Evolution, 59(4), 2005, pp. 849–857

RAPID EVOLUTION OF POSTZYGOTIC REPRODUCTIVE ISOLATION IN STALK-EYED FLIES

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Abstract.—We test the relative rates of evolution of pre- and postzygotic reproductive isolation using eight populations of the sexually dimorphic stalk-eyed flies *Cyrtodiopsis dalmanni* and *C. whitei*. Flies from these populations exhibit few morphological differences yet experience strong sexual selection on male eyestalks. To measure reproductive isolation we housed one male and three female flies from within and between these populations in replicate cages and then recorded mating behavior, sperm transfer, progeny production, and hybrid fertility. Using a phylogeny based on partial sequences of two mitochondrial genes, we found that premating isolation, postmating isolation prior to hybrid eclosion, and female hybrid sterility evolve gradually with respect to mitochondrial DNA sequence divergence. In contrast, male hybrid sterility evolves much more rapidly—at least twice as fast as any other form of reproductive isolation. Hybrid sterility, therefore, obeys Haldane's rule. Although some brood sex ratios were female biased, average brood sex ratio did not covary with genetic distance, as would be expected if hybrid inviability obeyed Haldane's rule. The likelihood that forces including sexual selection and intra- and intergenomic conflict may have contributed to these patterns is discussed.

Key words.—*Cyrtodiopsis*, Haldane's rule, hybrid sterility, postmating isolation, premating isolation, speciation.

Received May 7, 2004. Accepted January 12, 2005.

From a genetical perspective, understanding the process of speciation amounts to understanding the process of reproductive isolation. Factors inhibiting interspecies mating (premating prezygotic isolation), interspecies fertilization (postmating prezygotic or gametic isolation), and the fitness and fertility of hybrid organisms (postzygotic isolation) create barriers to gene flow that eventually lead to speciation. It can be difficult to determine the sequence of genetic changes causing each of these effects during the earliest stages of isolation because the species available for study have generally been separated for many thousands or even millions of years (Orr 1995) and may currently inhabit isolated locations. However, laboratory study of mating behavior and hybridization among multiple species or divergent populations has provided some insights into the early isolation process.

In a comprehensive meta-analysis, Coyne and Orr (1989, 1997) described several important features of reproductive isolation in *Drosophila*. First, allopatric species pairs tend to acquire prezygotic and postzygotic isolation at equal and constant rates. Subsequent studies on ducks (Tubaro and Lijtmaer 2002), doves (Lijtmaer et al. 2003), butterflies (Presgraves 2002), and frogs (Sasa et al. 1998) have also found evidence that postzygotic isolation evolves gradually over time, but a few studies have reached other conclusions. For example, Tilley et al. (1990) failed to detect a relationship between prezygotic isolation and allozyme differentiation in plethodontid salamanders, after taking into account geographic proximity. Moyle et al. (2004) reported that reproductive isolation correlates with genetic distance in *Silene* but not in two other angiosperm genera, possibly due to polyploidy events. Mendelson (2003) found evidence for accelerated evolution of prezygotic over postzygotic isolation in the fish genus *Etheostoma*, possibly as a result of sexual selection or the absence of degenerate sex chromosomes in these fish.

A second feature of reproductive isolation identified by Coyne and Orr (1989, 1997) was that sympatric species pairs accumulate prezygotic, but not postzygotic, isolation more rapidly than allopatric pairs, presumably as a consequence of reinforcement (Dobzhansky 1937). Similar results have been reported by Marin et al. (1993), who performed mass pairwise crosses of 30 populations from 10 species of the *Drosophila repleta* group. However, Moyle et al. (2004) found no such pattern in the three angiosperm genera studied. A third feature is that Haldane's rule, that is, a bias toward hybrid sterility or inviability in the heterogametic sex (Haldane 1922), appears during the earliest stages of postzygotic isolation in male-heterogametic *Drosophila*. This result has also been observed for female-heterogametic taxa (Presgraves 2002; Price and Bouvier 2002).

A potential weakness of meta-analyses is that they typically incorporate an isolation index that compresses information about multiple characters into a single categorical metric. Studies generally combine either male and female hybrid sterility or hybrid sterility and hybrid inviability to accommodate data from a variety of experimental designs. Such an index can, for example, allow a species pair that produces many sterile male hybrids to be scored identically to a species pair that produces no male hybrids at all. While it then may be possible to draw conclusions regarding the relative rates of evolution of prezygotic and postzygotic isolation, in general, or of hybrid sterility and hybrid inviability, differences between forms of isolation that have been combined into indices could confound interpretation. For example, Coyne and Orr (1989) at first failed to detect a difference between the rate of evolution of hybrid sterility and hybrid inviability using a categorical description of sterility and inviability and combining male and female hybrid sterility. They were later able to detect a difference (Coyne and

Orr 1997), as have other studies (Wu 1992; Sasa et al. 1998; Presgraves 2002) using different analyses.

In this paper, we measure premating isolation, postmating isolation, male hybrid sterility, and female hybrid sterility for a single study system. We estimate evolutionary rates for each of these forms of reproductive isolation by using mitochondrial DNA (mtDNA) sequence divergence between allopatric populations from two *Cyrtodiopsis* species of Southeast Asian stalk-eyed flies. To avoid problems associated with isolation indices, we scale measures of isolation relative to within-population measurements prior to comparing them to one another. We also test if hybrid sterility and hybrid inviability conform to Haldane's rule, that is, if male hybrids exhibit sterility and/or inviability before female hybrids.

