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Mutation and the experimental evolution of outcrossing in *Caenorhabditis elegans*

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Abstract

An understanding of the forces that contribute to the phylogenetically widespread phenomenon of sexual reproduction has posed a longstanding problem in evolutionary biology. Mutational theories contend that sex can be maintained when the deleterious mutation rate is sufficiently high, although empirical evidence is equivocal and experimental studies are rare. To test the influence of mutation on the evolution of obligate outcrossing, I introduced a genetic polymorphism for breeding system into populations of the nematode *Caenorhabditis elegans* with high- and low-mutation rate genetic backgrounds and tracked the change in frequency of females, hermaphrodites, and males over approximately 21 generations. Hermaphrodites invaded all populations, regardless of mutational background. However, experimental populations with elevated mutation rates experienced more outcrossing and greater retention of females. This provides experimental evidence consistent with deleterious mutational explanations for the evolution of sex in principle, but the action of other processes is required to explain the evolution of sex in entirety.

Introduction

The pervasive incidence of sexual reproduction – in the face of costs of sex associated with reproductive rate among other potential factors – has prompted scores of proposed explanations (Maynard Smith, 1978; Bell, 1982; Michod & Levin, 1988; Kondrashov, 1993). Deterministic and stochastic mutational models account for the cost of sexual reproduction at high mutation rates by the more efficient purging of deleterious mutations or the reconstitution of more-fit genotypes, respectively, from genetic exchange (Muller, 1964; Kondrashov, 1988; Charlesworth, 1990; Gabriel *et al.*, 1993). Although these models are often considered in terms of the evolution of recombination, they also apply to breeding system evolution (i.e. asexual vs. selfing vs. outcrossing; Kondrashov, 1985; Charlesworth *et al.*, 1990; Otto, 2003). In particular, the deterministic mutational hypothesis proposes that per-generation genomic deleterious mutation rates (U) of order one or higher should lead to a short-term advantage of obligate outcrossing over asexual or selfing reproduction, provided that deleterious mutations exhibit synergistic epistasis or are predominantly recessive (Kondrashov, 1988; Charlesworth, 1990; Chasnov, 2000; Otto, 2003). In these models, the selective advantage of an allele that confers asexual or selfing reproduction over an obligate outcrossing allele is offset by the genetic load of deleterious mutations when the mutation rate is sufficiently high, because of the more efficient elimination of deleterious mutations under outcrossing.

A plethora of empirical studies have targeted the assumptions about U and synergistic fitness interactions among loci, generally yielding results that do not endorse a dominant role for deterministic deleterious mutational models relative to other theories for the evolution of sex (Lynch *et al.*, 1999; West *et al.*, 1999; Otto & Lenormand, 2002; Rice, 2002). Despite the prevalence of studies that explore the assumptions of a deterministic explanation for the evolution of sex and despite their conflicting results (high and low U; positive, negative, weak, or no synergistic epistasis), little attention has focused on direct empirical tests of the consequences of mutation rate variation for the evolution of sex. This is in large part because of the rarity of

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organisms amenable to experimental analysis, although experimental evolution work with viruses has demonstrated an effect of stochastic processes in promoting recombination (Poon & Chao, 2004).

The present study departs from these earlier approaches by directly testing the invasive ability of different breeding systems in populations whose genomes experience different mutational environments. *Caenorhabditis elegans* provides an elegant experimental system to address this issue because both the breeding system and mutation rate can be manipulated genetically with single allelic differences. *Caenorhabditis elegans* typical breeding system is androdioecious – males and self-fertile hermaphrodites – where gender in the XO sex-determination system is controlled by the ratio of X chromosomes to autosomes (Meyer, 2000). This sex determination mechanism and the germline gametogenesis switch in hermaphrodites are highly amenable to genetic manipulation, with single alleles capable of transforming hermaphrodites functionally into females (Schedl & Kimble, 1988; Minniti *et al.*, 1996; Hodgkin, 2002; Stewart & Phillips, 2002). Such alleles may be thought of as extreme modifiers of the rate of outcrossing sex or selfing, and they allow the construction of populations that are polymorphic for breeding system (trioecious) by differentiation of females and hermaphrodites through a single locus (Uyenoyama & Waller, 1991; Otto, 2003). Therefore, alleles conferring divergent reproductive strategy phenotypes can be placed in direct competition under different treatments that are expected to yield contrasting selection pressures on the two modes of reproduction.

