Why Are There Males in the Hermaphroditic Species *Caenorhabditis elegans*?

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ABSTRACT

The free-living nematode worm *Caenorhabditis elegans* reproduces primarily as a self-fertilizing hermaphrodite, yet males are maintained in wild-type populations at low frequency. To determine the role of males in *C. elegans*, we develop a mathematical model for the genetic system of hermaphrodites that can either self-fertilize or be fertilized by males and we perform laboratory observations and experiments on both *C. elegans* and a related dioecious species *C. remanei*. We show that the mating efficiency of *C. elegans* is poor compared to a dioecious species and that *C. elegans* males are more attracted to *C. remanei* females than they are to their conspecific hermaphrodites. We postulate that a genetic mutation occurred during the evolution of *C. elegans* hermaphrodites, resulting in the loss of an attracting sex pheromone present in the ancestor of both *C. elegans* and *C. remanei*. Our findings suggest that males are maintained in *C. elegans* because of the particular genetic system inherited from its dioecious ancestor and because of nonadaptive spontaneous nondisjunction of sex chromosomes, which occurs during meiosis in the hermaphrodite. A theoretical argument shows that the low frequency of male mating observed in *C. elegans* can support male-specific genes against mutational degeneration. This results in the continuing presence of functional males in a 99.9% hermaphroditic species in which outcrossing is disadvantageous to hermaphrodites.

THE stability of a dioecious species with equal numbers of males and females requires explanation. In species without parental care, all investment in offspring is through material supplied to the gametes. Females invest significantly more resources in their gametes than males do in theirs, yet males contribute an approximately equal number of genes. A parthenogenetic female or a self-fertilizing hermaphrodite that produces no male offspring could potentially double her number of grandchildren (Maynard Smith 1978) and invade a dioecious population unless there are opposing selective forces. The problem of the maintenance of sexual outcrossing in a dioecious species is to identify and quantify these opposing forces. Mainstream theories divide possible forces into two broad categories: Either sexual outcrossing produces recombinant types that are better able to adapt to a changing environment, or sex more efficiently eliminates deleterious mutations (Crow 1994; Hurst and Peck 1996).

Although general theories for the maintenance of outcrossing are desirable, nature consists of special cases and it may be fruitful to study thoroughly particular examples. Natural species may not only confirm or refute various theories, but may also add important details missed by the wide theoretical brush. The phylum nematoda provides us with two closely related species that may be relevant to the problem of the stability of the dioecious mating system: *Caenorhabditis remanei* and *C. elegans*. The former nematode worm is dioecious with equal numbers of males and females, and the latter is a self-fertilizing hermaphrodite with males present at low frequency (Brenner 1974; Baird *et al.* 1994). In fact, the ability of *C. elegans* to both self-fertilize and outcross has rendered it a powerful genetic model for developmental studies, and well-established laboratory procedures as well as readily available mutants facilitate experimentation.

These two related species, *C. remanei* and *C. elegans*, are indistinguishable by their gross morphology, and their 18S rDNA sequences differ by only ~1.2% (Fitch *et al.* 1995; D. H. A. Fitch, personal communication). Phylogenetic evidence suggests that their common ancestor was dioecious (Fitch and Thomas 1997) so that an evolutionary study of these two species may serve as a useful model for understanding the maintenance of outcrossing against invasion by selfing hermaphrodites. Here, as a step in this direction, we consider the evolutionary status of *C. elegans*. Are *C. elegans* hermaphrodites descended from modified females capable of spermatogenesis that successfully invaded the ancestral dioecious species? If so, then why are there still males present in the *C. elegans* species?

Sex is determined in Caenorhabditis by the ratio of sex chromosomes to autosomes; females and hermaphrodites are XX and males are XO. With normal meiosis, females and hermaphrodites fertilized by males produce 50% males; self-fertilized hermaphrodites produce only hermaphrodites. Spontaneous nondisjunction of the

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sex chromosomes, however, can occur during meiosis (resulting in XX and O gametes) so that a self-fertilized wild-type hermaphrodite will produce males at low frequency. As observed in nature and in the laboratory, *C. elegans* males are present in populations at low frequencies, implying small levels of outcrossing between hermaphrodites and males. If males and hermaphrodites rarely mate, then why has natural selection not completely eliminated males from the species?

Two distinct possibilities present themselves. The first is that only a small amount of outcrossing may be required for sexual reproduction to yield an advantage (Hurst and Peck 1996). A selfing hermaphrodite that produces no males may be selected against in competition with hermaphrodites that produce males at low frequency and occasionally outcross. The second possibility is that *C. elegans* is essentially a self-fertilizing hermaphroditic species descended from protohermaphrodites that successfully invaded a dioecious ancestor. Here, nondisjunction and the production of males at low frequency by the hermaphrodite are nonadaptive to the hermaphrodite. We can rephrase these two possibilities as the following question: Is the frequency u of male offspring produced by nondisjunction in a selfing hermaphrodite optimum or minimum? An analogous question has also been asked of mutation rates in general (Maynard Smith 1978). If u is optimum, then natural selection has fine tuned its value to maximize the hermaphrodite's reproductive potential. Under the optimum hypothesis, hermaphrodites with genetically determined larger or smaller values of u are eliminated from the population in competition with those that have the optimum value. If u is minimum, however, then natural selection has favored hermaphrodites with genes that reduce u to a value as small as possible without incurring excessive costs in resources.

It is difficult to determine directly whether the value of u in *C. elegans* is optimum or minimum. In this article, however, guided by a mathematical model of the *C. elegans* genetic system, we perform laboratory observations and experiments that show that hermaphrodites have lost their attractiveness to males, resulting in a low frequency of outcrossing. We further argue that this implicates *C. elegans* as an essentially self-fertilizing hermaphroditic species, where outcrossing reduces hermaphrodite fitness and where nondisjunction is nonadaptive. Furthermore, any males present in the *C. elegans* population are under selection pressure to successfully mate with hermaphrodites, and we present theoretical results showing that the amount of outcrossing observed experimentally is sufficient to support male-specific genes against degeneration by deleterious mutations.

MATERIALS AND METHODS

Worm cultures: *C. elegans* strains were maintained and crosses were performed according to the standard laboratory procedures described by Brenner (1974). N2 was used as wild type. CB4088: *him-5* (*e1490*) V, which gives a high incidence of male progeny, was used to obtain sufficient male worms for mating assays (Hodgkin *et al.* 1979). Both *unc-24* (*e138*) and *unc-17* (*e245*) mutants were used as markers in some of the assays. Genetic variants of *C. elegans* used in this study include AB1 (Australia). *C. remanei* (EM464, Baird *et al.* 1994) was used as the dioecious species for comparisons of mating behavior and efficiency.