Diopsid stalk-eyed flies in the genus *Cyrtodiopsis* are well suited for studying the evolution of reproductive isolation for several reasons. First, because these flies are easily captured in the field and can be reared in captivity (Burkhardt and de la Motte 1983; Wilkinson 2001), many types of reproductive isolation can be measured in the laboratory. Second, both males and females are promiscuous and exhibit little or no precopulatory courtship behavior (Wilkinson et al. 1998a; Wilkinson et al. 2003). Thus, direct observations of copulation rates provide quantitative information on premating isolation. Third, while stalk-eyed flies are well known for having sexually dimorphic eyestalks that influence both female mate choice (Wilkinson et al. 1998a) and male-male competition (Panhuis and Wilkinson 1999), little to no divergence in eye-span allometry or body size has occurred between the populations used in this study (J. G. Swallow, L. E. Wallace, S. J. Christianson, P. M. Johns, and G. S. Wilkinson, unpubl. ms.). Thus, any reproductive isolation between populations is not likely to be the result of sexual selection on eye span. Fourth, robust phylogenetic hypotheses based on DNA sequence information are available for several species in the family (Baker et al. 2001) as well as among populations of two species in the genus (this paper). These populations occur on islands in the Sunda Shelf region of Southeast Asia and vary in geographic and genetic separation, but all exhibit evidence of X-chromosome meiotic drive (Wilkinson et al. 2003). Thus, they provide a natural experiment for inferring evolutionary change in reproductive isolation.

Materials and Methods

Study Populations

For this study we used six populations of *C. dalmanni* and two populations of *C. whitei* derived from flies captured in the Sunda Shelf region of Southeast Asia between January 1996 and September 2000 (Fig. 1). Populations were established with at least 50 adult individuals and subsequently kept at higher numbers in 30 × 30 × 30-cm Plexiglas population cages in the lab. *Cyrtodiopsis dalmanni* were captured near Cameron Highlands, Malaysia (4°15′N, 101°21′E); near Ulu Gombak, Malaysia (3°12′N, 101°42′E); near the Soraya field station, Sumatra (2°52′N, 97°54′E); near Bukit Lawang, Sumatra (3°35′N, 98°6′E); at a forestry research station in Bogor, Java (6°34′S, 106°50′E); and at the Kuela Belalong Field Station in Brunei, Borneo (4°30′N, 115°10′E). *Cyrtodiopsis whitei* were captured at Ulu Gombak and near Chiang Mai, Thailand (19°9′N, 98°7′E). We classified flies to species based on morphological comparisons to specimens housed at the National Museum of Natural History, Washington, D.C.

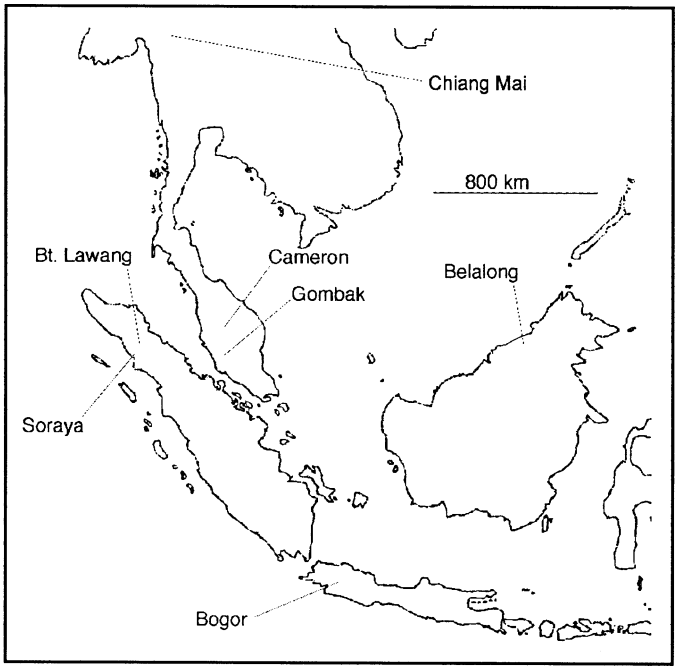

FIG. 1. Stalk-eyed fly collection localities in the Sunda shelf region of Southeast Asia. For site details and species collected, see text.

In the laboratory, stock populations were maintained in a humidified chamber at 25°C with a 12:12 h light-dark cycle. Adult animals were fed twice weekly with pureed corn treated with methyl paraben to inhibit mold. Flies used in mating trials were bred from stock populations by allowing females to oviposit on 50 ml of pureed corn in 100-ml plastic cups. Larvae were kept on the same light and temperature regime as their parents. After eclosion from the cups, flies were kept in single-sex cages for at least four weeks to ensure virginity and reproductive maturity (Lorch et al. 1993).

Phylogenetic Analysis

Phylogenetic relationships between the eight populations used in this study (Fig. 2) were inferred using partial gene sequences of two different mitochondrial genes, cytochrome oxidase II (COII) and 16S ribosomal RNA (16S). For each population we extracted DNA from five or more field-collected flies, which had been frozen or preserved in ethanol, using Qiamp tissue extraction kits (Qiagen, Valencia, CA). We then amplified the two gene fragments using primers and polymerase chain reaction (PCR) protocols optimized for diopsid flies (Baker and Wilkinson 2001). Using amplifying primers, we sequenced both strands of the products using BigDye cycle sequencing chemistry (PE Applied Biosystems, Foster City, CA) on an ABI 310 automated genetic analyzer. These partial gene sequences can be found in GenBank (COII: AY876495–AY876545; 16S: AY876546–AY876595). Sequence data for *Diopsis apicalis* (COII: AF304777; 16S: AF304742) and *Eurydiopsis argentifera* (COII: AF304764;