Under standard laboratory conditions, *C. elegans* hermaphrodites outcompete females (Stewart & Phillips, 2002). However, should the genomic deleterious mutation rate contribute to the evolution of breeding system, this finding is to be expected given the low rates of deleterious mutation inferred for *C. elegans* (Keightley & Caballero, 1997; Vassilieva *et al.*, 2000; Cutter & Payseur, 2003a). To investigate the overall effect of mutation rate, I propagated populations of *C. elegans* under a genetic background of normal or elevated mutation and monitored the evolution of breeding system (Table 1). The high mutation genetic background was imposed with a mismatch repair-deficient mutation, which induces a ≥28-fold higher incidence of recessive lethal mutations than wildtype, corresponding to a value of U near one (Tijsterman *et al.*, 2002). Across approximately 21 generations, mutation rate positively influenced the rate of outcrossing sex and retarded the loss of the obligately outcrossing allele, consistent with predictions of mutational theories of the evolution of sex. However, the inability of the obligately outcrossing breeding system to invade populations that experience extraordinarily high rates of mutation suggests that alternative explanations are required to account for the widespread gonochorism observed in rhabditid nematodes.

Table 1 Experimental design

	Mutation rate	
Breeding system	High: *msh-6* (*pk2504/pk2504*)	Low: *msh-6* (+/+)
Trioecious*	TH	TL
Androdioecious: *spe-27* (+/+)	AH	AL

*Trioecious populations: *spe-27* (*it132/it132*) 'females', *spe-27* (+/+, +/*it132*) hermaphrodites, males (all *spe-27* genotypes).

Materials and methods

Worm strains

The backcrossed and inbred strains BA963 *spe-27* (*it132*) (Minniti *et al.*, 1996) and NL2511 *msh-6* (*pk2504*) (Tijsterman *et al.*, 2002) were kindly provided by the *Caenorhabditis* Genetics Center and were crossed and inbred to generate the homozygous double-mutant BA1122. Hermaphrodites homozygous for the *it132* allele produce only nonfunctional self-sperm at the restrictive temperature 25 °C, at which all experiments were conducted, and have very low self-fertility at lower temperatures (Minniti *et al.*, 1996). Thus, the *it132* allele can be thought of as an 'obligate outcrossing' allele, because populations fixed for *it132* are composed only of 'females' and males. Male development is unaffected by this mutation and *it132* males exhibit fertility that is nearly the same as for wildtype males (Minniti *et al.*, 1996).

The NL2511 worms homozygous for the *pk2504* exonic-deletion allele of the *mutS* mismatch-repair gene homologue *msh-6* exhibit greatly increased rates of spontaneous mutation (Tijsterman *et al.*, 2002). The reported range of the per-generation genomic deleterious mutation rate (U) for wildtype N2 *C. elegans* varies from 0.005 to 0.03 (Vassilieva & Lynch, 1999; Keightley & Bataillon, 2000; Cutter & Payseur, 2003a). U for NL2511 can be roughly estimated to lie between 0.14 and 0.84 assuming it can be scaled by the 28-fold higher incidence of recessive lethals in NL2511 relative to N2 (Tijsterman *et al.*, 2002), although it may be approximately 25 times higher still (Davies *et al.*, 1999). By applying Haldane's (1966) relation $m = (U_L/s)^{1/2}$, the genomic lethal recessive mutation rate (U_L) is approximated as 1.2 for NL2511 [$m = (1.1$ based on 20 000 genes in genome/300 genes in lethal screen$) \times 1.65\%$ recessive lethals (Tijsterman *et al.*, 2002); selection coefficient $s = 1$] and as 0.002 for wildtype *C. elegans* (Rosenbluth *et al.*, 1983; Tijsterman *et al.*, 2002). If temperature positively influences the mutation rate, these may be underestimates for the elevated incubation temperature used in this study. The high mutation rate leads to a dramatic reduction in fitness (Tijsterman *et al.*, 2002) (Fig. 1a). A recent mutation accumulation study showed that a strain with a

