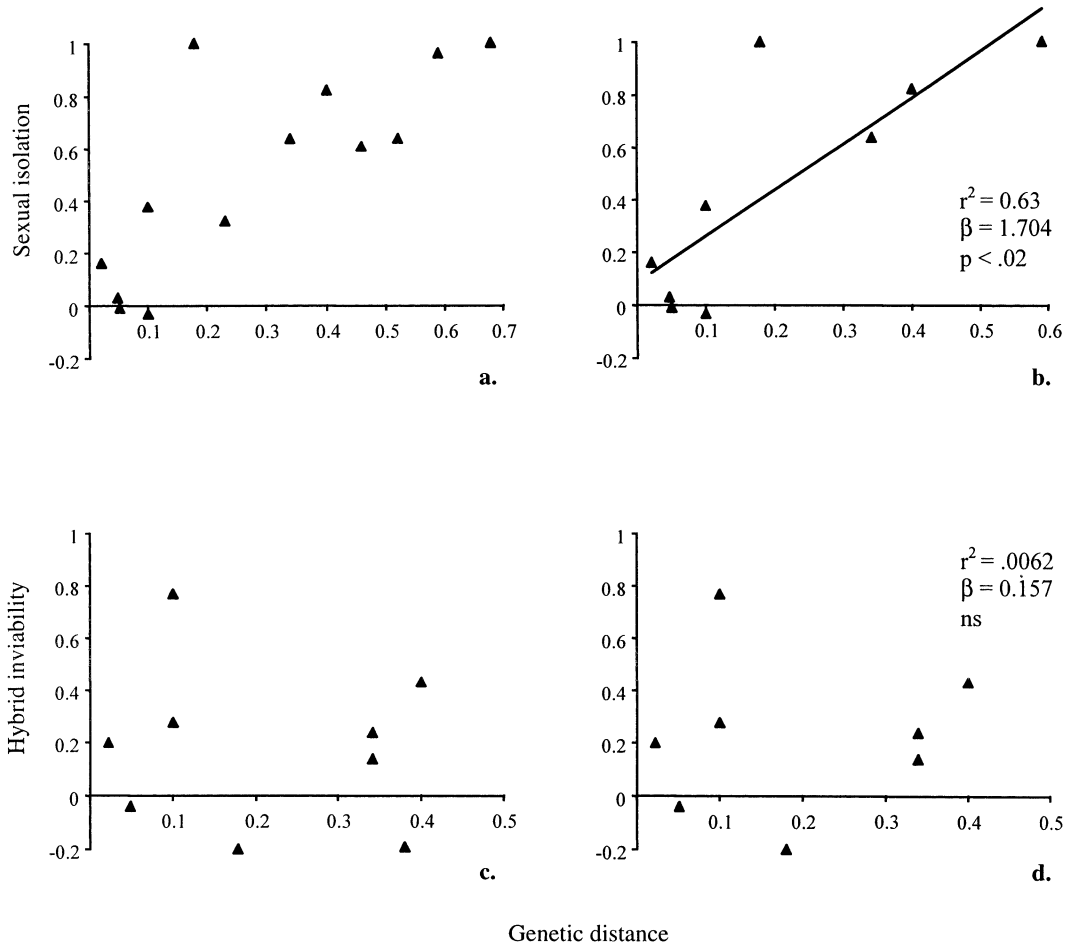

FIG. 2. (a) Relationship between genetic distance and the strength of sexual isolation for 13 pairs of *Etheostoma* species. Mean sexual isolation index (*SI*) from replicate mating trials is presented for each pair (Table 1). Genetic distance is based on sequence differences in the mitochondrial gene cytochrome *b* (GTR + I + Γ). (b) Relationship between genetic distance and sexual isolation for the subset of species pairs that are statistically independent (i.e., unique and phylogenetically independent). A significant positive correlation is detected ($r^2 = 0.63$, $\beta = 1.704$, $F = 11.67$, df = 1,8 $P < 0.02$). (c) Relationship between genetic distance and the strength of hybrid inviability for nine pairs of *Etheostoma* species (Table 2). (d) Relationship between genetic distance and hybrid inviability for eight statistically independent species pairs. No statistical correlation is detected ($r^2 = 0.00623$, $\beta = 0.1570$, ns), and no pair exhibits complete hybrid inviability. The slope of the regression of genetic distance on sexual isolation is significantly greater than that on hybrid inviability ($P < 0.05$, modified Student's *t*).

genus, which use very similar or indistinguishable habitat types (Page 1983). Although environment-dependent postzygotic isolation remains to be quantified, it is unlikely that hybrids of such ecologically similar species would be maladapted to either parent's environment. Thus, for *Etheostoma*, hybrid inviability in the F_1 generation is not likely to pose a significant barrier to gene flow before sexual isolation evolves to completion.

