Early events in speciation: Polymorphism for hybrid male sterility in *Drosophila*

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Capturing the process of speciation early enough to determine the initial genetic causes of reproductive isolation remains a major challenge in evolutionary biology. We have found, to our knowledge, the first example of substantial intraspecific polymorphism for genetic factors contributing to hybrid male sterility. Specifically, we show that the occurrence of hybrid male sterility in crosses between *Drosophila mojavensis* and its sister species, *Drosophila arizonae*, is controlled by factors present at different frequencies in different populations of *D. mojavensis*. In addition, we show that hybrid male sterility is a complex phenotype; some hybrid males with motile sperm still cannot sire offspring. Because male sterility factors in hybrids between these species are not yet fixed within *D. mojavensis*, this system provides an invaluable opportunity to characterize the genetics of reproductive isolation at an early stage.

Understanding the nature and number of genetic changes that underlie early events in speciation remains a major challenge in evolutionary biology. Experiments to identify "speciation genes" have become popular and often focus on loci affecting hybrid male sterility, an attractive trait because it is considered "discrete" and thus easy to score. Whereas sterility of the heterogametic sex (usually male) is one of the most common (1) and presumably earliest manifestations of postzygotic reproductive isolation (2), the genetic basis for its appearance remains obscure (3). According to the Dobzhansky–Muller view (4–6), postzygotic isolation is initiated by multiple loci, but most of the empirical support for this model involves species pairs between which time has permitted the accumulation of additional genetic changes subsequent to the initial speciation event (7). From such pairs of species, it therefore is impossible to disentangle which or how many factors arose first. Approaches to resolving this issue require two components. First, tests for hybrid sterility must employ species that are still at the early stages of divergence. Second, hybrid sterility must be measured in a way that can detect the action of multiple factors, if they exist.

To identify those loci at which the first incompatibilities arise, species still in the initial stages of differentiation must be examined. Ideally, factors causing postzygotic isolation would have arisen but would not yet have reached fixation in one or more separate populations. Unfortunately, most studies have not been conducted in ways that allow polymorphisms for hybrid male sterility to be detected because they use mass matings from multifemale cultures. There is some evidence that hybrid inviability, which tends to arise at greater genetic divergence than hybrid male sterility (8, 9), exhibits some natural variation in the *Drosophila melanogaster* species group. Mutations that rescue inviable hybrids have been found in some lines of *D. melanogaster* and its relatives (10–13), but the degree of polymorphism at loci responsible for hybrid rescue in these species has not been investigated. Some evidence exists, also, for polymorphism in hybrid sex ratio bias in *Tribolium* sp. (flour beetle) (14). Despite these suggestive findings, no study has yet characterized levels of naturally occurring variation for factors causing postzygotic isolation in any animal taxon. Knowing the nature and number of such factors would serve as the first step in identifying their functions and the nature of their interactions.

The North American species *Drosophila mojavensis* and its sibling *Drosophila arizonae,* are a relatively young pair of incipient species. The genetic distance (*D*) between *D. arizonae* and *D. mojavensis* is only 0.212 in contrast to *D. melanogaster–Drosophila simulans* at 0.550 and *Drosophila pseudoobscura–Drosophila persimilis* at 0.410, and on par with *Drosophila sechellia–D. simulans* at 0.280, other popular systems for the study of the genetics of speciation (8). In addition, pre- and postzygotic isolation between the species is incomplete (15). Hybrid male sterility originally had been thought to be exclusively unidirectional (16), because only the sons of *D. arizonae* females and *D. mojavensis* males lack motile sperm and thus are clearly sterile. The hybrid male sterility observed in sons of *D. arizonae* mothers and *D. mojavensis* fathers has been shown to result from an interaction between the *D. mojavensis* Y chromosome and the *D. arizonae* fourth chromosome (16–19). In sons of the reciprocal cross, no male sterility originally was observed (16–19). Subsequently, however, a strain of *D. mojavensis* was discovered on Santa Catalina Island, California, and when females of this population were crossed to *D. arizonae* males they were reported to produce sterile sons exclusively (20). Because it appears that hybrid male sterility depends on the source of the maternal population, the responsible factors clearly are not yet fixed in *D. mojavensis*, providing an unusual opportunity to examine the genetic basis for a postzygotic incompatibility arising early in speciation.