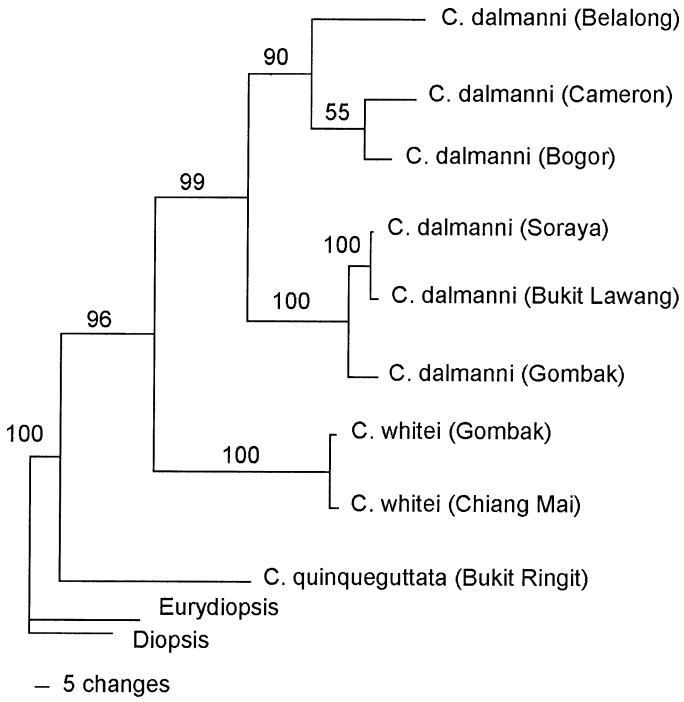

FIG. 2. Maximum-parsimony hypothesis for the phylogenetic relationships among populations using 889 bp of mitochondrial DNA (cytochrome oxidase II and 16S ribosomal RNA) from five flies per population. Numbers on each branch indicate bootstrap support.

16S: AF304729) were obtained from GenBank and included as outgroups to root the tree in the phylogenetic analyses.

Phylogenetic hypotheses were generated using maximum likelihood (ML) and maximum parsimony (MP) in PAUP* 4.0b10 (Swofford 2001) on the combined COII and 16S dataset. Exactly five animals per population were used for generating phylogenetic trees, but all available sequences were used for calculating genetic distances between populations. Optimal parameters of DNA substitution rates for ML searches were obtained using Modeltest 2.0 (Posada and Crandall 1998). The most appropriate model of DNA substitutions for this data set corresponded to a TIM model (transitional model: rAC = rGT ≠ rAT = rCG ≠ rAG ≠ rCT) with a transition: tranversion (ts:tv) ratio of about 1:4.4, rate heterogeneity (gamma distribution shape parameter = 4.85). Equal weighting and a ts:tv weighting scheme of 1:4 were used in the MP analyses. We conducted a single heuristic search using the ML criteria and 500 bootstrap replicates of heuristic searches using MP criteria. Gaps were treated as missing data and uninformative characters were excluded from analyses. Because all analyses resulted in similar phylogenetic trees, only the results of the parsimony analysis are presented.

Prezygotic Isolation

We tested for reproductive isolation by combining a male fly from one population with three female flies from another population in replicate cages. We used several, but not all, of the possible pairs of populations in an effort to maximize phylogenetic coverage without unnecessary replication of distant crosses (Table 1). We conducted experimental crosses

TABLE 1. List of crosses, sample sizes, and proportion nucleotide substitutions. Observation sample sizes before the slash are for the cross as listed in the left-hand column (male × female); those after the slash are for the reciprocal cross. For number of hybrids tested, the number before the slash represents flies whose male parent is listed first in the cross column; those after the slash are for flies whose female parent is listed first.

Cross	Observation sample size	Genetic distance	Number of hybrids tested	
			Male	Female
Within *Cyrtodiopsis dalmanni*				
Gombak × Soraya	16/15	0.0207	10/3	10/10
Gombak × Cameron	15/16	0.0576	0/0	0/1
Gombak × Belalong	14/15	0.0596	1/0	1/0
Gombak × Bukit Lawang	16/15	0.0211	10/10	10/10
Soraya × Cameron	15/15	0.0534	0/0	0/0
Soraya × Belalong	15/15	0.0533	0/0	2/0
Soraya × Bukit Lawang	16/16	0.0035	4/10	9/10
Cameron × Belalong	14/16	0.0399	3/0	10/4
Cameron × Bogor	15/15	0.0337	0/0	0/0
Belalong × Bogor	15/15	0.0376	10/10	10/10
Within *C. whitei*				
Gombak × Chiang Mai	15/17	0.0083	10/10	10/10
Between *C. dalmanni* and *C. whitei*				
Gombak × Chiang Mai	16/16	0.0783	0/0	0/0

in four rounds between May 2000 and March 2003. In the first round, we reciprocally crossed the two populations of *C. whitei*. In the second round, we crossed the Gombak, Soraya, Belalong, and Cameron populations of *C. dalmanni* in every possible combination. In the third round, we crossed Bukit Lawang and Bogor with a subset of the other *C. dalmanni* populations. In the final round, we tested the *C. dalmanni* Gombak and *C. whitei* Chiang Mai populations in reciprocal crosses. We also conducted all within-population crosses to estimate within-population fertility, behavior rates, progeny production, and copulation success.

To quantify reproductive isolation, we scored interest and success in mating using observations and female dissections. We observed flies in transparent Nalgene (Nalge Nunc International, Rochester, NY) mouse cages modified with ventilation and access holes and inverted on pans lined with moist cotton and blotting paper. The day before beginning observations we released three females individually marked with paint into a clean cage. Prior evidence indicates that mating behavior is most frequent at dawn and dusk (Lorch et al. 1993; Wilkinson et al. 1998a). Therefore, just before lights came on (0900 h) we released one unanesthetized male into each cage. We then timed all successful copulations and tallied all related male behavior not leading to successful mating for the ensuing 2.5 h using a hand-held tape recorder. We defined a successful copulation as one that exceeded 30 sec, which is long enough to transfer sperm in *C. whitei* (Lorch et al. 1993). Other behavior was recorded as ''pursuit,'' when a male chased or jumped toward a female but did not land on her, and ''copulation attempts,'' when a male alighted on a female but did not mate or mated for less than 30 sec. Observations on each day were balanced with respect to type of cross with either 15 or 16 cages observed at a time, depending on the round (Table 1).