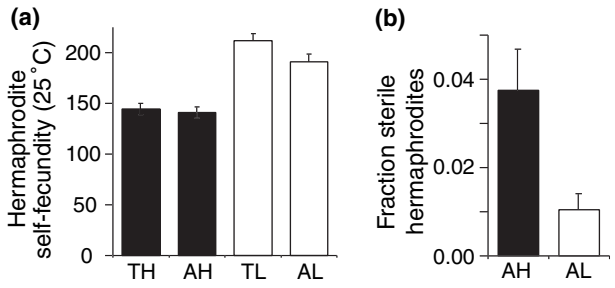

Fig. 1 Hermaphrodite self-fecundity (a) and self-sterility (b). (a) Self-fecundity is reduced in treatments experiencing high mutation rate (at transfer 21). (b) The incidence of hermaphrodite sterility (at transfer 23), which is higher in the treatment experiencing elevated mutation rate, was used to correct female frequency estimates. Treatment abbreviations as in Table 1. Bars indicate SEM.

similar mutator effect as NL2511 exhibits $U \sim 0.14$ for fitness-related traits, although U may be as high as 43 based on the incidence of new coding sequence mutations (Estes *et al.*, 2004).

Experimental populations

Ten initial populations were founded for each of the four treatments (Table 1). Trioecious populations were constructed by introducing the *spe-27* (*it132*) allele (Minniti *et al.*, 1996), such that 'females' (*it132*/*it132*), hermaphrodites (+/+, +/*it132*), and males (any *spe-27* genotype) coexisted in the founding populations. Homozygous *it132*/*it132* individuals (with two sex chromosomes) develop as hermaphrodites that fail to produce functional self-sperm under the experimental conditions and therefore are functionally females (Minniti *et al.*, 1996). At the first transfer, each replicate was split in quadruplicate to generate 40 replicate populations for each treatment. This large degree of replication was used to look for the possibility of mutational meltdown to extinction (Lynch *et al.*, 1993), which did not occur in any of the 160 populations. This lack of extinction is consistent with the maintenance of reasonably high fitness in populations of small size subject to elevated mutation rates (Estes *et al.*, 2004).

All populations were incubated at 25 °C (the restrictive temperature for *it132*) on 35 mm NGM agar Petri plates coated with a lawn of *E. coli* OP50 food source and transferred every third day (approximately one generation) by washing with 200 μL M9 buffer and subsequent transfer of 15 μL of suspended worms to a new plate. Although mixed-stage worms comprised the populations, the overwhelming majority of individuals were L1 or L2 larvae after three days and transfer aliquots contained >1000 individuals. At the experimental incubation temperature of 25 °C, egg-to-egg development takes approximately 60 h and L1 larvae take <35 h to molt into adults (Lewis & Fleming, 1995).

Androdioecious populations were initiated with 10 hermaphrodites and 10 males of strain N2 (low mutation rate; AL) or NL2511 (high mutation rate; AH). Initial high-mutation trioecious populations (TH) contained 10 BA1122 males, 10 BA1122 'females,' 10 NL2511 males, and 10 NL2511 hermaphrodites whereas low-mutation trioecious populations (TL) were founded with 10 BA963 males, 10 BA963 'females,' 10 N2 males, and 10 N2 hermaphrodites. Consequently, wildtype and *it132* alleles of *spe-27* were initially present at equal frequency in trioecious populations. The AL treatment is directly analogous to the experiment of Stewart & Phillips (2002) that followed the frequency of *fog-2* (*y71*), an allele that also confers a female phenotype when homozygous.