We used the opportunity presented by the Santa Catalina Island population of *D. mojavensis* to ask two critical questions. First, what is the extent of polymorphism within and among *D. mojavensis* populations for factors producing F_1 male sterility when crossed to *D. arizonae*? In other words, do all or just a proportion of *D. mojavensis* females from the Catalina Island population produce sterile sons in interspecific crosses? Furthermore, do any *D. mojavensis* females from other geographic populations produce sterile hybrid sons from matings with *D. arizonae* males? Second, does the F_1 male sterility, when observed, have a complex phenotypic and potentially genotypic basis; that is, does it initially arise as isolation due to breakdown at more than one aspect of the sperm morphological or functional phenotype?

An informative approach to identify within-population phenotypic variation is to test for hybrid sterility by using individual mothers from separate isofemale lines, as opposed to using mass matings from mass cultures. Previous studies of hybrid male sterility in *Drosophila* (8, 9) scored sperm motility of male progeny from mass interspecific matings, precluding the opportunity to detect among-mother variability in sterile son produc-

Abbreviations: DE, El Desemboque, Sonora, Mexico; TO, Torete, Baja California Sur, Mexico; OPNM, Organ Pipe National Monument, AZ; WC, Whitmore Canyon, AZ; CI, Santa Catalina Island, CA; PERA, Peralta Canyon Trailhead in the Superstition Wilderness, AZ.

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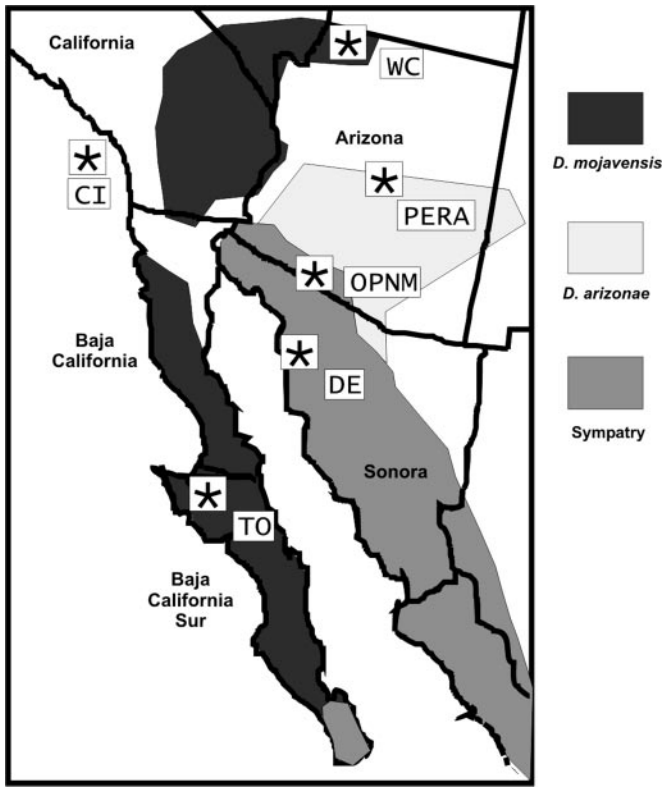

Fig. 1. Map of the southwestern United States and northwestern Mexico showing the distributions of *D. mojavensis* and *D. arizonae* and the areas of sympatry. The distribution of *D. arizonae* continues south of the range of the map to Guatemala. The populations used in this study are marked with asterisks. The *D. mojavensis* populations are from DE, TO, OPNM, WC, and CI. The *D. arizonae* tester population is from PERA.

tion. An isofemale line is comprised of the descendents of a single wild-caught female. Because each isofemale line represents a distinct genetic sample from the wild, variation found across isofemale lines reflects the phenotypic variation found in the wild. By using isofemale lines, the overall phenotypic variation in the species, therefore, can be partitioned into within population variation and variation occurring between genetically differentiated *D. mojavensis* populations.

