

Sexual Isolation Between Sympatric and Allopatric Populations of *Drosophila pseudoobscura* and *D. persimilis**

Wyatt W. Anderson^{1,2} and Yong-Kyu Kim¹

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According to reinforcement theory, sexual isolation between species in sympatry is strengthened by natural selection against maladaptive hybrids. *Drosophila pseudoobscura* and *D. persimilis* from four locations where these species are sympatric, and from three locations where only *D. pseudoobscura* has been found, were utilized in studies of sexual isolation. Multiple-choice observation chambers were used to record matings between sympatric and allopatric strains of the two species. There was a wide variation in sexual isolation between the two species in the four localities we studied. The average isolation index for sympatric strains of the species was not significantly different from the average index for allopatric strains. There were no meaningful differences between the isolation indices in sympatric and allopatric strains of the species. The failure to find a relationship is likely the result of gene flow among populations within the two species.

KEY WORDS: Allopatry; *Drosophila*; mating; reinforcement; sexual isolation; speciation; sympatry.

INTRODUCTION

Speciation was the process at the heart of Darwin's theory of evolution, and it remains at the core of evolutionary biology today. One of Dobzhansky's (1937) greatest contributions to the modern synthesis of evolutionary theory was the concept of reproductive isolating mechanisms that reduce or eliminate gene flow between species. Sexual isolation is a key isolating mechanism, and acoustic, olfactory, tactile, and visual signals are involved in sexual isolation between *Drosophila* species, playing different roles in different species (Spieth, 1968). Coyne and Orr (1997, 2004) review a number of studies with *Drosophila* of the degree of sexual isolation between pairs of closely-related species.

Dobzhansky (1940) proposed that natural selection should act to reinforce rudimentary sexual isolation between populations in the process of diverging towards status as separate species. Hybrids between different species are generally rare in nature, and often sterile or inviable as a consequence of genetic divergence between them. Sexual isolation may arise in such *allopatric* populations (Muller, 1942), but is reinforced by natural selection against less-fit hybrids in *sympatric* areas of overlap (see Howard, 1999, for a recent review).

In a preliminary experiment, Dobzhansky placed the closely related species *D. pseudoobscura* and *D. persimilis* together in a laboratory population to determine whether sexual isolation between them would be strengthened by natural selection against hybrids; he found that indeed it was (Dobzhansky, 1945). In larger-scale experiments, his student Koopman (1950) utilized different mutant markers in each species to recognize hybrids between them, which he then discarded each generation. Koopman observed an appreciable increase in sexual isolation between these species. There have been a number of other studies with *Drosophila* species demonstrating

¹ Department of Genetics, University of Georgia, Athens, GA, 30602-7223, USA.

² Address for correspondence: Wyatt W. Anderson, Department of Genetics, University of Georgia, Athens, GA, 30602-7223, USA. Tel: +706-542-7001. Fax: +706-542-3910. e-mail: wyatt@uga.edu

* This paper is dedicated to Prof. Lee Ehrman – excellent scientist, friend, and mentor.

reinforcement of sexual isolation by artificial selection in the laboratory (e.g., Dobzhansky and Pavlovsky, 1971; Ehrman, 1971, 1983; Kessler, 1966; Kim *et al.*, 1996).

Evidence for reinforcement of sexual isolation in nature is not as strong as that in laboratory populations (Marshall *et al.*, 2002). It has been reported in several *Drosophila* species (Dobzhansky and Koller, 1938; Ehrman, 1965; Noor, 1995; Wasserman and Koepfer, 1977), but not in all (Coyne *et al.*, 2002). Noor (1995) measured sexual isolation between *D. pseudoobscura* and *D. persimilis* from locations where the species were sympatric, and from locations where they were allopatric, and he reported stronger sexual isolation where the species were sympatric. Because sexual isolation between *D. pseudoobscura* and *D. persimilis* is an important case study for the reinforcement of sexual isolation, we have reexamined the sexual isolation between allopatric and sympatric populations of these species.