After observation the flies remained in their cages for one week, after which we dissected females and examined their spermathecae for sperm. We anesthetized a female with carbon dioxide or cold, pulled out the two terminal abdominal segments with spermathecae attached while the female was still alive, and deposited the tissue into a drop of phosphate-buffered saline (PBS, pH = 7.4) on a microscope slide. Spermathecae were then gently squashed under a cover slip and immediately examined for sperm at 400X magnification with dark-field illumination. If any female in a cage contained sperm, we scored the male as successfully transferring sperm. We then calculated the proportion of all replicates of a cross in which sperm was transferred.

Postmating and Postzygotic Isolation

To quantify postmating and postzygotic isolation, we used the cages described above to score progeny production and hybrid fertility. We collected eggs in food cups for one week after mating observations ended, counted all eclosing offspring by sex, and kept hybrids for four weeks to reach reproductive maturity. Then, treating hybrids from reciprocal crosses as different types, we crossed up to five flies of each sex from each hybrid type with each parental population (Table 1). Each hybrid fly was housed with two flies of the opposite sex to ensure a fertile partner. Females were allowed to oviposit in food cups for 10 days, after which we removed and examined their spermathecae for stored sperm. We also counted by sex any offspring that eventually eclosed from the food cups. We scored female hybrids sterile when they produced no offspring but stored motile sperm. We scored male hybrids sterile if no motile sperm were stored by their mates and no progeny were produced. Because we were not able to assess egg fertility rates, we cannot determine whether low progeny counts reflect failure of sperm to fertilize eggs or a failure of embryonic development. Thus, hybrid progeny counts potentially confound postzygotic isolation with postmating prezygotic isolation. Hybrid fertility, however, is purely a measure of postzygotic isolation.

Data Analysis

We first converted observations of pursuit, attempted copulation, and successful copulation into hourly rates. Then we performed linear regression on each of those measures, as well as the proportion of males that successfully transferred sperm and the number of progeny produced, against genetic distance. Genetic distance was measured as the average pairwise uncorrected proportion of nucleotide substitutions for the 889 bp of mtDNA between individuals from two populations (Fig. 1). Our observations are not independent because every datapoint is a property of two populations and each population is represented in several datapoints. Thus, we pooled the data from reciprocal crosses and used the regression version of the Mantel test as described in Smouse et al. (1986). We determined statistical significance of our regression coefficients using randomization procedures (Manly 1996), as have other studies of reproductive isolation (Tilley et al. 1990; Gleason and Ritchie 1998; Moyle et al. 2004). We chose to perform 10,000 replicates, which gives the *P*-value a standard error of 0.002 when it is near the alpha level of 0.05; 1000 replicates gives a standard error roughly three times larger. Prior to reporting the results of any parametric analyses, we examined the residuals to ensure compliance with the standard assumptions of normality and homoscedasticity. If an assumption was violated, we transformed the data to correct the problem.

Evidence of asymmetrical reproductive isolation may indicate that pooling data from reciprocal crosses would be inappropriate. We therefore tested each of our 12 pairs of reciprocal crosses for potential asymmetry in copulation, pursuit, and copulation attempt frequency and progeny production using *t*-tests. We also tested for asymmetry in sperm transfer frequency and hybrid fertility using association tests and estimated significance from either a chi-squared statistic or Fisher's exact test. We analyzed the hybrid sexes separately and only performed the analysis if at least six hybrids from each of the two reciprocal crosses had been tested for fertility. To correct for multiple testing, we applied the sequential Bonferroni correction for Type I error within each set of tests (Rice 1989).

To test if hybrid sterility obeys Haldane's rule, we performed a Wilcoxon signed-rank test on the percent fertility of male versus female hybrids. To test if hybrid inviability obeys Haldane's rule, we calculated the brood sex ratio (percent male) produced by every between-population cross and performed a Mantel test by regressing sex ratio on genetic distance. Although progeny counts could be influenced by either hybrid inviability or gametic isolation, these data can still be used to test for Haldane's rule for inviability because progeny sex ratio should be independent of sperm-egg incompatibility. We performed two tests to assess the potential influence of meiotic drive on the pattern of progeny sex ratios. In the first we used a regression Mantel test on the percent of drive males, that is, those producing biased sex ratios, against genetic distance. In the second we tested if male drive phenotype was independent of the type of cross (within or between population) using a chi-squared contingency table. We assessed drive phenotype using a chi-squared goodness-of-fit test for departure from a 50:50 sex ratio. Only those males producing at least 20 offspring were tested.

