BRIEF COMMUNICATIONS

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GENETIC VARIATION FOR OUTCROSSING AMONG *CAENORHABDITIS ELEGANS* ISOLATES

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Abstract.—The evolution of breeding systems results from the existence of genetic variation and selective forces favoring different outcrossing rates. In this study we determine the extent of genetic variation for characters directly related to outcrossing, such as male frequency, male mating ability, and male reproductive success, in several wild isolates of the nematode *Caenorhabditis elegans*. This species is characterized by an androdioecious breeding system in which males occur with hermaphrodites that can either self-fertilize or outcross with males. We find genetic variation for all characters measured, but also find that environmental variation is a large fraction of the total phenotypic variance. We further determine the existence of substantial genetic variation for population competitive performance in several laboratory environments. However, these measures are uncorrelated with outcrossing characters. The data presented here contribute to an understanding of male maintenance in natural populations through their role in outcrossing.

Key words.—*Caenorhabditis elegans*, genetic variation, outcrossing, self-fertilization.

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The evolution of breeding systems requires the existence of genetic variation, selection pressures favoring alternative reproductive modes (selfing or outcrossing), and differential sexual allocation among and within individuals. Extant diversity of breeding systems both between and within species attests for the existence of genetic variation for outcrossing rates (Jarne and Charlesworth 1993; Byers and Waller 1999; Pannell 2002). However, a few species have the potential to become experimental models to study breeding system evolution such as the nematode *Caenorhabditis elegans*, given the ease of laboratory cultivation and the ability to manipulate its breeding system (Hodgkin 1983; Stewart and Phillips 2002).

Caenorhabditis elegans is characterized by an androdioecious breeding system, in which hermaphrodites can either self-fertilize or outcross with males. Sex determination results from X-chromosome number; hermaphrodites having two and males only one. Males are either produced from male-hermaphrodite breeding or from the fertilization of aneuploid gametes where the meiotic nondisjunction of the X chromosome has occurred with normal gametes. Given this breeding system, outcrossing can be equated with male phenotypes.

It is thought that androdioecy is derived from dioecy (Kiontke et al. 2004), but it is an open question whether males are selectively maintained in natural populations (Chasnov and Chow 2002; Stewart and Phillips 2002; Cutter et al. 2003). Therefore, an important venue of research is characterization of genetic variation for characters related to outcrossing; in particular, those associated with male phenotypes. Numerous studies have described the developmental genetics of sex determination, although it is still difficult to relate them with natural genetic variation for outcrossing rates (see Hodgkin 1983, 2002; Nayak et al. 2005). The study of LaMunyon and Ward (2002) is notable since they describe significant quantitative genetic variation among some wild isolates of *C. elegans* for a character that may be related to outcrossing: male sperm size. In this report we extend the characterization of natural genetic variation to a larger number of characters associated with outcrossing and male reproductive success, to a larger number of wild isolates and laboratory environments.

MATERIALS AND METHODS

Genetic Variation in Male Proportions, Male Mating Ability, and Performance Characters

We obtained a total of 26 strains/isolates from the *Caenorhabditis* Genetics Center (CGC; AB1, AB3, CB4507, CB4852, CB4853, CB4854, CB4855, CB4856, CB4858, CB4932, MY1, MY2, MY3, MY6, MY16, N2 ancestral, PB306, RC301, and TR388), from M.-A. Félix (JU262, JU319, JU345, JU362, JU393, JU400, and JU440), and from B. White and P.C. Phillips (PX174, PX179; sampled in Oregon in 2002). These were inbred by single individual selfing for 10 generations to ensure isogenic backgrounds so that any phenotypic variation within strain could be attributed to environmental causes, with phenotypic variation among isolates due to genetic causes. After these 10 generations, cultures were expanded to high densities for two generations and cryogenically frozen for later use in the assays (Stiernagle 1999). The strains were chosen, based on their original geographical origin and described molecular diversity, to represent extant variation in this species (Hodgkin and Doniach 1997; Koch et al. 2000; Denver et al. 2003; Barrière and Félix 2005; Haber et al. 2005).