Fecundity score: Different genotypes of *C. elegans* were transferred individually onto an empty plate (one per plate). The brood size was scored over a period of 6 days with daily transfer of the parent. The number of progeny produced by each individual was averaged over the total test worms to reflect the fecundity of the parental worms of specific genotype.

Equilibrium cultures: An equilibrium culture of *him-5* or N2 worms was obtained by chunking a block of agar containing worms from an old plate onto a new plate seeded with bacteria once every 3–4 days over a period of >1 month. The incidence of males was scored over an additional six repeated passages of worms.

Observation of matings: Mating behavior in an equilibrium population of *C. elegans him-5* mutants and *C. remanei* was monitored by direct observation every 5 min under a dissecting microscope. Observed matings were timed. To obtain an estimation of the duration of matings, we doubled these timings because, on average, the observation period began halfway through the mating. For a further recording of the *C. elegans* male-hermaphrodite or *C. remanei* male-female association, five males were put on a fresh mating lawn (0.5 cm diameter) with five hermaphrodites or females. Their associations were timed and the averages from multiple observations were recorded.

Mating efficiency test for *C. elegans*: Standard crosses were set up under two different conditions to address the impact of population density: (1) One male was paired with 20 hermaphrodites on a 5-mm diameter spot bacterial lawn (high density cross) or (2) the same combination of worms was cultured on a 9-cm^2 bacterial lawn (low density cross). The hermaphrodites and males were picked as young adults less than half a day old, unless otherwise noted. Mating tests were performed using active and nonactive (*unc-24*) hermaphrodites to address the impact of hermaphrodite mobility on mating success. These matings were allowed to take place for 2 days; subsequently, the male or non-Unc progeny was scored.

Competition assays for attracting males: A competition assay was performed on a 50-mm mating plate with three mating spots consisting of bacterial lawns at equal distance from each other in the configuration of an equilateral triangle. A *C. remanei* female was put on one spot, a *C. elegans unc-24* hermaphrodite on a second, and the third spot was left empty. Ten to 12 young males (*C. elegans him-5* or *C. remanei*) were transferred to the center of the plate equally distant from the three bacterial spots. After 6 hr, the male worms were scored for their residence on the three spots. Plates where the female or hermaphrodite migrated from her home spot were discarded. Additional competition assays were also performed on 50-mm plates with two mating spots. Worms to be tested for the competition were placed on one of the two bacterial spots. The competitions were between (1) one *C. remanei* female *vs.* 10 *C. elegans unc-24* hermaphrodites; (2) a dead *C. remanei* female *vs.* an empty spot; (3) one *C. elegans unc-24* hermaphrodite *vs.* an empty spot; and (4) 10 *C. elegans unc-24* hermaphrodites *vs.* 10 *C. elegans unc-24; him-5* males. Ten to 12 *C. remanei* or *C. elegans him-5* males were placed midway between the two spots and after 6 hr their final positions were recorded. In these experiments, uncoordinated hermaphrodites were employed to inhibit migration. Some of the experiments were repeated using *unc-17* or wild-type hermaphrodites

to confirm that the results were independent of the specific Unc mutants employed in the assays.

Competition experiment between *him-5* and wild type: The population competition experiment on *C. elegans* was performed in triplicate with the starting culture composed of 100 *him-5* mutant hermaphrodites on a 100-mm culture plate. Two N2 wild-type hermaphrodites were added on day 0. After intervals of 2–3 days, when the food was finished or the cultures had a large number of L1 larvae, the worms were washed off the plate into a 1.5-ml Eppendorf tube. The mixed culture was allowed to stand for ~15–20 sec so that a majority of the old or dead adults settled to the bottom. The young smaller larvae and actively wiggling adults still in suspension were transferred to a new tube and washed twice with M9 buffer. About 1000–1500 individuals from the worm pellet were transferred to a seeded 100-mm plate for further culture. This procedure was repeated multiple times and the presence of males in the cultures was scored every 2–3 days over a period of 2½ months until the incidence of males had fallen to a level consistent with the entire population being wild type.

RESULTS

A mathematical model for the mating system of *C. elegans*: We first develop a general mathematical model for the *C. elegans* mating system that will be useful in suggesting and interpreting subsequent experiments. Consider a worm population consisting of H hermaphrodites and M males; the population frequencies of hermaphrodites P and males S are given by

$$P = \frac{H}{H + M}, \quad S = \frac{M}{H + M}. \tag{1}$$

Furthermore, we denote the average number of fertilized eggs per hermaphrodite by h and the average number of fertilizing sperm per male by m. An important model parameter will be the mating efficiency

$$b = m/h, \tag{2}$$

where b is unity for a dioecious species with hermaphrodites replaced by females and males and females in equal numbers. As a consequence of selfing, the total number of fertilizing male sperm mM is less than or equal to the total number of fertilized eggs hH, so that (1) and (2) imply $bS/P \leq 1$.

A deterministic model is constructed for the evolution of the frequency of males in the population under the usual simplifying assumptions of very large population size and discrete generations. We assume that one-half the offspring from male-fertilized eggs and a fraction u from self-fertilized eggs are male. To further simplify the model, we make the reasonable assumption that selfed offspring of genotype other than XO and XX (*e.g.*, XXX), which occur at low frequency due to nondisjunction of the sex chromosomes, are nonviable. The fractions of hermaphrodite and male zygotes from selfed and male-fertilized eggs are presented in Table 1. We further assume that offspring from self-fertilized eggs have fitness $1 - d$ relative to those from male-fertilized eggs. The parameter d models inbreeding depression (Charlesworth and Charlesworth 1978), and in general $0 \leq d \leq 1$.

The evolution equation for the male frequency, with a prime denoting the frequency in the next generation, is determined to be

$$S' = \frac{u(1 - d)(1 - bS/P) + bS/2P}{1 - d + bdS/P}, \tag{3}$$

with $P = 1 - S$. Our model (3) differs significantly from that of Hedgecock (1976) in that no *a priori* equilibrium assumption has been made in its construction. In (3), a population equilibrium $S' = S$ is obtained by iteration once u, d, b, and the initial male frequency $S = S_0$ are specified.

The mating efficiency b is in general expected to depend on the male frequency S when males compete for mates. Male competition acts to reduce b by decreasing the average number m of fertilizing sperm per male.

The mating efficiency b, valid only for equilibrium populations, may be determined from (3) with $S' = S$:

$$b = \frac{2(1 - d)(S - u)(1 - S)}{S[1 - 2u - 2d(S - u)]}. \tag{4}$$

We note that the functional dependence $b = b(S)$ for nonequilibrium populations may be quite different from (4).