To compare the rate of evolution between different types of reproductive isolation, we first converted all measurements to a common, continuous scale. For premating isolation we divided the mating frequency for each replicate of a between-population cross by the average of all replicates of the two corresponding within-population crosses. Similarly, for postmating isolation we divided the observed progeny count of each between-population replicate by the average progeny count for the maternal population. Then, to compare premating isolation (copulation rate) directly with postmating isolation (progeny production), we performed another Mantel test. In this test for each cross combination we subtracted scaled progeny production from scaled mating rate, and then regressed the resulting differences against genetic distance. A significant slope would, therefore, indicate that premating isolation evolves either more slowly or more rapidly than postmating isolation. Hybrid sterility could not be analyzed using linear regression methods due to poor fit of the data to a linear model. Instead, we compared male and female hybrid sterility separately to both progeny production and

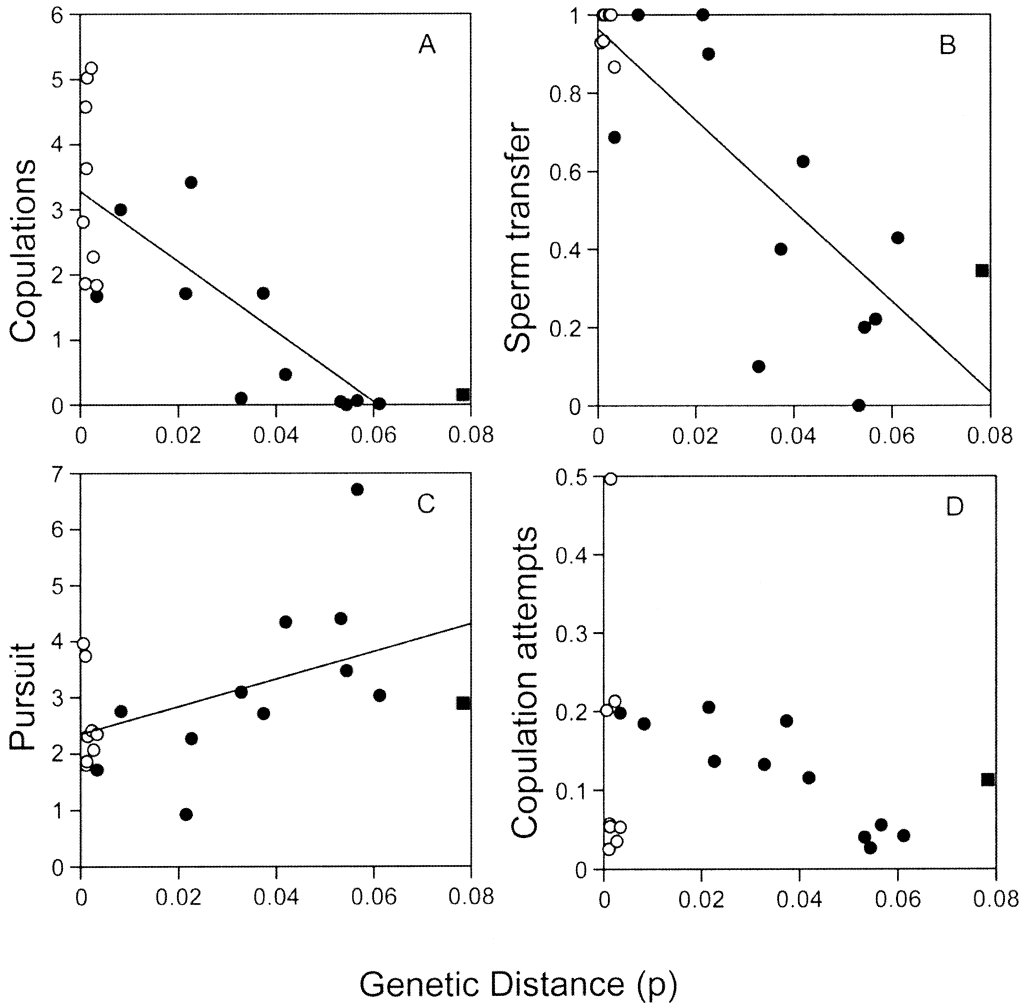

FIG. 3. Prezygotic isolation measures are plotted against genetic distance (p, the proportion uncorrected DNA sequence changes). Open circles show within-population values, filled circles show comparisons between conspecific populations, and the filled square represents the single between-species comparison. Within-population values indicate the average percent sequence divergence among five flies from each population. (A) Number of copulations observed per hour in cages containing one male and three females ($R^2 = 0.74$, $b = -22.21$, 95% confidence interval = −28.54, −15.86). (B) Proportion of replicate cages in which at least one female contained sperm in her spermathecae after one week ($R^2 = 0.68$, $b = -11.63$, 95% CI = −15.45, −7.85). (C) Number of times males chased females per hour ($R^2 = 0.20$, $b = 30.10$, 95% CI = 3.55, 46.00). (D) Number of unsuccessful copulation attempts per hour ($R^2 = 0.01$).

copulation rate using Wilcoxon signed-rank tests. Because the tabular *P*-values for this test are based on all possible permutations under the binomial distribution, a randomization procedure is unnecessary.

RESULTS

Phylogenetic Relationships

The combined COII and 16S dataset included 889 characters, 184 of which were phylogenetically informative. The weighted analysis (i.e., 1:4 ts:tv) resulted in two most parsimonious trees; a phylogram of the bootstrap consensus tree is presented in Figure 1. Each of the three described species of *Cyrtodiopsis* forms a monophyletic unit, with bootstrap support ranging from 96% for *C. dalmanni* to 100% for the other two species. In addition, all five individuals from each population form a monophyletic unit with significant bootstrap support. The six populations of *C. dalmanni* form two distinct clades separated by 5.3% sequence difference.

Prezygotic Isolation

Prezygotic isolation increases monotonically with genetic distance as evidenced by a decline in copulation frequency with increasing population divergence ($P < 0.002$, Fig. 3A). Between-population copulation frequencies are always below the within-population averages (Table 2), and the incidence of mating falls nearly to zero at 5% sequence divergence. No pairs of reciprocal crosses exhibited significant asymmetry in copulation frequency (Table 3). The frequency of sperm storage in females (Fig. 3B) exhibits a pattern that is similar to copulation frequency in that it declines with increasing genetic distance ($P < 0.002$), but for some between-population crosses sperm storage occurs more often than within

TABLE 2. Within-population average (± 1 SE) for hourly behavior frequencies, sperm transfer rate, and progeny production over a one-week period.