We measured phenotypic variation in the frequency of nondisjunction of the X chromosome in hermaphrodite gametogenesis in this set of 26 strains by determining the proportion of males segregating in the progeny of unmated her-

maphrodites. Samples from the frozen stocks were obtained and maintained under a standard laboratory environment for two generations. Briefly, the standard laboratory environment consists in the maintenance of approximately 1000 individuals in a 9-cm-diameter petri dish with NGM-light agar (US Biological, Swampscott, MA) covered with a lawn of HT115 *Escherichia coli* as food source. Each generation, gravid adults are killed by a hypochlorite/sodium hydroxide solution. Under this treatment only eggs survive (Stiernagle 1999). Eggs are then maintained in an M9 buffer solution for 16–18 h until all individuals arrest at a synchronized starved first larval stage (L1). Completion of this life cycle takes four days at 20°C and 80% RH controlled conditions. To propagate the next generation, L1 individuals are placed onto fresh petri dishes with bacteria at the appropriate density. Male proportions were estimated by counting the number of males relative to total number of individuals in eight petri dishes for each strain, for an average of 8000 individuals per observation. Binomial distributions were used to estimate sampling errors for each strain on the likelihood of being a male or a hermaphrodite, and no further analysis was done.

Male mating ability was also measured in the 26 isogenic strains. Stocks enriched with males were obtained by keeping an excess of males relative to hermaphrodites for three generations after thawing of frozen stocks. After this period, gravid hermaphrodites were killed by a bleach/NaOH solution, as above. Male mating ability was assayed by placing, three days later, six immature (L4) wild-type males competing for the fertilization of 22 hermaphrodites over a 24-hr period. Hermaphrodites were marked using the transgenic expression of a green flourescent protein (GFP) under a myosin-3 promoter integrated into a N2 strain background (named tester strain; CGC PD4251). Competition took place in two replicate 6-cm-diameter petri dishes seeded with a 4-mm-diameter lawn of bacteria. After the 24-h period, 20 hermaphrodites from each plate were killed with the bleach/NaOH solution. The progeny of these hermaphrodites that survived was then scored for gender. Male proportions were taken as a measure of male mating ability, which is thought to be maximized at these relative densities (Stewart and Phillips 2002). Since data was obtained as proportions, it was angular-transformed to comply with linear model assumptions and analyzed using an analysis of variance (ANOVA) with strain as a fixed factor.

Competitive population performance was estimated by placing 500 individuals of the wild strains together with 500 L1 individuals from the tester GFP strain in two replicate petri dishes. After three days the standard bleach/NaOH treatment was imposed and, at day 4, surviving L1s were placed in fresh petri dishes at a density of 1000 individuals. Again, three days later, the petri dishes were transferred to 4°C until scoring. Estimates of performance were obtained for each strain as the ratio of wild-type individuals to GFP marked individuals, assuming no outcrossing. Another competitive population performance assay was performed, which was similar to the standard competition except that the first generation adults were not bleached but instead left to lay eggs and exhaust the bacterial food source. After two weeks, surviving individuals, which were mostly at the stress-resistant dauer stage, were transferred to new petri dishes at 1000 density, allowed to grow to adulthood, and scored for GFP expression. As for male mating ability, competitive performance data was angular-transformed, and an ANOVA with strain as a fixed factor was performed.

Correlations among male frequency, male mating ability, and performance in several environments were estimated as the Pearson correlation coefficient using a single average estimate per strain.

Genetic Variation in Male Proportions and Performance Characters at Three Temperatures

To address the replication of our estimates and the existence of phenotypic plasticity with temperature for male proportions and competitive performance, we assayed a subset of the original 26 strains (AB1, CB4852, CB4856, PX174, N2, JU440), about six months later than the first set of assays. Two single hermaphrodites per strain were sampled from the frozen stocks and allowed to expand in numbers separately during two generations in a common environment. Maintenance during the two generations and estimates of male proportions and competitive performance under standard conditions were conducted at 15°C, 20°C, and 25°C.