Phenotype scoring

The sex composition of each population was scored at transfers 1, 4, 8, 16, and 21. For scoring, 48 L1 or L2 individuals from each TL and TH population were picked to individual OP50 seeded NGM agar-filled wells on 24-well plates and incubated until adulthood, when self-fertility or -sterility could be rapidly scored to assess the number of hermaphrodites or 'females.' An additional sample of 100–300 L1 or L2 individuals from each population of all four treatments were picked to a single 35 mm plate, incubated until adulthood, and scored for the number of males and nonmales (hermaphrodites plus 'females' for the trioecious populations, hermaphrodites only for the androdioecous populations). Worms in high-mutation treatments were excluded when they exhibited gross morphological or developmental defects or intersex phenotypes that made gender identification or scoring of self-sterility ambiguous.

Because hermaphrodites in populations with a high mutation rate experienced an elevated incidence of self-sterility (Fig. 1b), 48 hermaphrodites from each of 10 replicates for treatments AL and AH were scored for sterility at transfer 23 from which I subsequently adjusted for the 'false-positive' rate in calculations of female frequency in trioecious populations for statistical analyses. Self-fecundity of five hermaphrodites from each of 10 populations of AH, AL, TH and TL was scored at transfer 21. Repeated measures ANOVA models were used to test for the independent effects of mutation rate, time, and breeding system on the frequency of males and the corrected fraction of self-sterile individuals among nonmales. Arcsin-square-root transformed frequencies were used in ANOVA, but the figures show untransformed data.

Results

Males and outcrossing

Males occurred at significantly higher frequency in treatments with a high mutation rate ($F_{1,156} = 35.2$,

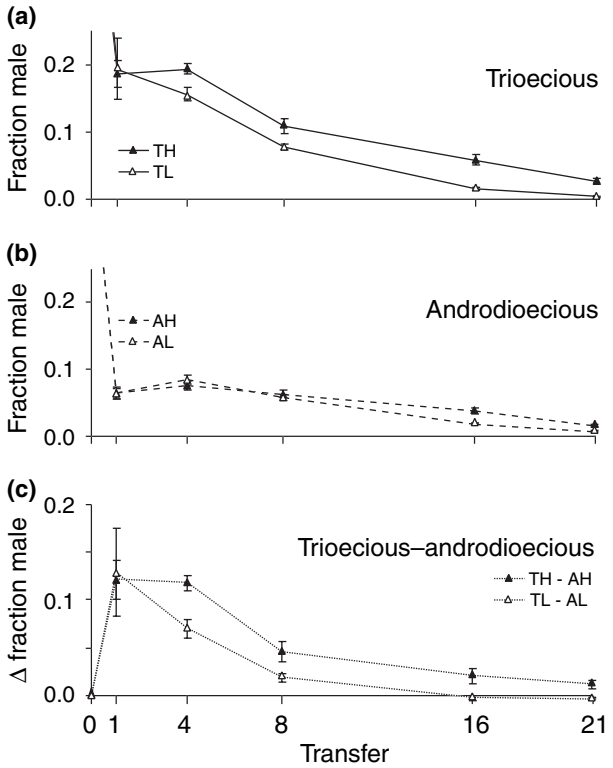

Fig. 2 Changes in male frequencies over time. In both trioecious (a) and androdioecious populations (b), males persist at greater frequencies under high mutation conditions. (c) Analysis of 'Δ fraction male' (TH − AH, TL − AL) controls for the possibility of elevated X-nondisjunction in high mutation treatments. Treatment abbreviations as in Table 1. Bars indicate SEM.

$P < 0.0001$; Fig. 2), indicating that outcrossing occurred more frequently in populations with a high mutation genetic background. Elevated male frequencies arose by transfer 4 and persisted for the remainder of the experiment (Fig. 2). Males also were more common in the trioecious treatments, independently of the effect of mutation ($F_{1,156} = 124.4$, $P < 0.0001$; Fig. 2). The XO sex-determination mechanism in *C. elegans* dictates that males may be produced only by crossing or rare self-fertilizations with X-nondisjoined gametes (Hodgkin *et al.*, 1979); thus male frequency is directly related to the rate of outcrossing (Chasnov & Chow, 2002; Stewart & Phillips, 2002; Cutter *et al.*, 2003).