Population	*n*	Copulation	Sperm transfer	Pursuit	Copulation attempts	Progeny count
Cyrtodiopsis dalmanni						
Bukit Lawang	15	1.9 ± 0.61	1.000	10 ± 4.1	0.07 ± 0.067	36 ± 8.6
Bogor	15	5.0 ± 0.42	1.000	6 ± 1.3	1.3 ± 0.34	130 ± 14
Belalong	14	2.8 ± 0.64	0.929	9 ± 1.4	0.5 ± 0.20	28 ± 8.3
Cameron	15	1.8 ± 0.26	0.867	5.4 ± 0.63	0.13 ± 0.091	24 ± 6.0
Gombak	15	4.6 ± 0.76	0.933	4 ± 1.1	0.13 ± 0.091	60 ± 12
Soraya	15	3.6 ± 0.38	1.000	4 ± 1.0	0.13 ± 0.091	70 ± 13
C. whitei						
Gombak	15	2.3 ± 0.55	1.000	4 ± 1.8	0.07 ± 0.067	38 ± 7.5
Chiang Mai	17	5.2 ± 0.47	1.000	4.8 ± 0.83	0.4 ± 0.24	65 ± 8.1

populations. This result indicates that sperm were transferred during the week of cohabitation between male and females in at least some cages where no mating was observed during the 2.5-h observation period. Two pairs of reciprocal crosses showed significant asymmetry in this measure (Table 3).

In contrast to the pattern exhibited by copulation and sperm storage, pursuit frequency (Fig. 3C) increases with genetic distance ($P = 0.010$) and often exhibits a higher rate of occurrence between than within populations. We failed to detect an effect of genetic distance on copulation attempts ($P = 0.29$, Fig. 3D). No significant asymmetry was detected between reciprocal crosses in either of these measures (Table 3).

Postmating Isolation

Progeny counts (Fig. 4A) decline with genetic distance ($P < 0.002$), with cross averages falling to zero at 5% sequence divergence. No pairs of reciprocal crosses exhibited significant asymmetry in progeny production (Table 3). Brood sex ratio does not vary with genetic distance ($P = 0.92$), indicating that no sex bias in hybrid viability could be detected. Brood sex ratio does appear to be affected by the presence of males carrying meiotic drive in all populations. Of 166 males that produced enough progeny to be tested for departure from a 1:1 brood sex ratio, 41 (24.7%) had significantly female-biased brood sex ratios. However, the fraction of males expressing female-biased brood sex ratios does not covary with the genetic distance of the cross ($P = 0.155$). Furthermore, the drive phenotype of a male is independent of the type (within or between populations) of cross (chi square = 0.076, $P = 0.783$).

Hybrid Sterility

Hybrid males were sterile (Fig. 4B) except for two crosses involving recently isolated populations. In *C. whitei*, Gombak males crossed with Chiang Mai females (0.83% sequence divergence) produced seven fertile and three sterile males, and the reciprocal cross produced three fertile and seven sterile males. In *C. dalmanni*, Gombak males crossed with Bukit Lawang females (2.2% sequence divergence) produced six fertile and four sterile males. The reciprocal cross between Gombak and Bukit Lawang produced no fertile males, making this the only pair of crosses to show significant asymmetry (Table 3). In contrast, female hybrid fertility was generally high, averaging 71 ± 11.9% until genetic divergence exceeded 5%, above which no fertile hybrids of either sex were found. Hybrid fertility of females is higher than males (Wil-

TABLE 3. Results of asymmetry tests. Table values are *t* (first four columns) or chi-square (last two columns) statistics. Numbers before the slash in the last column are for male hybrid fertility, after are for female hybrid fertility. Significance was calculated using a sequential Bonferroni correction applied to each column of *P*-values (Rice 1989).

Cross	Progeny count	Copulation frequency	Pursuit frequency	Attempt frequency	Sperm transfer	Hybrid fertility
Bukit Lawang × *Cyrtodiopsis dalmanni* Gombak	1.91	−0.91	−1.46	−1.59	[2]	10.77*/1.05
Bukit Lawang × Soraya	0.18	3.05	−2.17	1.25	5.24	[2]/1.72
Bogor × Belalong	2.71	3.06	1.84	−0.49	5.00	[2]/1.05
Bogor × Cameron	1.00	−0.87	0.79	−1.05	0.37	[3]
Belalong × Cameron	−3.11	−3.24	−2.34	1.47	16.63***	[3]
Belalong × *C. dalmanni* Gombak	−1.51	−1.00	−1.35	−0.65	0.58	[3]
Belalong × Soraya	−1.46	[1]	−1.07	[1]	7.50	[3]
Cameron × *C. dalmanni* Gombak	1.00	1.00	−0.28	0.15	4.21	[3]
Cameron × Soraya	[1]	1.86	1.17	−0.59	[2]	[3]
C. dalmanni Gombak × Soraya	0.11	−0.08	0.70	−0.58	[2]	[3]/1.17
Chiang Mai × *C. whitei* Gombak	−0.71	1.87	1.39	−0.72	[2]	3.20/[2]
Chiang Mai × *C. dalmanni* Gombak	[1]	1.89	1.46	−2.20	16.76***	[3]

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.
[1] Statistic < 0.001.
[2] Test not performed because one column of the contingency table contained only sperm transfer or hybrid fertility rates of zero.
[3] Test not performed because there were not enough surviving hybrid offspring.