We also sought evidence that hybrid male sterility has a complex basis. Specifically, we asked whether, in addition to the phenotype of sperm immotility, sterility is observed in hybrid males whose sperm are motile.

Materials and Methods

Strain of Flies. Isofemale lines of *D. mojavensis* were established from wild-caught females at four localities (Fig. 1): El Desemboque, Sonora, Mexico (DE), in November 2000; Torete, Baja California Sur, Mexico (TO), in January 2001; Organ Pipe National Monument, AZ (OPNM), in October 2000; Whitmore Canyon (near the Grand Canyon), AZ (WC), in March 2002, and Santa Catalina Island, CA (CI) in April 2001. A tester population of *D. arizonae*, derived from a multifemale collection made in April 1997 at the Peralta Canyon Trailhead in the Superstition Wilderness, AZ (PERA) (Fig. 1) was used in all interspecific crosses. Flies were maintained on banana-opuntia culture medium and, with the exception of *D. arizonae*, which has been maintained in the laboratory for 4 years, were used in experiments within a year and a half of their collection. We screened lines of both species for *Wolbachia* endosymbionts with specific PCR primers (Table 1) and found no evidence that it was present.

Table 1. Primers used for *Wolbachia* screens

Primer name	Primer sequence
wspF	TGGTCCAATAAGTGATGAAGAAAC
wspR	AAAAATTAAACGCTACTCCA

Data are from ref. 21.

Experimental Crosses. Virgin males and females were sorted by sex while anesthetized under light CO_2 for a maximum of 3 min, then stored with 20 or fewer flies per yeasted food vial until they were sexually mature, at 9 days of age. Mature virgin females from each of the *D. mojavensis* isofemale lines were mated to males from the PERA strain of *D. arizonae.* Because interspecific matings are most successful when set up with groups of flies rather than single pairs, multiple mature virgin females from a single isofemale line (*D. mojavensis*) were placed in a common container with multiple mature males (*D. arizonae*) and were allowed to mate for 24 h. Each female was then placed in an individual yeasted food vial to lay eggs. In this way, the number of interspecific matings obtained was maximized, but at the same time, we kept track of individual female's hybrid male offspring. As a control, *D. mojavensis* females were crossed to males of their own strain, as were female *D. arizonae*, providing baseline data for levels of within-strain male fertility.

Scoring Hybrid Male Fertility Phenotypes. The hybrid offspring of individual female *D. mojavensis* were collected, sexed, and counted. F_1 hybrid males were held in yeasted food vials for 9 days to assure that they had reached sexual maturity (22). A subset of males were aged longer before dissection to control for any delays in spermatogenesis as observed with hybrid males in another pair of *Drosophila* species (23). Sperm motility has been the standard criterion for determining fertility in *Drosophila* speciation studies (8, 9). Hybrid males thus were dissected and the motility of sperm in the seminal vesicle and testis was scored as either motile (if one or more sperm was observed to move) or nonmotile. Dissected material was observed for 60 sec before assigning a score, although if sperm were not observed to be motile after 10 sec, their score was not found to change. All male offspring from a given female, up to $n = 5$, if available, were assayed. Males from control crosses were processed in an identical fashion. In all, 1,433 hybrid males from 394 mothers and 63 isofemale lines were scored. There was no evidence of hybrid dysgenesis in the form of gonadal abnormalities or aspermia.