The higher frequency of males under high mutation conditions is not because of some artefact of more X-nondisjunction. Differential nondisjunction rates between mutation regimes would be expected to be equivalent in both breeding system treatments, so androdioecious populations can be used as a control for trioecious populations by comparing the differences in male frequency (TH% male − AH% male) to (TL% male − AL% male) ($F_{1,78} = 36.8$, $P < 0.0001$; Fig. 2c). The observation that (TH% male − AH% male) is greater than (TL% male − AL% male) demonstrates that males are more abundant in the high mutation treatment even after accounting for any potential effects of mutation rate that are common to populations in different breeding system treatments (e.g. by affecting X-nondisjunction). Furthermore, *msh-6* (*pk2504*) has been shown previously to exhibit no effect on X-nondisjunction (Tijsterman *et al.*, 2002). This analysis would be inappropriate only in the case that hermaphrodite viability suffers disproportionately from the effects of a higher mutation rate, resulting in a greater incidence of males in the high mutation rate treatments. However, male fitness should be impacted to a greater degree than hermaphrodite or female fitness because (i) the hemizygosity of the X chromosome in males will make every X-linked deleterious mutation visible to selection, (ii) male-related genes are uniformly distributed among all chromosomes (Reinke *et al.*, 2004), and (iii) early embryo-expressed genes are represented disproportionately on the X chromosome (Baugh *et al.*, 2003). Consequently, the elevation in male frequency when faced with a high mutation rate is most likely because of increased outcrossing than to decreased fitness or to an altered X-chromosome nondisjunction rate.

The elevated incidence of males in the high mutation treatments indicates that mutation rate positively influences the rate of outcrossing. The 2–5% elevation in male frequency under high mutation conditions in the trioecious treatment corresponds to a approximately 4–10% higher incidence of outcrossing (Cutter *et al.*, 2003); likewise, the last two sample points in the androdioecious populations suggest approximately 2–4% more outcrossing in the high mutation treatment. These observations are consistent with the prediction that greater inbreeding depression, because of an elevated mutation rate, should lead to more outcrossing and higher frequencies of males (Chasnov & Chow, 2002; Stewart & Phillips, 2002; Cutter *et al.*, 2003).

Females in trioecious populations

Females were significantly more common under high mutation rate conditions ($F_{1,78} = 54.6$, $P < 0.0001$; Fig. 3a), although they declined in overall frequency over the course of the experiment. The significance of the effect of mutation on female frequency declines when adjusted values are used ($F_{1,78} = 6.9$, $P = 0.011$; Fig. 3b). This retardation of female loss by an elevated mutation rate is consistent with the notion that the cost of obligate outcrossing is reduced as the influx of deleterious mutations rises. In other words, selection favouring selfing is stronger when the deleterious mutation rate is low. The deterministic mutational model for the evolution of sex predicts that an advantage of outcrossing should occur in the short-term (Kondrashov, 1988), consistent with the rapid origin of the differences between mutation treatments in the frequencies of males

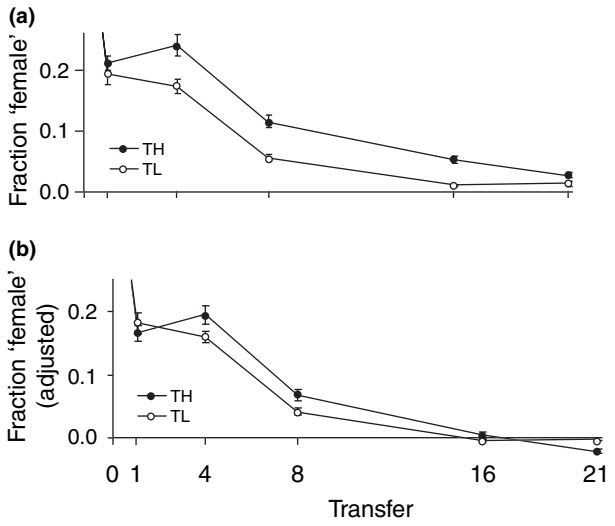

Fig. 3 Changes in 'female' frequencies over time. 'Females' are more common in the high mutation treatment at intermediate sample points. (a) Fraction of nonmales scored as 'female' and (b) adjusted for baseline sterility rates. Treatment abbreviations as in Table 1. Bars indicate SEM.