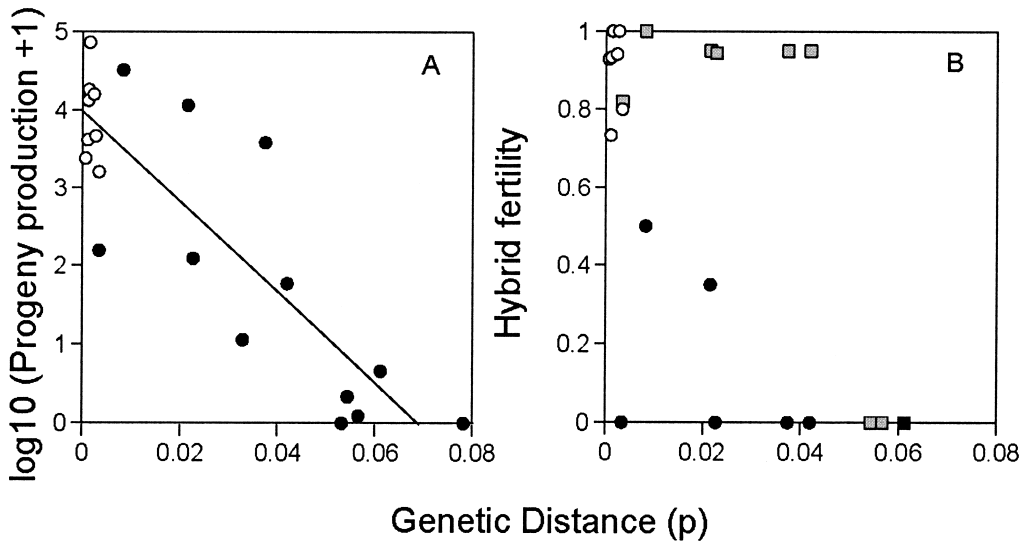

FIG. 4. Postzygotic isolation measures are plotted against genetic divergence (p, the proportion uncorrected mtDNA sequence changes). (A) Progeny production, after $\log_{10}$ transformation, with symbols as in Figure 3 ($R^2 = 0.74$, $b = -57.61$, 95% CI -73.95, -41.24). (B) Proportion of hybrids that produced progeny when mated. Open circles represent within-population males, closed circles represent male hybrids. Squares represent female hybrids. Within-population female values could not be calculated from these data.

coxon signed-ranks test, $S = -21$, $P = 0.031$), consistent with Haldane's rule for sterility.

Relative Rates of Evolution

We failed to find a difference between the rates at which prezygotic isolation (mating frequency) and postmating isolation evolve (Mantel test, $P = 0.705$). However, male hybrid sterility evolves sooner than both prezygotic isolation (Wilcoxon signed-ranks test, $S = -14$, $P = 0.016$) and postmating isolation ($S = -14$, $P = 0.016$). Female hybrid sterility evolves later than prezygotic isolation ($S = 15$, $P = 0.0391$), but we failed to detect a difference between female sterility and postmating isolation ($S = 5.5$, $P = 0.570$).

DISCUSSION

Prezygotic Isolation

Our results indicate that in *Cyrtodiopsis*, as in *Drosophila*, Lepidoptera, and other taxa, prezygotic isolation increases monotonically over time. This pattern is indicative of a gradual accumulation of multiple factors of small effect. Copulation and sperm transfer frequencies both exhibit a decrease with increasing genetic distance, indicating that as populations become isolated from each other the probability of successful mating decreases. This result indicates that either male or female mating interest is depressed in crosses between genetically distant populations. Because male pursuit behavior increases with genetic distance, it appears that males continue to seek mating opportunities from females that reject them. These results are consistent with mating discrimination evolving more rapidly in females than in males.

The cause of the observed prezygotic isolation is not yet known, because little obvious morphological evolution has taken place between these populations (J. G. Swallow, L. E. Wallace, S. J. Christianson, P. M. Johns, and G. S. Wilkinson, unpubl. ms.). Divergence between populations in traits affecting mate choice appears to be insufficient to explain the observed relationship between mating frequency and genetic distance. Neither body size nor eyestalk allometry differs between populations, but the Belalong population does have a distinctive eyestalk phenotype: instead of protruding horizontally from the head, the eyestalks are at a visibly higher angle. If mating interest was affected by this difference, we would expect to observe asymmetries in behavior in most crosses involving the Belalong population, but this did not occur. Divergence in courtship behavior (Price and Boake 1995) and cuticular hydrocarbons (Coyne et al. 1994; Coyne and Charlesworth 1997) have been shown to produce reproductive isolation in *Drosophila*. However, *C. whitei* and *C. dalmanni* display no obvious courtship behavior (Wilkinson et al. 1998a), that is, there is no ritualized precopulatory touching that might allow flies to sense nonvolatile waxes on the cuticle of a potential partner. Whether volatile compounds or other behavioral or morphological cues affect prezygotic isolation in these flies deserves further study.

Postmating and Postzygotic Isolation

We found evidence for Haldane's rule with respect to hybrid sterility but not hybrid inviability. Over the range of genetic distances we sampled, progeny sex ratio did not change as a function of genetic distance, as would be expected if male hybrids were less viable than female hybrids. Also, the frequency of males expressing meiotic drive was consistent with previously published estimates for *Cyrtodiopsis* (Wilkinson et al. 2003). In contrast, sex bias in hybrid sterility was apparent even for populations with very little sequence divergence, before complete sterility became the rule. The lowest sequence divergence we sampled at which all female hybrids were sterile was 5.4%, whereas the lowest value at which all male hybrids were sterile was 2.3%, a 2.4-fold difference. Thus, hybrid male sterility has evolved considerably faster than hybrid female sterility in these animals.