Statistical Analyses. Statistical analyses were performed by using the GLIMMIX macro (SAS Institute, Cary, NC), which allows for nested variables, a mixed model, and a binomial error distribution. This approach to the analysis was guided by the work of Krakow and Tkadlec (24) and uses an iterative search to fit parameter estimates. The analysis was performed on binary data, treating each F_1 son as a separate binary point (motile or nonmotile) with a logit link function, nested within mother, which was nested within isofemale line which, in turn, was nested within population. Mother nested in isofemale line and population, and isofemale line nested in population, were modeled as random effects, whereas population was treated as a fixed effect. Similar results are obtained from other methods of analysis, such as by using all fixed effects, a normal error distribution, or pooling F_1 son data within mother.

The effects of male age on average proportion of hybrid males with motile sperm was tested by using a paired two-tailed Student's t test on arcsin-transformed proportion data.

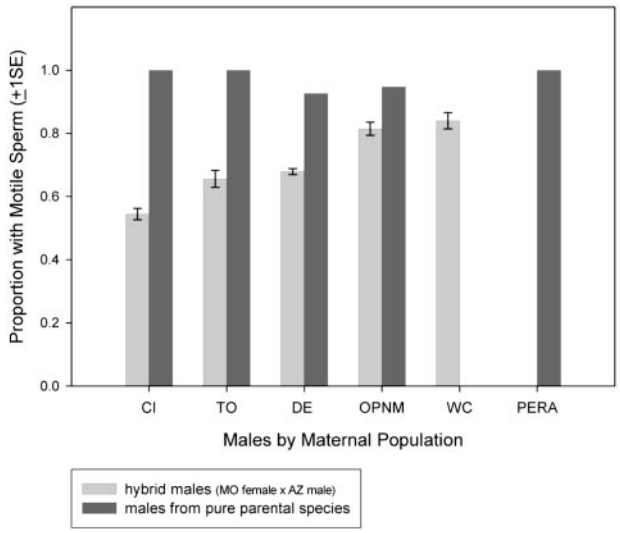

Fig. 2. The average proportion of males with motile sperm (±1 SE) by population. Light bars represent the average across isofemale lines for hybrid F_1 males from the cross of female *D. mojavensis* from the population shown and male *D. arizonae* (PERA) (number of isofemale lines: CI $n = 15$; TO $n = 13$; DE $n = 14$; OPNM $n = 6$; WC $n = 8$). Dark bars are the average motility of males from the pure species cultures (number of males per population: CI $n = 30$; TO $n = 30$; DE $n = 68$; OPNM $n = 95$; PERA $n = 77$). Average male sperm motility drops from between 92% and 100% in all of the pure species stocks to between 54% and 84% when males are hybrids. There is also significant variation between populations for hybrid male motility ($F = 3.06$, df = 4/50.9, $P = 0.0246$).