Within-strain structure in these temperature assays can be modeled because two independent hermaphroditic lineages per strain were measured. Assay plate was taken as the observational unit. Analyses of variance were performed with temperature treatment as a fixed effect, strain also as a fixed effect, and hermaphrodite lineage as a random nested within-strain effect. The interactions between temperature treatment and strain, and between temperature and hermaphrodite (within strain) were also assessed. For male proportions there was unequal within-strain replication: although strains CB4856 and CB4852 were only assayed in four and eight plates at 15°C, respectively, most strains were assayed in 11 plates at 15°C, and all strains assayed in nine plates at 20°C and 25°C. Because there were two experimenters scoring male proportions, this factor was first assessed for a differential effect before proceeding with the full models, which in no case was found to be significant. For competitive performance in standard conditions, three petri dishes per hermaphrodite lineage and per strain were assayed.

Replicability of male production estimates among the two 20°C assays performed six months apart was assessed with a categorical linear analysis, in which strain effects were tested for heterogeneity using a χ^2 distribution on the log-likelihoods of being hermaphrodite or male across assays, taking a single average estimate per strain (Sokal and Rohlf 1995).

Genetic Variation for Male Reproductive Success

Stewart and Phillips (2002) have shown selection against males in the laboratory, when using the reference N2 strain, by observing that populations that start with close to 50% males rapidly lose males in the span of less than 10 generations (see also Cutter 2005). Here, we repeated these experiments using four different wild strains (CB4856, PX174, N2, JU440) and employing a nonoverlapping generation protocol maintenance, as described above for the standard laboratory environment, for 11 generations. For each isogenic