That we observed Haldane's rule for hybrid sterility and not inviability may not be surprising. Although both types of postzygotic isolation are thought to be caused by the accumulation of deleterious epistatic (Dobzhansky-Muller) incompatibilities (Dobzhansky 1937; Muller 1940, 1942; Orr and Turelli 2001), separate genetic causes may be involved (Presgraves and Orr 1998). Sterility-causing incompatibilities accumulate more rapidly in male than female *Drosophila* (the faster-male theory: Wu and Davis 1993), and by extension other male-heterogametic taxa, possibly due to sexual selection (Wu et al. 1996). Inviability-causing incompatibilities become visible first in the heterogametic sex due to the expression of deleterious X-linked recessive mutations when in the hemizygous state (the dominance theory: Orr 1993; Turelli and Orr 1995). If one mechanism operates faster than the other, it should be possible to detect species pairs that diverged in the time period between the appearance of Haldane's rule for sterility and inviability. Some of the *Cyrtodiopsis* populations examined in this study are consistent with this prediction. However, even though we found no change in the brood sex ratio of hybrids with genetic distance, we did find a highly significant decline in progeny production. As Haldane's rule is an early and nearly ubiquitous form of postzygotic isolation (Coyne and Orr 1989), this decline may reflect gametic isolation instead of or in addition to hybrid inviability. Further study to determine the fertilization rate of eggs in between-population crosses is needed to separate hybrid inviability from gametic isolation.

Relative Rates of Evolution

We found two differences between hybrid sterility and postmating isolation in *Cyrtodiopsis*. First, hybrid male sterility, but not hybrid female sterility, has evolved faster than postmating isolation. Even if we have completely confounded sperm-egg incompatibility with hybrid inviability, our measure of postmating isolation sets an upper limit on the amount of hybrid inviability. Thus, we can be certain that male hybrid sterility evolves faster than hybrid inviability. This result shows the advantage of analyzing forms of reproductive isolation separately rather than combining traits into a composite isolation index. Second, hybrid sterility in *Cyrtodiopsis* shows a different pattern of evolution from postmating isolation. Postmating isolation increases gradually with no detectable change in brood sex ratio over the range of sequence divergence in this study. Sterility, however, evolves abruptly in a sex-specific pattern. Male hybrid sterility is incomplete at low genetic distance, but quickly becomes complete. Female hybrids all have similar fertility at genetic distances below 5% but are sterile above that level.

Whether these results differ from other taxa is difficult to determine with certainty because other studies have either lacked hybrid sterility information (Mendelson 2003), combined sterility and inviability data into one isolation index to compare prezygotic to postzygotic effects (Zouros 1973; Coyne and Orr 1989; Sasa et al. 1998), or lacked prezygotic isolation data (Sasa et al. 1998; Presgraves 2002; Price and Bouvier 2002). However, a genetic study of postzygotic isolation in *Drosophila* that tested many small introgressions found more male hybrid sterility loci than either hybrid inviability or female hybrid sterility loci (Tao and Hartl 2003). Moreover, hybrid sterility evolves faster than inviability in many taxa (Wu 1992; Sasa et al. 1998; Presgraves 2002; Price and Bouvier 2002). These studies also showed compliance with Haldane's rule, indicating faster evolution of sterility in the heterogametic hybrid sex. Thus, other taxa seem to follow the same pattern of evolution seen in *Cyrtodiopsis*: first male hybrid sterility, then female hybrid sterility, then hybrid inviability. How the rates of evolution of male and female hybrid sterility and inviability considered separately compare to prezygotic isolation in these other taxa is not known. In addition, quantitative measurement of postzygotic isolation will be necessary to determine if hybrid sterility also evolves abruptly in those taxa.

Accelerated Evolution of Male Hybrid Sterility

Postzygotic isolation could evolve rapidly by more than one mechanism. Possibilities include endosymbionts such as *Wolbachia* (Werren et al. 1986; Breeuwer and Werren 1990), intragenomic conflict from selfish genetic elements (Frank 1991; Hurst and Pomiankowski 1991), antagonistic coevolution (Rice 1996, 1998), and sexual selection (Wu and Davis 1993; Wu et al. 1996). However, not all mechanisms will produce accelerated male hybrid sterility in relation to female hybrid sterility, hybrid inviability, and prezygotic isolation. Tao and Hartl (2003) argued that experimental data supports a new faster-heterogametic-sex hypothesis of Haldane's rule. This hypothesis, which is related to an earlier hypothesis that divergent sex chromosome meiotic drive systems contribute to Haldane's rule (Frank 1991; Hurst and Pomiankowski 1991), suggests a major role of genomic conflict in the rapid evolution of male hybrid sterility. Intragenomic and cytoplasmic-nuclear genome conflicts affecting progeny sex ratio may be particularly important. At least two types of conflict, meiotic drive and sperm competition, are present and affect one another in *C. whitei* (Wilkinson and Fry 2001; Fry and Wilkinson 2004) and are intimately linked to sexual selection (Wilkinson et al. 1998b; Wilkinson et al. 2003). Future research is needed to determine how much influence these forms of conflict and sexual selection have on speciation in this genus.

Acknowledgments

We are grateful to M. Muscedere, E. Kelleher, E. Lichtenberg, and A. Phillips for assistance in the lab; E. Russek-Cohen, L. Douglass, and E. Martins for statistical advice; and P. M. Johns, M. Noor, and two anonymous reviewers for comments on the manuscript. This work was supported by a predoctoral fellowship from the National Science Foundation and a National Science Foundation Research Training Grant fellowship to SJC, by a National Research Service Award from the National Institutes of Health (F32 GM20377–01) and grants from the American Philosophical Society and the National Science Foundation (MRI/RUI-0216473) to JGS, and by grants DEB-0077878 and DEB-0343617 from the National Science Foundation to GSW.

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Corresponding Editor: M. Noor