MATERIALS AND METHODS

Drosophila Collections and Culture

Drosophila pseudoobscura and *D. persimilis* collected by WWA and his research colleagues during 1993–1995 were utilized in this study, along with a strain of *D. pseudoobscura* collected by Drs. Diana Alvarez and Manuel Ruiz-Garcia, near Bogota, Colombia in 1997. *D. pseudoobscura* ranges from Canada southward through Mexico and Guatemala. It has not been collected in Central America, but was first collected in South America, near Bogota, Colombia in 1959 (Hunter, 1960). The distribution of *D. persimilis* is included within that of *D. pseudoobscura*, ranging from British Columbia in the north to Mt. San Jacinto in southern California. Isofemale lines were set up with fertilized females from nature and maintained thereafter at 16 °C in the laboratory. Males collected in nature were mated to female *D. pseudoobscura* from a lab stock. *Drosophila pseudoobscura* and *D. persimilis* differ by a fixed inversion on chromosome XL, and the species were identified from salivary chromosome preparations as described by Anderson *et al.* (1977).

Sympatric strains of both *D. pseudoobscura* and *D. persimilis* were collected at Death Valley, California; James Reserve (adjacent to Mt. San Jacinto), California; Mather (near Yosemite National Park), California; and Spray, (in north-central) Oregon. The frequency of *D. persimilis* in the females and males

collected at these sites provides a measure of sympatry which we have put into three categories: *low* (James Reserve, 2.4% of 167 flies); *moderate* (Spray, 7.9% of 89 flies, and Death Valley, 9.4% of 117 flies); and *high* (Mather, 58.0% of 88 flies). The allopatric strains of *D. pseudoobscura* came from locations where *D. persimilis* has not been collected in the past: Mesa Verde, Colorado; Flagstaff, Arizona; and Sutatausa, near Bogota, Colombia. Sexual isolation was measured between *D. persimilis* and *D. pseudoobscura* from each of the four locations where both species were collected, and then with *D. pseudoobscura* from the three locations where *D. persimilis* has never been collected. We are designating these two combinations of the species as “sympatric” and “allopatric”, although we realize no truly allopatric populations of *D. persimilis* are known, where *D. persimilis* but not *D. pseudoobscura* has been collected.

In these experiments, all *Drosophila* cultures were kept at 22 °C in half-pint bottles on a food medium made of cornmeal, brewer’s yeast, molasses, and agar, to which a small amount of propionic acid was added to reduce the growth of molds. Virgin males and females were collected every 8 hours. To reduce effects of density during early development on mating behaviors of adults, a maximum of 20 virgin flies of the same sex were kept in each holding vial, where they were aged for seven days before experimentation.

Observations of Matings

Noor (1995) placed a single male and a single female in a vial for 24 hours. He dissected the female and recorded a mating if sperm were found in spermathecae or seminal receptacles. This technique is designated “no-choice” because there is no alternative mate available. Mating during the 24 hours of confinement reflects courtship by the male and acceptance by the female, but it is not clear how long the male courted and whether acceptance occurred within a short time period or took longer. The failure of a pair to mate may reflect continued rejection of the male by the female, a failure of the male to court, or a failure of the male to continue courtship after rejection.

We wanted to measure matings when a choice of mates from these two species was available. *Drosophila* often mate where they feed (Carson, 1971), so a multiple-choice measurement seems more realistic than a no-choice one. Thus, we chose to

observe matings in chambers containing equal numbers of males and females of *D. pseudoobscura* and *D. persimilis*. The multiple-choice observation chambers we used are like those described by Elens and Wattiaux (1964), except that ours are built of clear plastic instead of glass and wood. These observation chambers are circular, approximately 12 cm in diameter and 1.3 cm deep, with a transparent top and paper towel across the bottom. An aspirator is used to introduce flies through a hole into the chamber.

Groups of 12 virgin males and 12 virgin females of each species, 7 days old, were introduced into a single mating chamber. Species were distinguished by minimally notching the wings of only one species, so that mating pairs could be identified. Four replicate chambers were observed for each combination of species. In two replicates the wings of *D. pseudoobscura* males and females were notched, and in the other two the wings of *D. persimilis* males and females were notched. In these species notching has not been found to affect male activity during courtship or mating, nor is notching correlated with female discrimination of mates (Dobzhansky and Powell, 1975). Observations were carried out at 22 °C. All matings were recorded for 60 min. Females mated only once during our observations, and copulations lasted approximately 4–5 min.

Casares *et al.* (1998) have carefully examined the techniques used to measure sexual isolation in *Drosophila*. They note a problem when multiple-choice experiments continue so long that flies of all genotypes are not available for mating. They recommend that observations be discontinued when either 50% of potential matings have occurred or after 30 min. Many of the interspecific matings in our experiment occurred after the first half of matings, but before 30 min, so we chose to base statistical analyses on data for 30 min of observation. We present data for both the first 30 min and for the entire 60 min of observations.