We can further determine the conditions under which males will be maintained in the worm population in the absence of sex chromosomal nondisjunction ($u = 0$). If males are present in the population at low frequencies, then b can be taken to be independent of S. An equilibrium solution of (3) with $u = 0$ is $S = 0$. When this solution is stable, males are eliminated from the population; otherwise males are maintained. The stability of the solution $S = 0$ is readily determined, and we find that males will be eliminated from the worm population when

$$\frac{1}{2}b + d < 1 \quad \text{(males eliminated)}. \tag{5}$$

The above stability condition is similar to one found previously (Otto *et al.* 1993) in a study of the mating system of the clam shrimp *Eulimnadia texana*, in which both selfing and outcrossing occurs, and our interpretation of (5) follows theirs. The factor of ½ can be attributed to the cost of males. Males can be maintained if inbreeding depression is absent ($d = 0$) and males are more than twice as productive as hermaphrodites ($b > 2$) or if males and hermaphrodites are equally productive ($b = 1$) and inbreeding depression is large ($d > ½$).

When sex chromosomal nondisjunction occurs ($u > 0$) and the inequality (5) is satisfied, males are maintained in the population at a frequency proportional to u. For poor mating efficiency ($b \ll 1$), the male equilibrium frequency S is given to leading order in b as

$$S = u\left(1 + \frac{(1 - 2u)b}{2(1 - u)(1 - d)}\right) + O(b^2). \tag{6}$$

TABLE 1

Mating table for *C. elegans*

Mating	No. of zygotes	Frequency	Progeny frequency ☿♂	Progeny frequency ♂
☿♂ ⊗	hH-mM	$1 - bS/P$	$(1 - u)(1 - bS/P)$	$u(1 - bS/P)$
☿♂ × ♂	mM	bS/P	$bS/2P$	$bS/2P$

Hermaphrodite and male frequencies satisfy $P + S = 1$.

When u, $d \ll 1$, S is insensitive to b over a wide range of values. Larger values of u, however, increase the sensitivity of S to b, and this observation will be helpful in an experimental determination of the mating efficiency.

Inbreeding depression is not found in laboratory *C. elegans* strains: Inbreeding depression can be caused either by loss of heterozygosity at individual loci or by deleterious mutations being made homozygous. Inbreeding depression has been clearly demonstrated in Drosophila (Hollingsworth and Maynard Smith 1955). Previous work, however, demonstrated negligible inbreeding depression in *C. elegans* (Johnson and Hutchinson 1993). We made additional tests by crossing two isolates of *C. elegans*: the AB1 strain from Australia and N2 from Bristol, England. Using the average self-fertilized brood size as a parameter for comparison, we scored the number of progeny of the parental F_0, F_1 heterozygous, and F_2 variants. Our parental strain, N2, had an average brood size of 195 ± 26 (134–266, N = 40), and AB1 had an average of 182 ± 27 (148–251, N = 50). The average brood size of heterozygous AB1/N2 F_1 worms was 175 ± 24 (138–227, N = 40); the F_2 worms had an average brood size of 176 ± 45 (71–269, N = 50). From these results, the F_1 worms inheriting one chromosome from each parental strain did not have an increased fecundity, and neither did the genetically heterogeneous F_2 worms. Considering only brood sizes of selfed hermaphrodites, outcrossing seemed to be of little benefit to hermaphrodites, and we subsequently assume $d = 0$ in the mathematical model.

Measurement of *u* and *S* and determination of *b* for a mutant *him-5* strain: To obtain an estimate of the mating efficiency b used in the mathematical model, we took advantage of the mutant *him-5* strain, which had been reported to produce ~30% males among the progeny of selfed hermaphrodites due to a high nondisjunction rate (Hodgkin *et al.* 1979). We measured u by counting 5695 males out of 17,488 offspring from virgin hermaphrodites, resulting in $u = 0.3257 \pm 0.0035$. The errors here and subsequently are estimated using the binomial distribution, *i.e.*, $\sigma_u = \sqrt{u(1 - u)/N}$, where N is the total number of worms counted. We then counted 9929 males out of 30,008 total worms present in equilibrium cultures, yielding a value of $S = 0.3309 \pm 0.0027$, slightly higher than the value of u as expected if there is only a small amount of outcrossing. Using these values of u and S in (4), taking $d = 0$, and propagating the errors in the usual way results in $b = 0.06 \pm 0.05$. Since the mating efficiency b is much less than unity, *C. elegans* males are not nearly as productive as the males of a dioecious species. The inefficiency of *C. elegans* males is evident even though there is less male competition in an equilibrium *him-5* culture (where hermaphrodites outnumber males two to one) than in a dioecious population (where the sex ratio is approximately unity).

Measurement of *S* and determination of *u* for a wild-type N2 strain: Male worms were counted in a number of parallel cultures of *C. elegans* wild-type N2 worms. Twenty-two males were recorded after counting 28,473 worms, yielding an equilibrium male frequency of $S = 0.00077 \pm 0.00016$, or $0.077 \pm 0.016\%$. A somewhat larger value of approximately 0.2% was reported previously for the equilibrium male frequency of N2 (Hodgkin 1983). Assuming the same value of the mating efficiency b for N2 as *him-5*, we can estimate u from the N2 population by solving (4) for u (with $d = 0$),

$$u = \frac{S(2(1 - S) - b)}{2(1 - S(1 + b))} \approx S(1 - \frac{1}{2}b), \qquad (7)$$

the last approximation being valid for $S \ll 1$. We find $u = 0.075 \pm 0.016\%$, which is only slightly lower than the value of S because of the rarity of outcrossing.

In Figure 1, using (4) we plot the values of b, which could occur in equilibrium populations, *vs.* S, for $d = 0$, $u = 0.00075$ (N2), and $u = 0.3257$ (*him-5*). Also shown by the dotted line is the measured value $b = 0.06$. From the figure it is evident that b is best measured using the *him-5* strain.