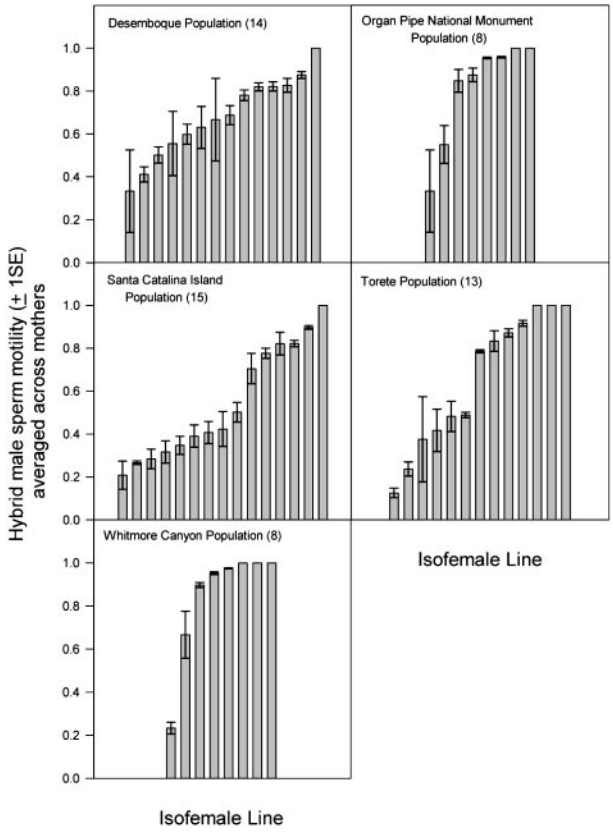

Fig. 3. The fraction of F_1 hybrid sons with motile sperm from the cross of female *D. mojavensis* and male *D. arizonae* averaged by mother within isofemale line. Isofemale lines are shown in ascending order of the average fraction of sons with motile sperm organized by population. The error bars indicate 1 SE above and below the mean. The number of females tested per isofemale line in ascending order of the average fraction of sons with motile sperm are as follows. DE: 3, 8, 7, 3, 13, 4, 3, 11, 5, 5, 7, 12, 10, and 2; OPNM: 3, 8, 7, 4, 11, 4, 2, and 2; CI: 6, 5, 10, 7, 11, 16, 7, 5, 11, 6, 18, 8, 9, 15, and 2; TO: 2, 9, 2, 6, 11, 4, 2, 3, 7, 4, 2, 6, and 5; and WC: 12, 6, 23, 7, 8, 3, 6, and 5. There is significant variation within isofemale line ($Z = 6.86$, $P < 0.0001$) and between isofemale lines ($Z = 3.32$, $P = 0.0005$) for hybrid male motility.

Results and Discussion

Polymorphism for Factors Causing F_1 Male Sterility. We observed an extreme decrease in the number of hybrid males with motile sperm relative to pure species males (Fig. 2.). Furthermore, the frequency of hybrid males with immotile sperm varied within and among populations of *D. mojavensis* from which the mothers were derived (Figs. 2 and 3). These data demonstrate intraspecific polymorphism for factors that cause hybrid male sterility, and show that geographically separated populations of *D. mojavensis* differ in the frequency of these factors. The greatest decrease in hybrid male sperm motility was observed in crosses when mothers were from the CI population. Significant variation in hybrid male sperm motility was detected at every hierarchical level, within isofemale line (nested within population) ($Z = 6.86$, $P < 0.0001$), between isofemale lines (nested within population) ($Z = 3.32$, $P = 0.0005$), and between populations ($F = 3.06$, df = 4/50.9, $P = 0.0246$). The variation across isofemale line is shown in Fig. 3. Significant variation at each Hierarchal level is consistent with segregation, within all five *D. mojavensis* populations, for a genetic factor or factors causing hybrid male sperm motility. Contrary to an earlier report (16), the factor(s) is therefore neither fixed in, nor restricted to, the population of *D. mojavensis* from CI.

We asked also whether, as in male hybrids of the *D. melanogaster* species group (23), observed sperm immotility could be explained by delayed male sexual maturity. A subset of 60 F_1 males from 12 mothers (Table 2) were scored for sperm motility at a later age (18 instead of 9 days) than their brothers. Although there was a trend toward an increase in the average proportion of hybrid males with motile sperm with increasing age, the difference between older and younger males was not significant (paired $t = 1.755$, df = 11, $P = 0.107$).

Complex Nature of Hybrid Male Sterility. The second question we addressed was whether hybrid male sterility appears to be a complex trait, meaning, a trait with multiple, genetically-controlled phenotypic levels. As noted earlier, hybrid male sterility in *Drosophila* has typically been assessed by the presence of absence of motile sperm (8, 9). Although clearly, if sperm are immotile they will be nonfunctional, the reverse is not necessarily true. Male sterile mutations in *D. melanogaster* include some phenotypes with motile sperm (25). Thus, sperm function is genetically heterogeneous, influenced by different and distinctly controlled genetic attributes, some but not all of which may be manifest as motility. If motile hybrid male sperm are nonfunctional, it would provide the first evidence that when F_1 male sterility arises, it has a more complex basis than previously assumed.