Statistical Analyses

The joint isolation index for both sexes, (Malogolowkin-Cohen *et al.*, 1965) was used to measure degrees of sexual isolation in our multiple-choice tests. It is the fraction of homogamic matings minus the fraction of heterogamic matings. It is defined as

$$I = (X_{AA} + X_{BB} - X_{AB} - X_{BA})/N,$$

where X_{AA} , X_{BB} , X_{AB} , and X_{BA} represent the number of matings between females and males of strain A,

females and males of strain B, females of strain A and males of strain B, and females of strain B and males of strain A, respectively. N is the total number of matings. I ranges from 1.00 (total isolation, all homogamic matings), through 0 (random mating), to -1.00 (all heterogamic matings).

A two-factor analysis of variance was performed on the isolation indices from the four replicate chambers scored for each allopatric or sympatric combination of *D. pseudoobscura* and *D. persimilis* in each of the four localities studied.

RESULTS

Table I presents the data from observations of matings between *D. persimilis* and *D. pseudoobscura* in multiple-choice tests. The joint isolation index for males and females of both species is given. None of the sexual isolation indices from the full 60 min of observations differed significantly from the indices calculated for the first 30 min of observation.

As shown in Figure 1, the mean I for sympatric strains of *D. pseudoobscura* and *D. persimilis* is 0.75, and is lower than the mean of 0.78 for allopatric strains of the two species. This difference is not statistically significant. The analysis of variance gave an F -ratio of 0.43 for comparisons of sympatric and allopatric strains of the two species, which for 1 degree of freedom has an associated probability of 0.51. The analysis of variance gave an F -ratio for 3.51 for differences among localities, which for 3 degrees of freedom has an associated probability of 0.02. There were clearly significant differences among localities. The interaction of sympatric/allopatric strains and localities was highly significant, with an F -ratio of 4.36, which for 3 degrees of freedom has an associated probability of 0.01.

To get a better idea of what the data show, we calculated χ^2 for 2×2 tables of homospecific and heterospecific matings in the sympatric and grouped allopatric strains of *D. pseudoobscura* and *D. persimilis* for each locality. Only for James Reserve did the χ^2 indicate a statistically significant difference between sympatric and allopatric strains of the two species: $\chi^2 = 6.17$, $df = 1$, and $p < 0.025$. In this one case of a clear and statistically significant difference between sympatric and allopatric strains of the species, the allopatric strains showed *stronger* sexual isolation than the sympatric ones.

The joint isolation indices between *D. pseudoobscura* and *D. persimilis* from all the combinations

Table I. Sexual Isolation (Measured as the Joint Isolation Index *I*) Between *D. persimilis* (P) and *D. pseudoobscura* (S) in Sympatry and in Allopatry

Populations	Duration	<i>N</i>	Mating Combinations				<i>I</i>
			P♀ × P♂	P♀ × S♂	S♀ × P♂	S♀ × S♂	
<i>Death Valley, CA</i>							
Sympatry	60	96	46	2	2	46	0.92
	30	88	39	2	2	45	0.91
Allopatry (Mesa Verde, CO)	60	96	46	2	4	44	0.88
	30	91	43	2	4	42	0.87
Allopatry (Flagstaff, AZ)	60	96	47	1	4	44	0.90
	30	85	40	1	4	40	0.88
Allopatry (Bogota, Colombia)	60	96	46	2	9	39	0.77
	30	87	39	2	9	37	0.75
<i>James Reserve, CA</i>							
Sympatry	60	96	28	20	0	48	0.58
	30	80	18	17	0	45	0.58
Allopatry (Mesa Verde, CO)	60	79	23	8	0	48	0.80
	30	69	19	7	0	43	0.80
Allopatry (Flagstaff, AZ)	60	92	26	18	0	48	0.61
	30	82	26	13	0	43	0.68
Allopatry (Bogota, Colombia)	60	86	34	4	0	48	0.91
	30	64	16	2	0	46	0.94
<i>Mather, CA</i>							
Sympatry	60	96	44	4	2	46	0.88
	30	92	41	4	2	45	0.87
Allopatry (Mesa Verde, CO)	60	96	38	10	3	45	0.73
	30	92	35	10	3	44	0.72
Allopatry (Flagstaff, AZ)	60	96	37	11	1	47	0.75
	30	86	31	11	1	43	0.72
Allopatry (Bogota, Colombia)	60	96	42	6	7	41	0.73
	30	89	36	6	7	40	0.71
<i>Spray, OR</i>							
Sympatry	60	96	32	16	0	48	0.67
	30	88	25	15	0	48	0.66
Allopatry (Mesa Verde, CO)	60	74	22	4	1	47	0.86
	30	66	17	4	1	44	0.85
Allopatry (Flagstaff, AZ)	60	96	39	9	3	45	0.75
	30	92	38	9	3	42	0.74
Allopatry (Bogota, Colombia)	60	96	32	16	2	46	0.63
	30	82	26	10	2	44	0.71