Direct observation of mating: To confirm that outcrossing was infrequent, matings were observed in an equilibrium *him-5* mutant culture. Occasional matings were observed with males displaying an active vulva searching behavior when associated with hermaphrodites. When the number of males and hermaphrodites was sampled, we found 9.1% (N = 1315) and 4.0% (N = 2798) of males and hermaphrodites, respectively, engaged in mating. When the duration of the mating events was estimated, males and hermaphrodites stayed together an average 2.14 min (measured timings from 10 sec to 4 min, N = 40). Out of these 40 matings, copulation was observed in only 2, both of which had

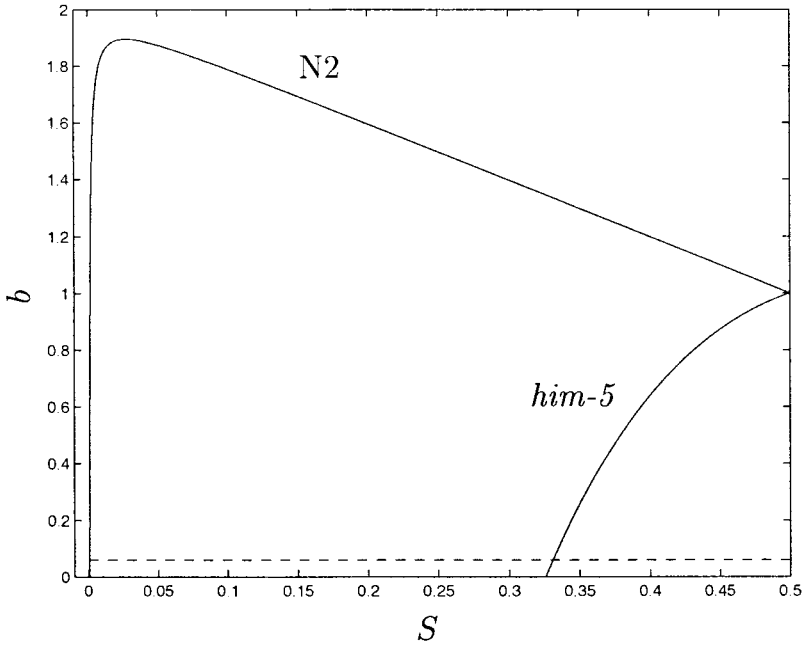

FIGURE 1.—The mating efficiency b *vs.* the male frequency S for populations in equilibrium. Solid curves represent the equilibrium solutions for N2 wild type with $u = 7.5 \times 10^{-4}$ and *him-5* mutant with $u = 0.3257$. The dashed line is the value for b obtained by measuring u and S from a *him-5* mutant population.

their measured times exceeding 2 min (very few matings lasted for >2 min). Thus, although multiple matings were observed, copulation was less frequent. To further investigate the lack of mating success, we examined the impact of density as well as hermaphrodite inactivity on matings. When 1 single male was used to mate with 20 active hermaphrodites on a 9-cm^2 lawn, successful matings occurred in only 6 out of 80 tests, with an average cross brood of 11 ± 10, estimated by doubling the number of male offspring. The overall average cross brood among the 80 tests was 0.83. When the mating lawn area was reduced to a 0.5-cm diameter circle, however, the successful matings increased to 17 out of 80 tests, with the average cross brood 10 ± 9 per successful cross. The overall average cross brood was more than doubled to 2.2. The mating success of males was further improved when 20 uncoordinated hermaphrodites were used to mate with a single male. In a 9-cm^2 lawn mating test, there were 23 successful matings in 30 tests, with an average cross brood per mating of 55 ± 23 and an overall average of 42. In a parallel test with a mating lawn of 0.5 cm diameter, 100% successful matings were achieved ($N = 30$), with an average cross brood of 120 ± 45. It should be mentioned that active mating behavior exhibited by males could be affected by their age. While the above tests were done with young males <1 day old, additional tests done with males older than 3 days showed extremely inefficient mating.

All of these results imply that *C. elegans* males cannot mate efficiently in a normal cultured equilibrium population. A similar observation was recently reported by STEWART and PHILLIPS (2002). The inability of uncoordinated hermaphrodites to migrate actively within the mating area enhances mating success 40- to 50-fold, and an increased density of hermaphrodites enhances mating success by 2-fold. Because the hermaphrodites exhibit no active seeking behavior, our mating efficiency results suggest that hermaphrodites are passive mating partners. The success of mating is determined primarily by the seeking behavior of the males, which is affected by the density of hermaphrodites on a plate as well as the ability of hermaphrodites to move.

***C. remanei* mates more efficiently than *C. elegans*:** In contrast to a hermaphroditic species, a dioecious species depends on successful male-female matings. A lack of mating vigor comparable to *C. elegans* would likely drive a dioecious species extinct. Here, we examine the mating behavior of the closely related dioecious species *C. remanei*. In equilibrium *C. remanei* populations, we determined 33.4% ($N = 2136$) males and 30.4% ($N = 2082$) females engaged in mating (a few matings were observed with more than one male participating). Thus the encounters required for the initiation of successful mating appeared to be more frequent in the dioecious species. For successful mating, the duration of coupling between the two sexes is important. In this regard, *C. remanei* outperformed *C. elegans* by >15-fold. The dioecious species had an average mating time of 41.6 ± 33.2 min, and copulation was observed in 100% (40/40) of the matings. In fact, in synchronized *C. remanei* populations, almost all females were found to be mated (by scoring the presence of a mating plug) within 1 hr after reaching maturity.

***C. elegans* males mate effectively with *C. remanei* females:** Our observations make it evident that the dioecious species *C. remanei* is more efficient at mating than *C. elegans*. Since both species are believed to share a common dioecious ancestor (FITCH and THOMAS 1997),

this suggests that a genetic mutation might have occurred during the evolution of *C. elegans* that reduced its mating efficiency. To explore this possibility, we conducted cross-species mating tests between males and females or hermaphrodites. Five *C. elegans* males were placed with five *C. remanei* females on mating plates, and their mating frequencies and duration of matings were scored. By observing the females at an instant in time, matings were found in 24.6% of the worms ($N = 275$). In opposite mating pairs with five *C. remanei* males and five *C. elegans* hermaphrodites, only 3.0% of the hermaphrodites were observed to be mating ($N = 201$). The duration of matings was estimated for observed couplings. The *C. elegans* male-*C. remanei* female pairs mated for an average of 34.0 ± 19.6 min ($N = 25$) and the *C. remanei* male-*C. elegans* hermaphrodite pairs mated an average of 1.4 ± 0.6 min ($N = 5$). The mating duration of the *C. elegans* male-*C. remanei* female pair is similar to that recorded for intraspecies *C. remanei* couplings, and the mating duration of the *C. remanei* male-*C. elegans* hermaphrodite pairs is similar to that for intraspecies *C. elegans* couplings. The differences in mating durations are thus determined by the *C. remanei* female or the *C. elegans* hermaphrodites and not by the males. The surprising result here is that *C. elegans* males mate more effectively with *C. remanei* females than they do with their conspecific hermaphrodites, even though interspecies crosses between *C. elegans* and *C. remanei* result in no viable progeny (Baird *et al.* 1994).