The prediction that sexual isolation will evolve earlier than other forms of reproductive isolation throughout taxa characterized by sexual selection has been supported primarily by comparative studies in birds (Barraclough et al. 1995; Mitra et al. 1996; Owens et al. 1999; Møller and Cuervo 1998). These studies demonstrate that clades characterized by sexual selection tend to contain a greater number of species than closely related clades that are not characterized by sexual selection. Additional support is based on evidence that many species of birds remain capable of hybridizing with species to which they are relatively distantly related (Prager and Wilson 1975). While highly suggestive, these studies do not directly compare the magnitude of different forms of reproductive isolation, and therefore cannot be used to rule out alternative hypotheses concerning rates of evolution (Panhuis et al. 2001). A direct comparison of the magnitude of different types of reproductive isolation across early stages of speciation provides a more explicit test of this widely held prediction.

The Role of Sexual Selection

Coyne and Orr (1989, 1997) compared the rates of evolution of different forms of reproductive isolation in *Drosophila* in a pair of landmark studies and found a pattern different than that demonstrated here for *Etheostoma*. Based on extensive literature surveys, they conclude that sexual isolation in *Drosophila* evolves faster than postmating iso-

TABLE 2. Number of viable eggs crossed and hatched per cross type for nine allopatric species pairs. Conspecific hatching success was determined by pooling the data from A × A and B × B crosses; heterospecific hatching success was determined by pooling the data from A × B and B × A crosses. Numbers in parentheses indicate the number of unique parental pairs that contributed to estimates of hatching success. $\overline{HI}$ indicates the strength of hybrid inviability for each species pair. Genetic distance is based on cytochrome *b* and represents the average number of nucleotide substitutions per site (GTR + I + Γ).

Species pair	A × A		A × B		B × A		B × B			Genetic distance (cyt *b*)
	Crossed	Hatched	Crossed	Hatched	Crossed	Hatched	Crossed	Hatched	$\overline{HI}$	
(A) *E. zonistium* (Tennessee R, TN)[1]	101 (2)	19	109 (2)	24	71 (2)	3	27 (1)	10	0.20	0.02
(B) *E. pyrrhogaster* (Obion R, TN)										
(A) *E.* cf *spectabile* (Caney Fork R, TN)[1]	241 (4)	38	232 (4)	59	178 (4)	42	42 (4)	27	−0.035	0.05
(B) *E. lawrencei* (Cumberland R, TN)										
(A) *E. raftnesquei* (Green R, KY)[1]	75 (2)	23	87 (2)	2	250 (5)	3	221 (6)	11	0.77	0.10
(B) *E. barrenense* (Barren R, KY)										
(A) *E. thalassinum* (Santee R, SC)[1]	119 (6)	42	108 (4)	24	16 (3)	7	41 (3)	29	0.28	0.10
(B) *E. inscriptum* (Savannah R, SC)										
(A) *E. blennius* (Buffalo R, TN)[1]	20 (1)	15	95 (2)	54	31 (1)	6	106 (5)	24	−0.21	0.18
(B) *E. swannanoa* (French Broad R, NC)										
(A) *E. caeruleum* (Osage R, MO)[1]	115 (4)	63	181 (6)	48	55 (1)	39	37 (1)	11	0.14	0.34
(B) *E. radiosum* (Red R, OK)										
(A) *E. coosae* (Coosa R, TN)[1]	254 (6)	84	275 (7)	27	126 (7)	58	100 (5)	39	0.24	0.34
(B) *E. duryi* (Tennessee R, TN)										
(A) *E. flavum* (Cumberland R)	162 (4)	44	13 (1)	0	71 (3)	28	47 (2)	3	−0.19	0.38
(B) *E. zonale* (White R, AR)										
(A) *E. simoterum* (Tennessee R, TN)[1]	166 (5)	36	211 (9)	18	85 (4)	11	129 (8)	37	0.43	0.40
(B) *E. baileyi* (Rockcastle R, KY)										

[1] Unique, phylogenetically independent pairs used in the regression analysis.

lation, but only between species that are currently sympatric. In contrast, for geographically isolated species, sexual and postmating isolation appear to evolve at roughly the same rate. These results have been interpreted to suggest that reinforcement is accelerating the evolution of sexual isolation between sympatric taxa (e.g., Andersson 1994). However, for allopatric species of *Drosophila*, sexual isolation does not appear to evolve to completion any earlier than postmating isolation.