of localities are large and statistically significant, as expected for different species. In Figure 2, these indices are depicted separately for matings between *D. pseudoobscura* and *D. persimilis* from the four locations where these species are sympatric. The data do not show greater sexual isolation where *D. persimilis* was rarest, as would be expected if contact with the much commoner *D. pseudoobscura* resulted in reinforcement of sexual isolation (Noor, 1995). Quite the opposite, sexual isolation was weakest in James Reserve, where *D. persimilis* was at its lowest frequency in the collections from nature. Sexual iso-

lation between the two species was considerably different for Spray and for Death Valley, two localities where the frequency of *D. persimilis* was nearly the same. Sexual isolation between the species was about as high in Death Valley, where the frequency of *D. persimilis* was 9%, as it was at Mather, where *D. persimilis* was 58% of the collection. In two populations the sexual isolation index was smaller in the sympatric strains than in the allopatric ones. In the other two, the sexual isolation index was higher in the sympatric strains than in the allopatric ones. Thus, there does not seem to be a meaningful association of

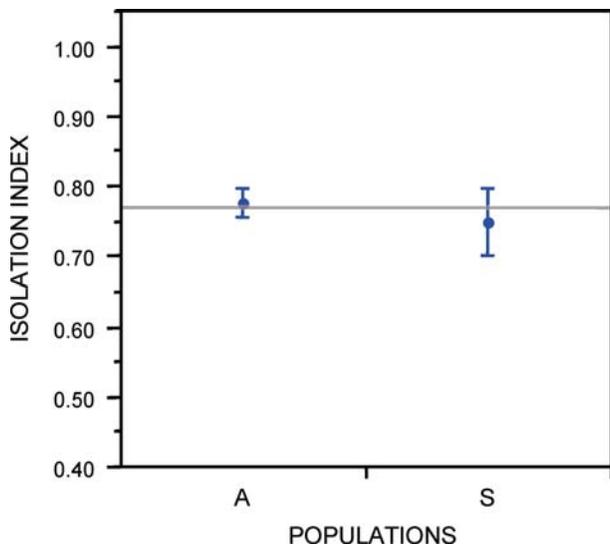


Fig. 1. The average isolation index for allopatric (A) and sympatric (S) populations of *D. persimilis* and *D. pseudoobscura*. The horizontal line is the grand mean of isolation indices.

the sexual isolation indices for the sympatric and allopatric strains of *D. pseudoobscura* and *D. persimilis*.

The matings between *D. pseudoobscura* and *D. persimilis* given in Table I show that the mating propensities of *D. pseudoobscura* females and males were higher than those for *D. persimilis* females and

males. In 30 min of observation, 54.7% of the matings involved *D. pseudoobscura* females, and 60.5% involved *D. pseudoobscura* males. *Drosophila persimilis* females mated with *D. pseudoobscura* males three times as frequently as *D. pseudoobscura* females mated with *D. persimilis* males.

DISCUSSION

Our measurements do not provide support for the hypothesis that selection has reinforced sexual isolation between *D. pseudoobscura* and *D. persimilis* to a greater extent in localities where they overlap, than in localities where they do not. The average isolation index for sympatric populations of the two species is actually *lower* than the average index for allopatric populations, although the difference is not statistically significant. We measured sexual isolation between the species in four locations where the two species are sympatric and compared them with measurements of sexual isolation between *D. persimilis* from these locations and *D. pseudoobscura* from three locations where *D. persimilis* is unknown. Our analyses of these populations show a wide variation in sexual isolation between the two species and no meaningful difference between the joint isolation indices in sympatric and allopatric strains of the two species.