***C. elegans* hermaphrodites no longer attract males:** To account for the difference in mating durations, we reason that male attraction to females might be due to a chemotactic process relying on sex pheromones produced by females and not by hermaphrodites. To explore this possibility, we first performed an assay for attracting males with a three-way competition between an empty spot with only a bacterial lawn, a second spot with a *C. elegans* hermaphrodite, and a third spot with a *C. remanei* female. The number of males attracted to each spot is shown in Table 2A. Both *C. elegans* and *C. remanei* males showed a preference to the spot where the *C. remanei* female was placed over the spot where the *C. elegans* hermaphrodite was placed, which in turn was slightly more attractive than the empty spot. In similar assays, two-way competitions were performed to compare directly the preference between sources of attractant. As shown in Table 2B, a single *C. remanei* female was much more attractive to both *C. elegans* and *C. remanei* males than were 10 *C. elegans* hermaphrodites, implying that a female produces at least an order of magnitude more attracting substance than a hermaphrodite. The null hypothesis that this result was due to a random choice between females and hermaphrodites (exact binomial test) is rejected by the very small P value. Also, no statistically significant difference (goodness-of-fit test: $P = 0.76$) was observed between *C. elegans* and *C. remanei* males in this assay. Furthermore, as shown in Table 2C, when the *C. remanei* female was killed by a hot worm pick the carcass did not elicit the same attraction, suggesting that the attractant was emitted only by live females.

Additional experiments were done to determine if *C. elegans* hermaphrodites secreted any attractive substance. Two two-way competition experiments were performed: one *C. elegans* hermaphrodite *vs.* an empty spot, presented in Table 2D, and 10 *C. elegans* hermaphrodites *vs.* 10 *C. elegans* males, presented in Table 2E. Our results indicate a small preference for live worms over an empty spot (the P values indicate only marginal significance), but no preference for hermaphrodites over males. In fact, on plates cultured with *him-5* mutants it is common to observe males mating with other males. All of these competition results considered together, as well as our direct observations of matings, strongly suggest that *C. elegans* hermaphrodites lack an attractant (perhaps a sex pheromone) expressed by *C. remanei* females.

Why are *C. elegans* males still attracted to *C. remanei* females if their conspecific hermaphrodites are no longer attractive? Maintaining this attraction would appear to be maladaptive to *C. elegans* males since copulation with *C. remanei* females is unproductive. Here, we can suggest only three possible reasons. First, a very small amount of attractant undetectable by our assays is produced by hermaphrodites; second, *C. elegans* male attraction to *C. remanei* females is maintained as a slightly deleterious side effect to some other more advantageous but unknown function; or third, continuing male attraction to *C. remanei* females is an evolutionary relic that has not been significantly selected against. Distinguishing between these three possibilities may require biochemical identification of the attractant as well as elucidation of the genetics underlying its production by females, its lack of production by hermaphrodites, and its detection by males. Of particular interest is whether a gene required for pheromone production in *C. remanei* females is mutated in *C. elegans* hermaphrodites.

A competition experiment between *him-5* and wild type: With negligible inbreeding depression and b small, the mathematical model predicts that natural selection should act to lower the nondisjunction rate and the corresponding u to as low a value as possible. As a test of this hypothesis, we conducted a competition experiment using a wild-type N2 strain to compete against a mutant *him-5* strain. The brood sizes of selfing N2 and *him-5* strains were measured prior to the experiment and found to be approximately equal: For N2, the brood size was 195 ± 26 (134–266, $N = 40$); for *him-5*, the brood size was 197 ± 28 (118–238, $N = 27$). (This result in itself is puzzling because the substantially larger nondisjunction rate for *him-5* should result in autosomal nondisjunctions as well as zygotes with sex chromosome genotypes O, XXX, and XXXX. In fact, XXX genotypes should occur with the same frequency as XO males, but in our *him* mutants very few XXX dumpy worms are observed.) Three competition experiments were initi-

TABLE 2

Competition assays for attracting males

	Choices				
Males	*remanei* (1 f)	*elegans* (1 h)	Empty	*N*	
A					
elegans	70	30	12	20	
remanei	63	28	24	23	
Males	*remanei* (1 f)	*elegans* (10 h)		*N*	*P* value
B					
elegans	274	126		37	<0.00001
remanei	270	132		38	<0.00001
Males	*remanei* (1 f, dead)		Empty	*N*	
C					
elegans	113		138	20	
remanei	97		102	19	
Males		*elegans* (1 h)	Empty	*N*	*P* value
D					
elegans		192	166	37	0.085
remanei		202	168	38	0.039
Males		*elegans* (10 h)	*elegans* (10 m)	*N*	
E					
elegans		109	114	20	
remanei		135	173	25	

The numbers of attracted males (*C. elegans* or *C. remanei*) are shown; *N* is the number of independent trials; *P* value indicates the significance of the result. (A) Three-way competition between one *C. remanei* female, one *C. elegans* hermaphrodite, and an empty spot. (B) Two-way competition between one *C. remanei* female and 10 *C. elegans* hermaphrodites. (C) Two-way competition between one dead *C. remanei* female and an empty spot. (D) Two-way competition between one *C. elegans* hermaphrodite and an empty spot. (E) Two-way competition between 10 *C. elegans* hermaphrodites and 10 *C. elegans* males.

ated with 100 *him-5* and 2 wild-type hermaphrodites, and subsequent measurements of average male frequency *vs.* the elapsed number of days are shown as the data points in Figure 2. The error bars represent the standard deviation determined from the three separately evolving cultures. It is observed that the *him-5* mutation was essentially eliminated from the populations in ~70 days (~23 generations).

The experimental results may be compared to a relatively simple mathematical model. We consider the dynamics of a population consisting of a mixture of *him* mutants and wild-type worms, assuming that both phenotypes have the same brood size and differ only in the frequency of selfed spontaneous males. The genotypes, associated phenotypes expressed as the spontaneous male frequency u, and corresponding genotype frequencies are shown in Table 3.

In general, a mating table may be constructed and evolution equations for the male and hermaphrodite frequencies determined. The resulting model, with parameters b, d, u_{AA}, u_{Aa}, and u_{aa}, consists of five independent evolution equations and requires numerical solution. Since the observed value $b = 0.06$ is small, however, we make the simplifying assumption that no mating occurs ($b = 0$), which permits an analytical solution of the model equations.