To test the assumption that hybrid males with motile sperm are fertile, we examined the relationship between motility and fertility in the F_1 hybrid sons of *D. mojavensis* mothers from the CI strain and fathers from the *D. arizonae* tester strain. First, we mated mature F_1 sons from CI mothers to their sisters and to females of both parental strains. Once mated, the hybrid males were dissected and their sperm motility was scored. An average of 17.5 hybrid males from each of eight isofemale lines were mated and dissected. Females mated to hybrid males (10 per isofemale line) were saved to see whether they produced progeny (Table 3). Matings of hybrid males from four of the isofemale lines produced offspring and all

Table 2. Effects of age on proportion of hybrid males with motile sperm

Population and isofemale line of mother	Proportion motile sperm in 9-day-old hybrids (*n*)	Proportion motile sperm in 18-day-old hybrids (*n*)
CI-4	0.5 (4)	0.51 (7)
CI-7	0.64 (11)	0.40 (5)
CI-8	0.75 (4)	0.67 (3)
CI-9	0.60 (5)	0.40 (5)
CI-10	0.00 (7)	0.00 (5)
CI-10	0.40 (5)	0.33 (3)
CI-10	0.50 (8)	1.00 (4)
CI-16	0.00 (3)	0.78 (9)
DE-6	1.00 (3)	1.00 (4)
TO-8	0.00 (4)	0.83 (6)
TO-13	0.33 (3)	0.67 (3)
TO-13	0.50 (6)	0.67 (6)
Mean proportion motile (±SE)	0.43 (±0.028)	0.61 (±0.024)

Sons from individual *D. mojavensis* females mated to *D. arizonae* males were dissected at 9 or 18 days post-eclosion and scored for motile sperm.

Table 3. Ability of hybrid males from the cross of *D. mojavensis* females from CI to *D. arizonae* males to sire progeny

CI isofemale line, no.	Proportion hybrid males with motile sperm (*n*)	Progeny produced
6	0.84 (38)	Yes
9	0.95 (22)	Yes
10	0.90 (21)	Yes
11	0.67 (15)	Yes
12	0.91 (23)	No
3	0.67 (12)	No
15	0.33 (3)	No
16	0.67 (6)	No

Hybrid males were mated to their sisters and/or females from their parental lines. Mated males subsequently were scored for the presence of motile sperm as well as whether their female mates produced larvae.

of these hybrid males, not surprisingly, were observed to have motile sperm. Also, as expected, F_1 hybrid males found to have no motile sperm fathered no offspring. Hybrid males from four of the eight isofemale lines that produced hybrid sons with motile sperm, however, were unable to sire offspring. The species of the female to which hybrid males were crossed had no effect on the outcome.

Motility, thus, is not a direct indicator of fertility in F_1 hybrid males. Furthermore, the fact that it is not suggests that hybrid male sterility does not have a simple basis. A locus or loci in addition to controlling motility could control another function connected with sperm storage, recovery from storage, or ability to penetrate the micropyle. It also is possible that a gene of major effect determines sperm motility in hybrid males but that it exhibits incomplete penetrance with respect to whether motile sperm are functional. Modifiers at other loci could influence the number of sperm that are motile as well as their vigor, none of which typically are measured in studies of hybrid male sterility in *Drosophila*. Examination of sperm by light microscopy and transmission electron microscopy could reveal the nature and number of defects in sperm of hybrid males.

The existence of within- and between-population variability for postzygotic incompatibility factors provides a system in which to test the dynamics (number, location, function, and population genetics) of speciation genes before their fixation and interaction with postspeciation mutations. Because the loci causing sterility in the reciprocal cross (*D. arizonae* females × *D. mojavensis* males) have already been assigned to particular chromosomes (17–19), an obvious question is whether the same or different chromosomes are implicated in the observations reported here. Also unclear is the role, if any, these factors play in reproductive character variation within and between *D. mojavensis* populations.