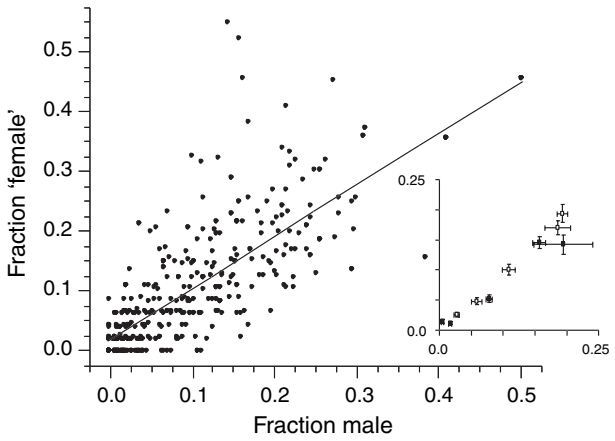

Fig. 4 Male and 'female' frequencies correlate positively. Inset shows mean gender frequencies for each sample point, separated by mutation treatment (TH filled, TL unfilled boxes). The line indicates least-squares regression fit. Bars indicate SEM.

and females. Furthermore, the strong correlation between the frequencies of females (which can reproduce only by crossing) and males (which only crossing can produce) attests to the association between outcrossing and the incidence of males in populations ($r^2 = 0.46$, $F_{1,398} = 338.9$, $P < 0.0001$; Fig. 4).

Hermaphrodites invade all populations

Despite elevated male (and female) frequency in high-mutation treatments, hermaphrodites invaded all 160

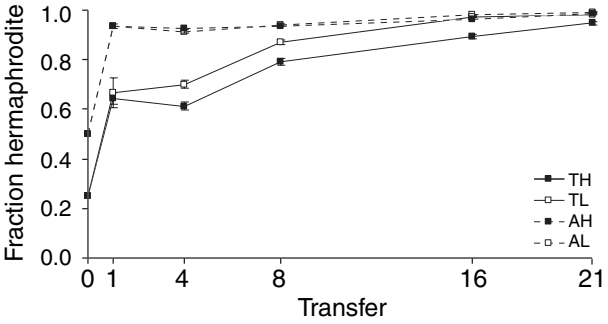

Fig. 5 Changes in hermaphrodite frequencies over time. Hermaphrodites invade populations with high or low mutation rate. Self-fecundity of hermaphrodites is significantly lower in high-mutation rate treatments (inset). Treatment abbreviations as in Table 1. Bars indicate SEM.

populations over the course of 21 transfers, irrespective of genomic mutational background or breeding system (Fig. 5). Thus, the initial observation by Stewart & Phillips (2002) that *C. elegans* hermaphrodites rapidly outcompete females in a wildtype genetic background also extends to populations with genomes subject to greatly increased deleterious mutational input. This dominant competitive ability of hermaphrodites indicates that the elevated mutation rate was insufficient to offset the cost of obligate outcrossing in the short-term.

Discussion

One explanation for the invasion of all populations by hermaphrodites is that the fitness effects of deleterious mutations may not interact synergistically or may only rarely be recessive, thus undermining the assumptions of deterministic mutational models (Kondrashov, 1988; Charlesworth, 1990; Chasnov, 2000; Otto, 2003). Although experimental studies in *C. elegans* indicate that the degree of epistasis between deleterious mutations is highly variable (Peters & Keightley, 2000), it is a long-held view that deleterious mutations exhibit primarily recessive phenotypic effects (Muller, 1950; García-Dorado *et al.*, 1999). Empirical support for recessive effects in *C. elegans* comes from both mutation accumulation experiments (Vassilieva *et al.*, 2000) and mutagenesis experiments (Peters *et al.*, 2003).