The assumption of no mating as well as the initial absence of heterozygous genotypes leads to the evolution equation

$$\left(\frac{P_{AA}}{P_{aa}}\right)' = \left(\frac{1 - u_{AA}}{1 - u_{aa}}\right)\left(\frac{P_{AA}}{P_{aa}}\right), \tag{8}$$

which permits analytical solution. The corresponding solution for the male frequency S at generation $n > 0$ is determined to be

$$S = \frac{cu_{AA}(1 - u_{AA})^{n-1} + u_{aa}(1 - u_{aa})^{n-1}}{c(1 - u_{AA})^{n-1} + (1 - u_{aa})^{n-1}}, \tag{9}$$

where c is the initial value of P_{AA}/P_{aa} when $n = 0$. In a competition between *AA* wild type and *aa him-5* mutants, we can approximate $u_{AA} = 0$ and $u_{aa} = U$, so that (9) simplifies further to

$$S \approx \frac{U(1 - U)^{n-1}}{c + (1 - U)^{n-1}}. \tag{10}$$

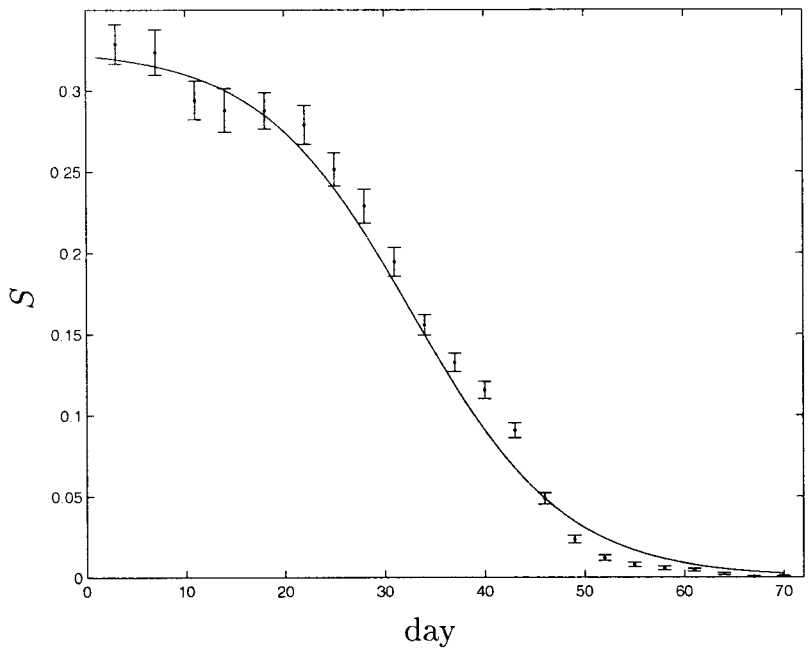

FIGURE 2.—Results of a competition experiment between N2 wild type and *him-5* mutant. Male frequency S (points with error bars) is plotted *vs.* the elapsed number of days. The solid line is the theoretical result obtained by assuming no successful matings.

The result (10), with $U = 0.3257$ and $n = D/3$, where D is the number of elapsed days in the experiment, 3 days is the estimate for the generation length of *C. elegans*, and $c = 0.02$ (2 wild-type and 100 *him-5* hermaphrodites at $n = 0$), can be compared directly to the laboratory measurements and is the solid curve in Figure 2. Good agreement is observed between the experimental data and the theoretical curve. Our results exemplify the simple idea that without a substantial number of matings between males and hermaphrodites, a hermaphrodite that produces a male instead of a hermaphrodite reduces its effective fecundity.

Why has the male phenotype not degenerated? The loss of sex pheromone expression in hermaphrodites may be due to natural selection favoring hermaphrodites that self-fertilize. Natural selection, however, also favors males that mate efficiently since this is the only route for male genes to pass into the next generation. Interestingly, the two sexes of *C. elegans* have conflicting interests: Hermaphrodites want to self and males want to mate. This is in contrast to a dioecious species where males and females must cooperate to reproduce.

Here, we further consider the evolution of genes that are expressed only in males, *e.g.*, the genes needed to build the *C. elegans* male sensory rays. All genes face a constant mutation pressure and those genes required to build the sensory rays, say, can be maintained only if they enhance the reproductive success of males. Complex adaptations that no longer contribute to an organism's reproductive success degenerate over evolutionary time: An often-cited example is eye loss in cave-dwelling animals (FONG *et al.* 1995). If, for instance, *C. elegans* males never successfully mate with hermaphrodites, then an adaptive organ such as the sensory rays would be expected to degenerate, and *C. elegans* might evolve into a hermaphroditic species without males.

C. elegans males, however, do occasionally fertilize hermaphrodites and obviously the genes specifically expressed in males have not degenerated. The question we address here is: How often must males successfully mate with hermaphrodites to prevent male genes from degenerating? An answer requires determining the relationship between a critical deleterious mutation rate v_c for a male-expressed gene and the mathematical model parameters u, b, and d, such that for a deleterious mutation rate $v > v_c$ the male gene becomes nonfunctional, resulting in male sterility. For simplicity, we consider only a deterministic model with infinite population size. Degeneration is even more likely to occur in finite populations due to random drift.

Accordingly, we consider a wild-type gene A expressed only in males such that male genotypes AA average m fertilizing sperm per male, and male genotypes Aa and aa average none; *i.e.*, these genotypes are sterile because of degeneration. Here, we assume a dominant mutation to simplify the algebra, but additional numerical computations (not shown here) demonstrate the same final result (13) for a recessive mutation. We further assume

TABLE 3

Genotypes, phenotypes, and frequencies of wild-type (*AA*, *Aa*) and *him* mutants (*aa*)

Genotype	AA	Aa	aa
Phenotype	u_{AA}	u_{Aa}	u_{aa}
☿ frequency	P_{AA}	P_{Aa}	P_{aa}
♂ frequency	S_{AA}	S_{Aa}	S_{aa}

Genotype frequencies satisfy $P = P_{AA} + P_{Aa} + P_{aa}$, $S = S_{AA} + S_{Aa} + S_{aa}$, and $P + S = 1$.

TABLE 4

Mating table—only males with genotype *AA* are fertile

Mating	Zygote frequency	Progeny frequency/zygote frequency ♀♂			♂		
♀♂ × ♂		*AA*	*Aa*	*aa*	*AA*	*Aa*	*aa*
AA ⊗	$(P_{AA}/P)(1 - bS_{AA}/P)$	$(1 - u)$	0	0	u	0	0
Aa ⊗	$(P_{Aa}/P)(1 - bS_{AA}/P)$	$\frac{1}{4}(1 - u)$	$\frac{1}{2}(1 - u)$	$\frac{1}{4}(1 - u)$	$\frac{1}{4}u$	$\frac{1}{2}u$	$\frac{1}{4}u$
aa ⊗	$(P_{aa}/P)(1 - bS_{AA}/P)$	0	0	$(1 - u)$	0	0	u
AA × *AA*	$(P_{AA}/P)(bS_{AA}/P)$	$\frac{1}{2}$	0	0	$\frac{1}{2}$	0	0
Aa × *AA*	$(P_{Aa}/P)(bS_{AA}/P)$	$\frac{1}{4}$	$\frac{1}{4}$	0	$\frac{1}{4}$	$\frac{1}{4}$	0
aa × *AA*	$(P_{aa}/P)(bS_{AA}/P)$	0	$\frac{1}{2}$	0	0	$\frac{1}{2}$	0