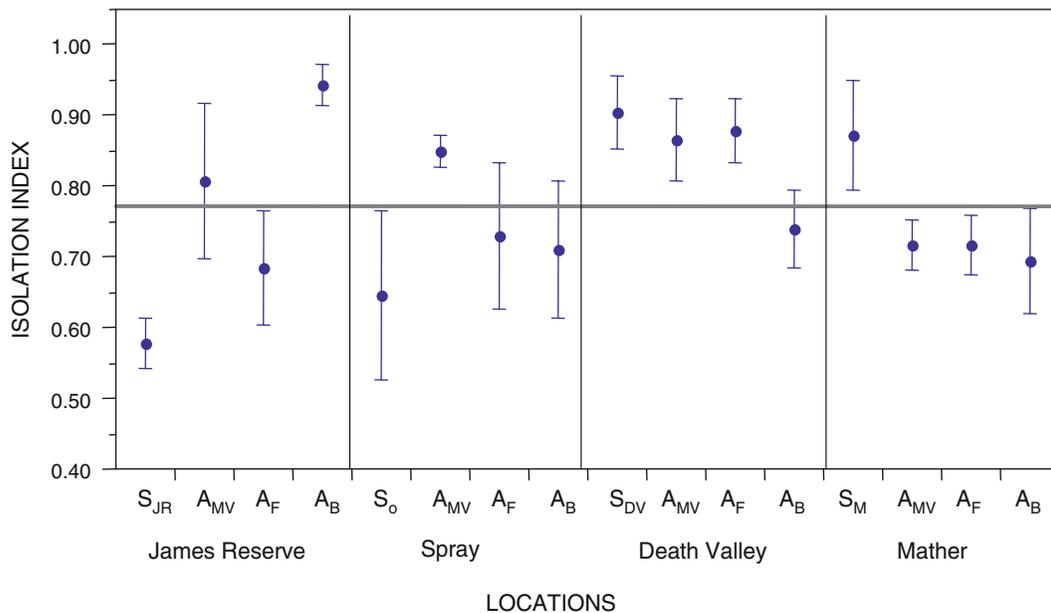


Fig. 2. Comparison of isolation indices among allopatric (A) populations and sympatric (S) populations of *D. persimilis* and *D. pseudoobscura* JR (James Reserve), O (Spray), DV (Death Valley), M (Mather), MV (Mesa Verde), F (Flagstaff), and B (Bogota). The bracket indicates one standard error above and below each isolation index. The horizontal line is the grand mean of isolation indices.

Drosophila pseudoobscura females strongly discriminated against *D. persimilis* males, while *D. persimilis* females were less discriminating. These observations are consistent with Kaneshiro's (1976) behavioral survey on *D. silvestris*, which showed that females of ancestral populations tended to discriminate in copulation tests against males of daughter populations due to the losses of courtship elements.

The *D. pseudoobscura* populations near Bogota constitute an interesting case, because *D. pseudoobscura* in Bogota seems likely to have been isolated from the mainbody of the species for 155,000 years (Schaeffer and Miller, 1991), or perhaps as much as 500,000 years (Wang and Hey, 1996). *D. pseudoobscura* and *D. persimilis* diverged some 600,000 (Hey and Nielsen, 2004) to 1,000,000 (Wang and Hey, 1996) years ago, so that sexual isolation from *D. persimilis* was achieved before the Bogota populations were founded. Thus, it is reasonable that the Bogota strain of *D. pseudoobscura* shows strong but variable sexual isolation from the strains of *D. persimilis* against which it was tested.

The reinforcement of isolating mechanisms by natural selection, as proposed by Dobzhansky (1940), is a natural outgrowth of the central role played by isolating mechanisms in creating species as distinct gene pools, and of natural selection as the agency responsible for the isolating mechanisms. Interest in reinforcement has grown in the last few years (Marshall *et al.*, 2002; Noor, 1999; Servedio and Noor, 2003), and Noor's (1995) study of sexual isolation between *D. pseudoobscura* and *D. persimilis* has figured prominently in this renewed interest in reinforcement. He and his colleagues (Ortiz-Barrientos *et al.*, 2004; Dixon Schully and Noor, 2004) have gone well beyond initial analyses of sexual isolation between populations of *D. pseudoobscura* and *D. persimilis* to clarify the genetic basis for the sexual isolation between these two species. Noor's (1995) measurements of sexual isolation do not involve a choice of mate, however, and we feel the multiple-choice technique better represents the mating process as it occurs in nature. Spieth and Ringo (1983) have proposed that the best design for measuring mating behavior is the one that imitates nature most closely, so that the multiple-choice design is preferable to others (see also Casares *et al.*, 1998). With this experimental design, females and males are free to choose and to copulate with preferred partners, rather than being forced to copulate with a given partner or not to copulate at all, as in a no-choice test. Ortiz-Barrientos *et al.* (2004) reported that a single *D. pseudoobscura* female was surrounded by multiple