One explanation for the observed difference between *Etheostoma* and *Drosophila* is that sexual selection for exaggerated mate-recognition characters may be more intense in *Etheostoma*, driving the rapid evolution of mating behavior even in the absence of reinforcement. Except for the Hawaiian species, the genus *Drosophila* as a whole is not characterized by particularly striking sexual dimorphism or highly exaggerated secondary sexual characters (Ringo 1977). In contrast, *Etheostoma* is considered highly dimorphic throughout the genus, with males greatly ornamented relative to females. Although quantifying and comparing the magnitude of sexual dimorphism across divergent taxa is not straightforward, these data are nonetheless consistent with the hypothesis that a greater intensity of sexual selection in *Etheostoma* causes the relatively rapid evolution of sexual isolation, strictly as a by-product of divergence in geographically isolated populations.

Additional Forms of Reproductive Isolation

Another explanation for the observed difference between *Drosophila* and *Etheostoma* is that the magnitude of postmating isolation quantified in the *Drosophila* studies includes estimates of hybrid sterility, as well as hybrid inviability. Hybrid sterility is an important form of postmating isolation whereby hybrid offspring fail to produce viable gametes (e.g., Mayr 1963), and it may evolve at the same rate as or earlier than sexual isolation in *Etheostoma*. However, preliminary evidence from the literature suggests sexual isolation is likely to evolve to completion earlier than hybrid sterility.

Hybrid sterility has been examined in four pairs of *Etheostoma* species. *Etheostoma grahami* and *E. lepidum*, two closely related species, produce fully viable and fertile offspring (Strawn 1961). Three more distantly related pairs (*E. spectabile–E. lepidum*, *E. spectabile–E. grahami*, and *E. spectabile–E. radiosum*) also produce viable offspring; however, whereas females of these crosses are fully fertile, males appear to be sterile (Hubbs 1958, 1967; Linder 1958). A hybrid sterility index for the latter three pairs is inferred to be *HS* = 0.5, because half the hybrids are sterile (see Coyne and Orr 1989).

The magnitude of sexual isolation is available for direct comparison in two of these species pairs. For *E. spectabile* and *E. lepidum*, Hubbs (1960) found that control (i.e., conspecific) matings occurred readily in the laboratory, whereas heterospecific matings were never observed. Sexual isolation is therefore inferred to have evolved to completion, and hybrid sterility is still of intermediate magnitude. *Etheostoma radiosum* and *E. spectabile* exhibit an isolation index of *SI* = 0.61. It is unclear whether this value indicates the more rapid evolution of sexual isolation (*HS* = 0.5); however, three

additional species pairs of a smaller genetic distance than *E. radiosum* and *E. spectabile* exhibit sexual isolation indices greater than $SI = 0.5$ (Table 1). Although hybrid sterility remains to be more thoroughly examined, preliminary evidence suggests the magnitude of sexual isolation in early stages of divergence is greater than that of hybrid sterility and may therefore tend to evolve to completion earlier.

To address the prediction that sexual isolation will be the first form of reproductive isolation to evolve in *Etheostoma*, the rate of evolution of additional forms of reproductive isolation must be compared. For example, habitat isolation occurs when lineages have diverged in habitat preference, such that populations occupy different habitats within the community and therefore fail to encounter one another. Another form of premating isolation is seasonal (temporal) isolation, whereby lineages have diverged with respect to the time of year (or day) during which they breed; these populations fail to encounter one another in breeding condition (Mayr 1963; Futuyma 1986). Neither habitat nor seasonal isolation is likely to have evolved in the species pairs examined here. As noted, the two species of each pair are closely related members of the same subgenus. Based on natural-history observations, they occupy very similar or indistinguishable habitats and also breed at the same time, both seasonally and daily (Page 1983). Although these forms of isolation remain to be quantified, it is likely that upon secondary contact, reproductively active individuals in each pair would regularly encounter one another.

Gametic incompatibility is a form of postmating-prezygotic isolation (Markow 1997; Howard 1999) prevalent in marine systems (Palumbi 1994) whereby sperm of one species are unable to successfully penetrate eggs of another. Gametic compatibility is unlikely to pose a barrier to gene flow in early stages of *Etheostoma* speciation, because fertilization has proven successful across genera (Hubbs 1967). Successful fertilization across widely divergent species in this group also suggests that variation in resistance to *Wolbachia* bacteria, which appears to restrict gene flow in some taxa (e.g., Werren 1998; Bordenstein et al. 2001), is not likely to play a role in this system.