Genotype frequencies satisfy $P = P_{AA} + P_{Aa} + P_{aa}$, $S = S_{AA} + S_{Aa} + S_{aa}$, and $P + S = 1$.

that hermaphrodites of all genotypes average h fertilized eggs per hermaphrodite and that the chance of an *AA* male fertilizing an oocyte is independent of the genotype of the hermaphrodite. To develop our model equations, consider a worm population consisting of H_{AA}, H_{Aa}, and H_{aa} hermaphrodites of genotype denoted by the subscript and M_{AA}, M_{Aa}, and M_{aa} males. With $H = H_{AA} + H_{Aa} + H_{aa}$ and $M = M_{AA} + M_{Aa} + M_{aa}$, the population frequencies of hermaphrodites and males of the various genotypes are given by

$$P_{AA} = \frac{H_{AA}}{H + M}, \quad P_{Aa} = \frac{H_{Aa}}{H + M}, \quad P_{aa} = \frac{H_{aa}}{H + M},$$
$$S_{AA} = \frac{M_{AA}}{H + M}, \quad S_{Aa} = \frac{M_{Aa}}{H + M}, \quad S_{aa} = \frac{M_{aa}}{H + M}. \tag{11}$$

The number of zygotes from different matings can be calculated. As an example, the number of self-fertilized zygotes from *AA* hermaphrodites is given by

$$hH_{AA} - mM_{AA}H_{AA}/H,$$

where the first and second terms are the total number of zygotes from *AA* hermaphrodites and the number that are male-fertilized, respectively. Division by the total number of fertilized zygotes hH determines the various zygote frequencies. These frequencies and the frequencies of the various progeny are given in Table 4, where as before we define $b = m/h$. Finally, we assume that wild-type gene *A* has probability v of mutating to gene *a* each generation. For further simplicity, we assume that $v \ll 1$ so that terms of order v^2 are negligible.

General evolution equations for the various genotype frequencies may now be constructed. Here, however, we are interested in the stability of a population in which all genes *A* have mutated; that is, $P_{aa} = 1 - u$, $S_{aa} = u$, with all other genotype frequencies equal to zero. We thus perturb this equilibrium state and linearize the evolution equations about the infinitesimal frequencies P_{AA}, S_{AA}, P_{Aa}, and S_{Aa}, and the deviations of P_{aa} and S_{aa} from equilibrium. Only the evolution equations for P_{AA}, S_{AA}, and P_{Aa} are coupled, and these equations written in matrix form are given by

$$\begin{pmatrix} P_{AA} \\ S_{AA} \\ P_{Aa} \end{pmatrix}' = \begin{pmatrix} 1 - 2v & 0 & \frac{1}{4}(1 - 2v) \\ \frac{(1-2v)u}{1-u} & 0 & \frac{(1-2v)u}{4(1-u)} \\ 2v & \frac{b(1-v)}{2(1-u)(1-d)} & \frac{1}{2} \end{pmatrix} \begin{pmatrix} P_{AA} \\ S_{AA} \\ P_{Aa} \end{pmatrix}. \tag{12}$$

The eigenvalues of the coupling matrix may be computed; the degenerated equilibrium state is unstable when an eigenvalue becomes larger than unity. Setting the maximum eigenvalue to unity and solving for v determines the critical mutation rate v_c. To leading order in u, the analytical result is

$$v_c = \frac{ub}{4(1 - d)} + 0(u^2). \tag{13}$$

When $v > v_c$, the equilibrium state where all genes *A* have mutated to *a* is stable; all males are sterile in this population. When $v < v_c$, at least some males in the population are fertile. With $u = 0.00075$, $b = 0.06$, and $d = 0$, we obtain the numerical value $v_c = 1.1 \times 10^{-5}$. An estimate for the deleterious mutation rate per haploid genome in *C. elegans* is $U = 0.0026$ (Keightley and Caballero 1997), and with an estimate of 19,000 genes (*C. elegans* Sequencing Consortium 1998), the mutation rate per gene is $0.0026/19{,}000 \approx 1.4 \times 10^{-7}$, almost two orders of magnitude smaller than our estimate for v_c.

A further simplifying approximation considers all the male-only expressed genes to be tightly linked. For this approximation, male fertility will be maintained only if males express $< \sim 1.1 \times 10^{-5}/1.4 \times 10^{-7} \approx 80$ genes that have no phenotypic expression in hermaphrodites. For the more realistic situation of less tightly linked genes, substantially more male-only expressed genes could be maintained. Recent work by Jiang *et al.* (2001) found 1651 male-enriched genes with expression ratios

between males and hermaphrodites ranging from 1.5 to 110. Our model applies to male genes that have no phenotypic expression in hermaphrodites. The *unc-68* gene known to affect both males and hermaphrodites has an expression ratio of 5.3 and the *pkd-2* gene known to be expressed only in males has an expression ratio of 7. Accordingly, we assume that genes with an expression ratio >6 are expressed only in males. If we count only those genes thought to be expressed in the male soma (478) rather than in spermatogenesis (also required by hermaphrodites), then an estimate of 50–60 male-only genes is obtained. It would thus appear that the amount of gene flow from males to future generations is sufficient to prevent the mutational degeneration of the male phenotype even for the limiting case of tight linkage.

DISCUSSION

We begin with some words of caution. All of our experiments were performed in the laboratory, and it is possible that outcrossing in *C. elegans* occurs more frequently in the natural environment. Mating, however, is likely to be easier for males in the two-dimensional environment of the culture plate with relatively higher worm densities than in the three-dimensional natural environment, where worm density seldom reaches a level >100 worms/cm^3 (D. H. A. Fitch, personal communication). It is also possible that we have missed some advantage of outcrossing that appears intermittently and perhaps only in the natural environment. This advantage, however, would have to be large to have an impact, and the low incidence of *C. elegans* males typically found in natural compost piles (D. H. A. Fitch, personal communication) argues against it.