Alternatively, the benefit of more efficient purging of deleterious mutations may not exceed the cost of obligate outcrossing even in the context of an elevated rate of mutation. This could occur for two reasons: (i) the estimate of U conferred by *msh-6* (*pk2504*) might be lower than that required to give outcrossing a net advantage over pure selfing (Charlesworth, 1990) and (ii) hermaphrodite *C. elegans* can facultatively mate with males. Theory suggests that obligate outcrossing can invade a partially selfing population less readily than a purely

selfing population and a small amount of outcrossing at equilibrium can be favoured under some conditions (Charlesworth & Charlesworth, 1987; Charlesworth, 1990; Charlesworth *et al.*, 1991; Uyenoyama & Waller, 1991). Thus, a small amount of outcrossing by hermaphrodites – as predicted for *C. elegans* in nature and the laboratory (Hedgecock, 1976; Chasnov & Chow, 2002; Stewart & Phillips, 2002; Cutter *et al.*, 2003; Cutter & Payseur, 2003b) – may provide sufficient benefits in terms of mutation elimination to preclude the evolution of obligate outcrossing even in populations faced with an unusually high mutation rate. The higher incidence of males, and therefore outcrossing, observed in populations experiencing elevated mutation rates regardless of breeding system treatment argues for an important role for the cross-fertilization of hermaphrodites.

Traditional models of the evolution of breeding system that focus on inbreeding depression indicate that a number of factors can influence the evolution of selfing in complicated ways. Depending on the average strength of selection against new mutations, purging of deleterious mutations by inbreeding (i.e. selfing) may or may not be sufficiently effective to allow a selfing genotype to invade an outcrossing population (Lande & Schemske, 1985; Charlesworth *et al.*, 1990). The ability for inbreeding depression to describe the relative fitness of self- and cross-progeny also depends on mutational dominance such that, for example, selfing can invade a population even under extreme inbreeding depression because of recessive lethals (Holsinger, 1988). However, selfing should incur less of an advantage relative to outcrossing in the presence of 'pollen discounting' (Holsinger *et al.*, 1984) – such discounting is complete in *C. elegans* where hermaphrodites cannot inseminate other hermaphrodites.

The finding that selfing hermaphrodites invade populations under conditions of both low and high mutation rate suggests that the selfing advantage might result from reductions in fitness that are predominantly caused by large-effect deleterious mutations, which can be purged effectively by inbreeding and can lead to an advantage of selfing even with >50% reduction in fitness relative to outcrossers. Such an explanation is consistent with the observations that (i) no heterosis or inbreeding depression is found in natural strains of *C. elegans* and that (ii) large-effect detrimental mutations induce most of the mutational variance in a strain that experiences a high mutation rate (*msh-2*) (Johnson & Hutchinson, 1993; Chasnov & Chow, 2002; Estes *et al.*, 2004). Mutant alleles of *msh-2* and of *msh-6*, used in this study, have very similar effects (Tijsterman *et al.*, 2002). Most mutations induced by ineffective DNA repair (e.g. *msh-2*) or chemical mutagenesis (e.g. EMS) have small deleterious fitness effects (Davies *et al.*, 1999; Estes *et al.*, 2004). However, it is not clear experimentally whether such slightly deleterious mutations are important for breeding system evolution in *C. elegans*, as selfing cannot efficiently purge this class of mutations (Charlesworth *et al.*, 1990). Theory also shows that extreme modifiers of the selfing rate (like those alleles used in the experiments in this study) can often invade populations more easily than subtle modifiers (Charlesworth *et al.*, 1990). Thus, it will be important to study the experimental evolution of modifiers with a range of effects on the selfing rate.

In conclusion, previous studies have demonstrated wide variation in genomic deleterious mutation rates and mixed evidence for the occurrence of synergistic epistasis (Drake *et al.*, 1998; Lynch *et al.*, 1999; Rice, 2002). This study presents a direct test for an effect of mutation on the genetic invasibility of different types of breeding system. Although these data demonstrate the plausibility of a role for deleterious mutational explanations in the evolution of outcrossing, the persistence of obligate outcrossing throughout most of the *Caenorhabditis* genus in the face of low genomic rates of deleterious mutation suggests that deleterious mutational explanations alone can not fully account for the evolution of different breeding systems (Cutter & Payseur, 2003a). Additional explanations, such as environmental-ecological models, may need to be invoked to help explain the evolution of breeding system in combination with deleterious mutational theories (West *et al.*, 1999).

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