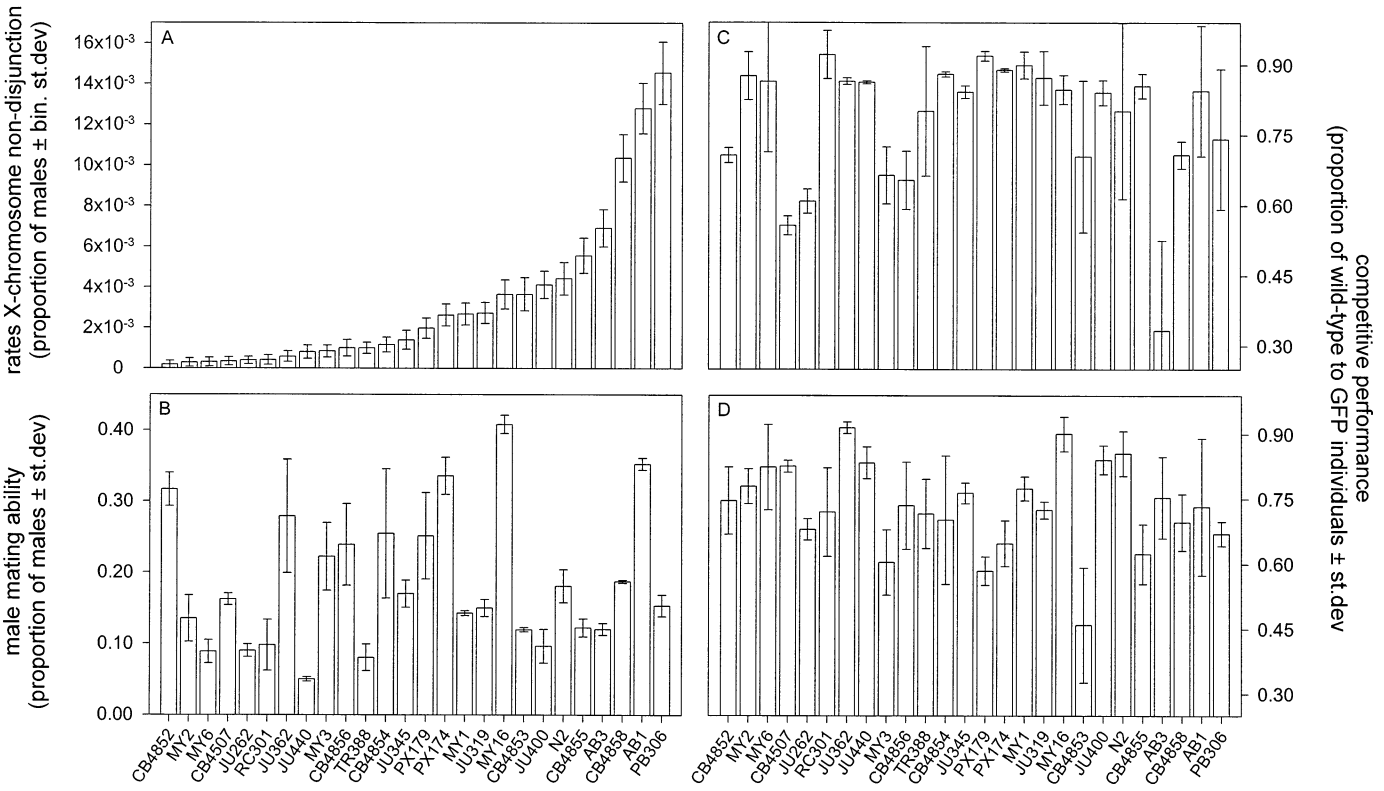

FIG. 1. Strain characterization for (A) male proportions, (B) male mating ability, (C) competitive population performance under standard conditions, and (D) competitive population performance under starvation conditions. Each strain is represented by a mean bar with associated standard deviations as error bars. For male proportions, error bars indicate binomial sampling errors.

strain, two replicate lines were obtained by placing several hermaphrodites with an excess of males to ensure outcrossing and a high proportion of males, at generation zero of the experiments. Each of the two replicates was further subdivided in two the following generation, for a total of 16 independent lines maintained. Male proportions were observed at generations 1, 2, 3, 4, 5, 7, 9, and 11 of maintenance.

Each of the four replicate populations within each of the strains were the units of observation for the analysis (16 datapoints at each generation). A repeated-measures ANOVA was fitted to the data, with strain as a four-level fixed factor (CB4856, JU440, PX174, and N2) and generation as an eight-level fixed factor.

RESULTS AND DISCUSSION

Male production in *C. elegans* is a rare event because it results from the meiotic nondisjunction of the X chromosome during the gametogenesis of hermaphrodites. Nevertheless, we found genetic variation for male production among the wild isolates that were studied (Fig. 1A). The average male frequency resulting from X chromosome nondisjunction among the 26 isolates was 0.33% ± 0.015% SD, with the lowest frequency observed in strain CB4852 (0.019%) being close to two orders magnitude lower than the highest frequency from strain PB306 (1.5%). Taking into consideration the binomial sampling errors, it appears that there are strain-specific differences. Retesting a subset of these lines over a set of three different temperatures reveals that male production is highest at intermediate temperatures (Fig. 2, Table 1). These temperature-related effects tended to dominate any strain-specific differences, and no significant among-strain variation was detected in this subset of lines even though a relatively large fraction of the total variance is explained by this factor (see Table 1). Further, the among-hermaphrodite variance within each strain was highly significant, indicating substantial local environmental variation.

Despite the large environmental components on male production, we tested specifically for the among-line component of variance using a categorical linear analysis with the six strains tested twice at 20°C in the two experiments. This revealed highly replicable estimates. The estimates obtained from the two assays performed six months apart were not significantly different, but the log-likelihoods of being a male or an hermaphrodite differs significantly among strains ($\chi^2_5 = 343$; $P < 0.001$). Based on the combined results of these two assays, we conclude that there is genetic variation for rates of X-chromosome nondisjunction in hermaphrodites.

Significant genetic differences among strains in male mating ability are revealed in the tester hermaphrodite fertilization experiment (Fig. 1B; $F_{25,51} = 16.3$, $P < 0.001$). There can be as much as an eightfold difference in the fraction of offspring sired by a male of a particular strain. Despite being circumstantial, we notice that both male proportions and male mating ability in the strain AB1 is higher than in the strain N2, as found for sperm size differences between these strains by LaMunyon and Ward (2002). We were not able to detect