Another form of postmating-prezygotic isolation for which no data are yet available in *Etheostoma* is conspecific sperm precedence (CSP), whereby conspecific sperm will outcompete heterospecific sperm for fertilization (Howard 1999; Birkhead 2000). In *Etheostoma*, it is possible that eggs exposed simultaneously to conspecific and heterospecific sperm are more likely to be fertilized by conspecific sperm. Laboratory experiments, as well as field observations assessing the extent of simultaneous fertilization, will help determine whether CSP occurs between closely related species of *Etheostoma*. With regard to the stated prediction, it will be necessary to determine whether CSP restricts gene flow at early stages of divergence, before sexual isolation has begun to evolve.

Finally, hybrid inviability and hybrid sterility in the F_2 generation and beyond remain to be quantified. Hybrid breakdown in generations succeeding the F_1 have been suggested to play an important role in preventing gene flow between species (Wu and Palopoli 1994) and may contribute to the maintenance of species boundaries in *Etheostoma*.

Comparing Different Forms of Reproductive Isolation

Understanding the rates at which different forms of reproductive isolation evolve may elucidate the relative contributions of different evolutionary forces to species formation. For example, sexual isolation arises as a result of evolutionary divergence in mating behavior, and in sexually dimorphic clades it is thought to evolve primarily as a result of sexual selection for exaggerated mate-recognition characters. In contrast, hybrid inviability and hybrid sterility are thought to evolve most commonly according to a model proposed by Muller (1942) and Dobzhansky (1936), in which allelic substitutions at coadapted loci lead to incompatibility between the genomes of diverging lineages (e.g., Orr 1997). According to this model, substitutions that lead to postmating isolation may result from either genetic drift or natural selection. Thus, if sexual isolation is indeed the first form of reproductive isolation to evolve in *Etheostoma*, then sexual selection is likely to play a primary role in species formation in this group.

For some questions, however, comparing different forms of reproductive isolation may not be appropriate, because their magnitudes may not be equivalent. The magnitude of reproductive isolation is taken to represent the extent to which gene flow will occur between diverging populations. However, it is difficult to infer, both quantitatively and qualitatively, how the strength of reproductive isolation will translate into gene flow, particularly at intermediate levels of reproductive isolation. For example, from a quantitative perspective, it is not clear whether a sexual isolation index of $SI = 0.5$ reflects the same total amount of gene flow as a hybrid inviability or sterility index of the same magnitude. From a qualitative perspective, it is unclear whether the same alleles will be prevented from introgressing given different forms of reproductive isolation. These are empirical questions to which answers may vary from case to case; as yet, too few data exist with which to address them.

The aim here is to determine which form is the first to evolve to such a magnitude that populations will remain distinguishable in sympatry. Distinctiveness in sympatry may not require complete reproductive isolation (i.e., the total absence of gene flow); however, complete reproductive isolation should be comparable across all forms of reproductive isolation. Thus, this study is conservative, in that it identifies which of two forms will be the first to evolve to completion. Whether intermediate levels of reproductive isolation are sufficient to maintain distinctiveness in sympatry for this group remains to be determined.

Finally, the question of which single form of reproductive isolation evolves to completion first may in some cases be inappropriate. The first opportunity for populations to remain distinct may come not from a single form of reproductive isolation, but rather a combination of forms, none of which have evolved to completion. If each form of reproductive isolation is characterized by a particular probability of gene flow, then taken together, the product of these probabilities could result in an amount of gene flow sufficient to maintain a bimodal distribution of phenotypes. As yet, too few data are available to indicate whether and how often this is the case. Continued research into the interaction and coevolution

of different forms of reproductive isolation may begin to address this question.

Conclusion

Results of this study demonstrate that sexual isolation in a major taxon will tend to evolve to completion before hybrid inviability, strictly as a by-product of divergence in geographically isolated populations. Additional evidence from the literature suggests sexual isolation will also evolve earlier than other forms of reproductive isolation in this genus, although these additional forms remain to be quantified and explicitly compared. These results are consistent with the more general prediction that sexual isolation will be the first form of reproductive isolation to evolve throughout taxa characterized by sexual selection.

Because darters are highly sexually dimorphic, these results are also consistent with the hypothesis that sexual selection for exaggerated mate-recognition characters has led to the relatively rapid evolution of sexual isolation. Comparing results of this study with those of *Drosophila* further supports this hypothesis. In *Drosophila*, a less dimorphic genus, pre- and postmating isolation between allopatric populations evolve at similar rates. This study therefore provides evidence of the relatively rapid evolution of sexual isolation throughout a clade and further suggests that the mechanism responsible is sexual selection.

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