males in many areas at Mather, a situation that clearly provides a choice of mates for the female. Of course, any experimental study is somewhat artificial, and rearing and handling conditions may also affect behavior. Spieth and Ringo (1983) point out that it may be worthwhile to attempt measurements under more natural conditions, and we agree that doing so is an important goal for future studies. Sexual isolation is influenced not only by experimental design, but also by male and female mating propensities, experience-mediated courtship modification (Siegel *et al.*, 1984), frequency-dependent mating propensities (Ehrman, 1966; Spiess, 1970), and rare male mating advantage (Anderson and Brown, 1984; Ehrman, 1970; Knoppien, 1985).

Our study is close in concept to that of Dobzhansky and Koller (1938). They measured sexual isolation between strains of *D. miranda* from the Puget Sound area of the Pacific Northwest and *D. pseudoobscura* from the Puget Sound area, as well as *D. pseudoobscura* from elsewhere. *D. miranda* is closely related to *D. pseudoobscura* and *D. persimilis*. In the same way, they measured sexual isolation between strains of *D. miranda* from Lone Pine Canyon in the Sierra Nevada (referred to as "Whitney" in Dobzhansky and Koller, 1938) and *D. pseudoobscura* from Lone Pine Canyon and elsewhere. They reported stronger isolation between *D. miranda* and *D. pseudoobscura* from the Pacific Northwest than between *D. miranda* from the Northwest and *D. pseudoobscura* from other localities. They did *not*, however, find this same relationship at Lone Pine Canyon. Thus, it is hard to say that *D. miranda* and *D. pseudoobscura* show greater isolation in general where they are sympatric, since this relationship was found in one location where both occur, but not in another.

Issues of gene flow are important to considerations of the reinforcement of sexual isolation. *Drosophila pseudoobscura* shows a geographical pattern of frequencies for inversions of the third chromosome that appears to be tied to environmental differences (Dobzhansky, 1944). A good example, among many, is a very high frequency of the inversion Arrowhead across the vast Great Basin of the western United States, while Standard, Treeline, and others are found more frequently along the Pacific coast (Anderson *et al.*, 1991; Dobzhansky, 1944). *D. pseudoobscura* marked with paint or with mutant alleles and released into nature did not disperse very far (Dobzhansky and Wright, 1947), indicating relatively low gene flow by active dispersal

among populations. Dobzhansky (1974) later recognized the importance of passive transport, probably by air currents, to gene flow in *D. pseudoobscura*.

Studies of allozymic variation (Jones *et al.*, 1981) and of DNA sequence variation for several molecular markers (Schaeffer and Miller, 1992; Wang *et al.*, 1997) have provided a very different picture than did release–recapture studies. DNA sequence variation does not show the same geographic pattern as inversions, and there appears to be enough gene flow to tie the mainbody of *D. pseudoobscura* together as essentially one panmictic unit. Only a few migrants among populations each generation are required to achieve such a continuous population structure. Machado *et al.* (2001) have even provided evidence from analysis of DNA sequence variation that gene flow has occurred between *D. pseudoobscura* and *D. persimilis* after these species separated, although not in the recent past.

Hey and Nielsen (2004) reported that loci inside or near the inversions that separate *D. pseudoobscura* and *D. persimilis* (see Noor *et al.*, 2001) show little evidence of gene flow, and they propose that natural selection has opposed gene flow at these loci. The geographical differentiation of inversion frequencies is very likely the result of strong local selection acting on large blocks of genes held together by chromosomal inversions as “supergenes.” It is not clear that the genes responsible for sexual isolation would be locally differentiated by selection to reinforce sexual isolation, or whether this character would spread across the species range by gene flow. There is evidence that geographic populations of *D. pseudoobscura* do not show sexual isolation from each other. Anderson and Ehrman (1969) used the multiple-choice technique to examine possible sexual isolation among five geographically distant populations of *D. pseudoobscura*. They found no significant sexual isolation in either the data for the entire 75 min of observation or for the first 30 min.

Experimental studies like that of Koopman (1950) make it clear that natural selection can indeed reinforce the sexual isolation between *D. pseudoobscura* and *D. persimilis*. The studies cited above, however, make it likely that gene flow within each species has been sufficient to infuse geographic populations across the mainbody of the species with genes fostering sexual isolation. Our failure to find a clear relationship between the degree of sexual isolation between sympatric and allopatric strains of the two species is likely a result of this gene flow.

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