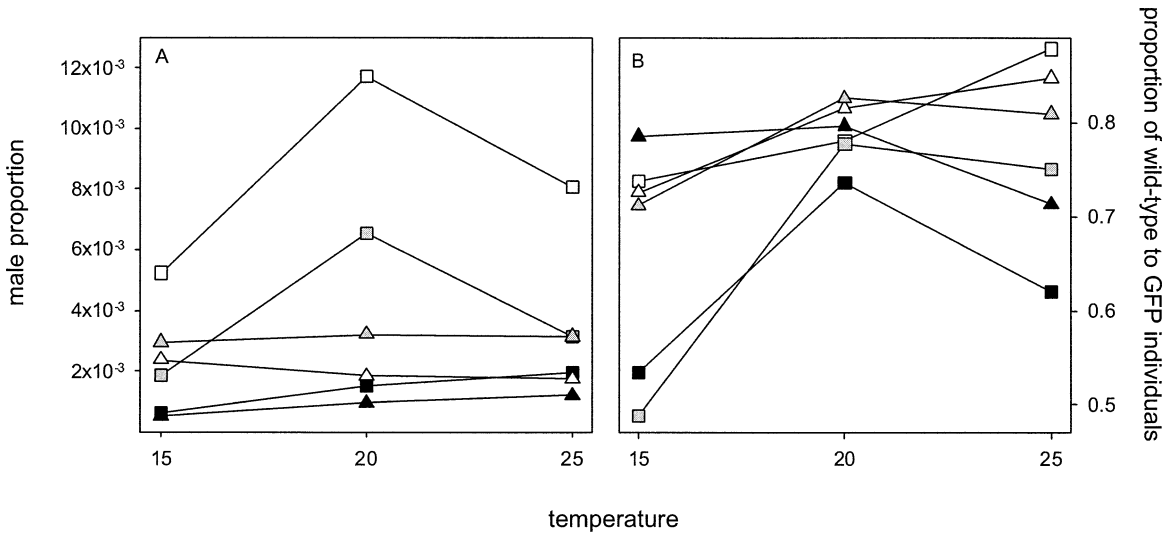

FIG. 2. Isogenic strain characterization for male proportions (A) and competitive population performance in standard conditions (B) at three different temperatures. Only mean values are shown for clarity: white squares for strain AB1, gray squares for CB4856, black squares for CB4852, white triangles for JU440, gray triangles for PX174, and black triangles for N2.

a significant correlation among male proportions and male mating ability ($r = 0.143$, $P = 0.466$), which probably reveals a lack of genetic association among these characters.

Competitive population performance also reveals genetic differences among the *C. elegans* strains studied, in particular in the first set of assays with 26 strains (Fig. 1C, D). Significant differences are apparent under both standard ($F_{25,51} = 3.88$, $P = 0.001$) and starvation ($F_{25,51} = 3.87$, $P = 0.001$) conditions. In Table 1 we show the results obtained for competitive performance at three different temperatures. It is clear that performance increases with increasing temperature and that variance among hermaphroditic lineages of the same strain is strongly dependent on temperature. This last observation indicates a high environmental variance component, as for male proportions (above). As for male proportion and male mating ability, we do not find any significant correlations between male production and competitive population performance within each temperature treatment ($r_{15oC} = 0.244$, $P = 0.641$; $r_{20oC} = -0.074$, $P = 0.890$; $r_{25oC} = 0.620$, $P = 0.189$, respectively).

In our last set of experiments we characterized an outcrossing rate function as measured by the male frequencies over 11 generations of laboratory maintenance. As in previous studies (Stewart and Phillips 2002; Cutter 2005), we find that the frequency of males in androdioecious populations tends to be rapidly driven to low levels (Fig. 3, Table 2). Most significant for this study, however, is the fact that there are large differences in male frequencies maintained among different natural isolates. Males are quickly eliminated from the standard lab strain, N2, as well as the French isolate JU440, whereas the rate of decline in males in populations of natural isolates from Hawaii (CB4856) and Oregon (PX174) is moderate (Fig. 3). These results are confirmed by a repeated-measures ANOVA, which shows highly significant strain and strain-by-generation effects (see Table 2). Posterior contrasts using Tukey's test reveal differences among all strains, except between N2 and JU440 (not shown).

Despite the lack of correlation between male mating ability from the first set of assays (Fig. 1B) and the outcrossing rate function (Fig. 3)—we only have four datapoints (four strains)

TABLE 1. Analysis of variance for male proportion and competitive performance (both angular transformed) contrasted among six isogenic strains and three different temperatures. Hermaphrodite refers to the independent lineages derived from two sampled hermaphrodites within each strain. Mean squares are based on type III sum-of-squares.