Finally, given our observations, earlier *Drosophila* speciation studies probably tell only a partial story about hybrid male sterility in those systems (8, 9). First, crossing parents from strains derived from mass collections, as opposed to the more labor-intensive method of crossing isofemale lines, masks within-species variability for the very factors of interest. Previous studies (8, 9) of closely related taxa may have missed existing polymorphism for hybrid sterility factors. Second, the fact that hybrid males with motile sperm are often unable to produce offspring suggests that the numerous *Drosophila* studies that used motility as the hybrid male fertility measure may be severely underestimating the actual degree of hybrid male sterility. Hybrid sterility frequencies may actually be several fold higher, as a rule, than previously reported, and therefore this trait may be evolving even more rapidly relative to hybrid inviability than has been previously estimated.

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1. True, J. R., Weir, B. S. & Laurie, C. C. (1996) *Genetics* **142,** 819–837.
2. Haldane, J. B. S. (1922) *J. Genet.* **12,** 101–109.
3. Orr, H. A. & Presgraves, D. C. (2000) *BioEssays* **22,** 1085–1094.
4. Muller, H. J. (1942) *Biol. Symp.* **6,** 71–125.
5. Muller, H. J. (1940) in *The New Systematics*, ed. Huxley, J. (Clarendon, Oxford), pp. 185–268.
6. Dobzhansky, T. (1937) *Genetics and the Origin of Species* (Columbia Univ., New York).
7. Orr, H. A. (1995) *Genetics* **139,** 1805–1813.
8. Coyne, J. A. & Orr, H. A. (1989) *Evolution (Lawrence, Kans.)* **43,** 362–381.
9. Coyne, J. A. & Orr, H. A. (1997) *Evolution (Lawrence, Kans.)* **51,** 295–303.
10. Hutter, P., Roote, J. & Ashburner, M. (1990) *Genetics* **124,** 909–920.
11. Lachaise, D., David, J. R., Lemeunier, F., Tsacas, L. & Ashburner, M. (1986) *Evolution (Lawrence, Kans.)* **40,** 262–271.
12. Sawamura, K., Watanabe, T. K. & Yamamoto, M. T. (1993) *Genetica (The Hague)* **88,** 175–185.
13. Davis, A. W., Roote, J., Morley, T., Sawamura, K., Herrmann, S. & Ashburner, M. (1996) *Nature* **380,** 157–159.
14. Wade, M. J. & Johnson, N. A. (1994) *Heredity* **72,** 155–162.
15. Markow, T. A. & Hocutt, G. D. (1998) in *Endless Forms: Species and Speciation*, eds. Howard, D. J. & Berlocher, S. H. (Oxford Univ. Press, New York), pp. 234–244.
16. Zouros, E. (1981) *Drosophila Inf. Serv.* **56,** 167–168.
17. Zouros, E., Lofdahl, K. & Martin, P. A. (1988) *Evolution (Lawrence, Kans.)* **42,** 1321–1331.
18. Pantazidis, A. C. & Zouros, E. (1988) *Heredity* **60,** 299–304.
19. Vigneault, G. & Zouros, E. (1986) *Evolution (Lawrence, Kans.)* **40,** 1160–1170.
20. Ruiz, A., Heed, W. B. & Wasserman, M. (1990) *J. Hered.* **81,** 30–42.
21. Braig, H. R., Zhou, W., Dobson, S.I. & O'Neill, A. L. (1998) *J. Bacteriol.* **180,** 2373–2378.
22. Markow, T. A. (1982) in *Ecological Genetics and Evolution: The Cactus-Yeast-Drosophila Model System*, eds. Barker, J. S. F. & Starmer, W. T. (Academic, London), pp. 273–287.
23. Naviera, H. F. & Maside, X. R. (1998) in *Endless Forms: Species and Speciation*, eds. Howard, D. J. & Berlocher, S. H. (Oxford Univ. Press, New York), pp. 330–338.
24. Krackow, S. & Tkadlec, E. (2001) *Behav. Ecol. Sociobiol.* **50,** 293–301.
25. Wakimoto, B. T., Lindsley, D. L. & Herrera, C. (2004) *Genetics* **167,** 207–216.