We now review the laboratory facts before addressing the question posed by this article's title:

1. No obvious fitness advantage of outcrossed offspring is observed. Brood sizes from self-fertilizing hermaphrodites are independent of whether these hermaphrodites developed from self-fertilized or outcrossed zygotes. Self-fertilized offspring from *C. elegans* appear to suffer little inbreeding depression, certainly not the factor of two required to repay the cost of males.
2. The mating efficiency of *C. elegans* is poor. In our laboratory culture of *him-5* mutants, each male had on average six productive sperm for every 100 fertilized hermaphrodite eggs, even though hermaphrodites outnumber males by two to one in the population. By direct observation, successful copulation by males is very infrequent.
3. Direct comparison of mating behavior shows that the frequency is much lower and the duration of mating for *C. elegans* is much shorter than that for *C. remanei*.
4. Competition assays between *C. elegans* hermaphrodites and *C. remanei* females to attract males of both species demonstrate that *C. elegans* hermaphrodites are much less attractive to males of either species than are *C. remanei* females. In fact, *C. elegans* males are significantly more attracted to *C. remanei* females than they are to hermaphrodites of their own species.

Some additional experimental facts are also relevant:

5. Hermaphrodites produce fewer sperm than oocytes. Self sperm are utilized with almost 100% efficiency and the additional oocytes produced are laid unfertilized unless the hermaphrodite is mated. As a consequence, under laboratory conditions hermaphrodites that both self and outcross produce substantially more progeny than hermaphrodites that only self (Ward and Carrel 1979).
6. Natural selection acts more strongly on earlier than later produced progeny. A mutation that results in a 50% increase in hermaphrodite sperm production is outcompeted by wild-type worms. Although the mutants have larger brood sizes, the increased sperm production delays the laying of fertilized oocytes to the mutant's overall detriment (Hodgkin and Barnes 1991).

It may be impossible to reconstruct the evolutionary history of *C. elegans*. That said, it is still illuminating to speculate on a plausible evolutionary path from the ancestral dioecious species to *C. elegans* that is consistent with the observations and experiments. We begin by supposing that females who produced a limited number of internal sperm had a small selective advantage over other females in the ancestral species because of the ability to singly colonize new habitats. These protohermaphrodites still required male sperm to fertilize the bulk of their oocytes, so natural selection would support hermaphrodite attractiveness. These protohermaphrodites may have evolved, however, to self-fertilize more of their own oocytes as the deleterious effects of inbreeding depression diminished, and selection for attractiveness could have turned from positive to negative. This reversal in selection is due to an important tradeoff. Attractive hermaphrodites more efficiently obtain sperm from conspecific males and can produce larger brood sizes than nonattractive hermaphrodites. Attractive hermaphrodites, however, may be mated quickly with the disadvantage that early progeny are 50% males, whereas early progeny of unattractive hermaphrodites are likely to be selfed and are thus 100% hermaphrodites. As soon as hermaphrodite attractiveness became disadvantageous, any mutation that eliminated sex pheromone expression would have rapidly swept through the hermaphrodite population. Although the optimum reproductive strategy for a *C. elegans* hermaphrodite is to first self-fertilize its oocytes until all internal sperm are used and then outcross, natural selection acting on attractiveness alone cannot attain this optimum. A single

loss-of-function mutation could conceivably result in loss of attractiveness, whereas delaying the onset of attractiveness may require a specific regulatory mutation, if such a mutation exists at all, with much lower probability of occurring. Loss of attractiveness is likely to have become fixed in the population before the regulatory mutation occurred, and it would then be impossible to move from the adaptive peak of no attraction to the theoretical optimum peak of a well-timed attraction.

With the frequency of male-hermaphrodite matings suppressed, males exist mainly because of the nondisjunction of the sex chromosomes in self-fertilizing hermaphrodites. Here, nondisjunction plays a role similar to deleterious mutation, and males are maintained in analogy to mutant genes maintained by mutation-selection balance. In fact, if there were no successful matings between males and hermaphrodites, the male phenotype would be genetically lethal and the frequency of males in the population would be identical to the rate at which males are born to selfing hermaphrodites. With small levels of outcrossing, the male phenotype is maintained in the population at slightly higher frequencies.

Furthermore, we hypothesize that the nondisjunction rate found in *C. elegans* is as low as possible without incurring excessive costs. A possible test of this hypothesis would be a comparison of X chromosome nondisjunction rates in *C. elegans* with that in its dioecious relative *C. remanei*. If natural selection favored hermaphrodites with an increased level of X chromosome nondisjunction, resulting in the birth of a significant number of spontaneous males, then the rate of nondisjunction in *C. elegans* hermaphrodites should be significantly higher than that found in *C. remanei* females, for which X chromosome nondisjunction has no obvious adaptive purpose. On the other hand, if the two nondisjunction rates are similar, then the reasonable inference is that the rate of nondisjunction has been minimized by natural selection in both species.

We are unaware, however, of any data on nondisjunction rates in *C. remanei* or other dioecious nematodes. A relevant study, however, of spontaneous X chromosome nondisjunction in *Drosophila melanogaster* was undertaken almost 40 years ago (Merriam and Frost 1964). Using the X-linked recessive mutation *y* (yellow) and X-linked dominant mutation *B* (Bar) as markers, XX females (genotype *y/y*) were crossed with XO males (genotype *yB/*O) and 26 yellow-non-Bar females out of a total of 45,112 female progeny were scored. The X chromosome nondisjunction rate in *D. melanogaster* was thus determined to be 0.00058 ± 0.00011, where we have estimated the error from the binomial distribution. Our result is for the frequency u of spontaneous male births by *C. elegans* hermaphrodites. Male births may be due to either a nullo-X egg fertilized by a normal X sperm or a normal X egg fertilized by a nullo-X sperm. Since we do not know the relative frequency of nondisjunction in the formation of eggs or sperm, we assume here that these frequencies are equal. Therefore, for comparison, the X chromosome nondisjunction rate in *C. elegans* is $u/2 = 0.00038 \pm 0.00008$. In mammals, however, the nondisjunction rates are substantially higher. In humans, aneuploidy—the most common types being trisomy 21 (causing Down's syndrome) and sex chromosome trisomies—occurs in 0.3% of all newborns, and it is estimated that $\geqslant 5\%$ of all human conceptions are aneuploid (Hassold and Hunt 2001). The closeness of the two nondisjunction rates for the worm and the fly, both below that of mammals, lends some support to our claim that the nondisjunction rate in *C. elegans* is not significantly elevated. We further note that the observed nondisjunction rate in *C. elegans* is at a level similar to errors occurring in other cellular functions, *e.g.*, an error rate of 0.03–1% for amino acid incorporation during translation (Freist *et al.* 1998).

Despite the obstacles imposed by indifferent hermaphrodites, natural selection still favors males that successfully mate. In this battle between the sexes, males manage to fertilize hermaphrodites often enough to support a small number of male-only genes against degeneration by deleterious mutations.

We have thus argued that males are not present in the *C. elegans* species because of any advantage to outcrossing, as is usually supposed. Rather, hermaphrodites no longer attract males and obtain higher fitness by selfing.

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