Character	Source	df[1]	MS	*F*	*P*
Male proportion	Strain	5, 6	0.02652	2.55	0.143
	Hermaphrodite(strain)	6, 12	0.01043	6.52	0.003
	Temperature	2, 12	0.00887	5.57	0.019
	Temperature × hermaphrodite(strain)	12, 304	0.00161	2.83	0.001
	Strain × temperature	10, 12	0.00150	0.93	0.538
	Error	304	0.00057		
Performance	Strain	5, 6	0.118440	2.27	0.174
	Hermaphrodite(strain)	6, 12	0.052279	1.51	0.254
	Temperature	2, 12	0.202522	5.87	0.017
	Temperature × hermaphrodite(strain)	12, 72	0.034523	11	<0.001
	Strain × temperature	10, 12	0.034929	1.01	0.485
	Error	72	0.003147		

[1] Degrees of freedom for the numerator, denominator.

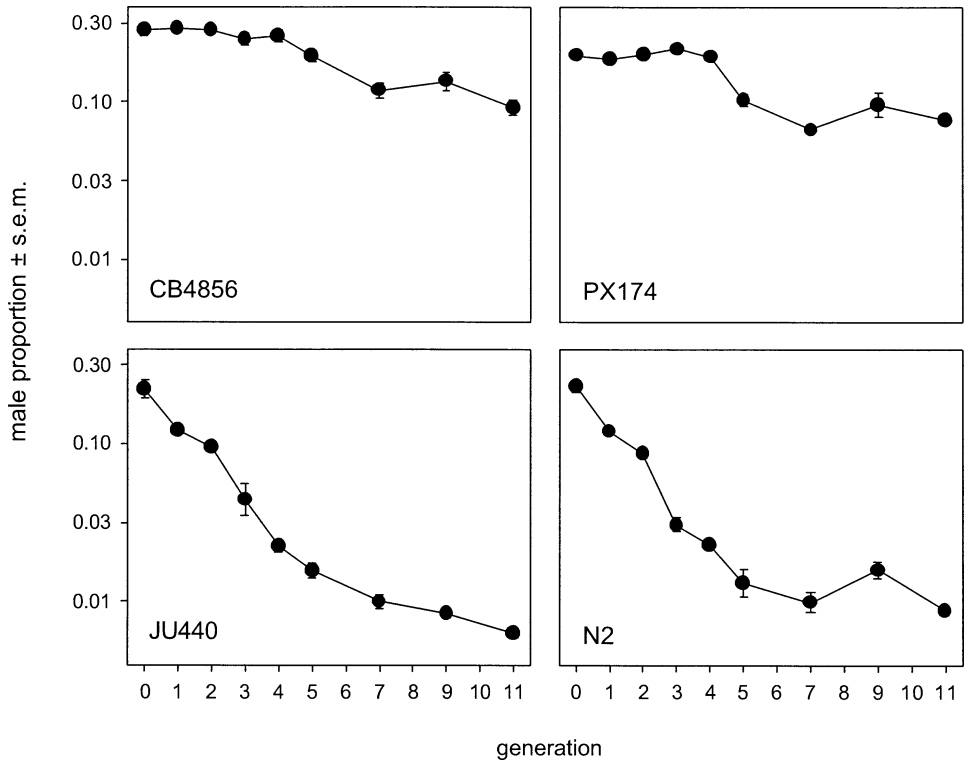

FIG. 3. Male frequency decrease in four isogenic strains during 11 generations of laboratory maintenance after initial set-up at high frequencies. In all plots, data are shown as mean values of the four replicates with standard error of the mean as the error bars. For clarity, data is shown in a $\log(10^2 \times$ male proportions) scaling, and labeling is shown in proportions.

for comparison, which would make such a correlation difficult to detect—the two strains that have higher outcrossing rate functions, CB4856 and PX174, also have higher male mating ability than the two strains that have a lower outcrossing rate function. We thus interpret the differences in the male frequency decrease among strains as a consequence of differences in male reproductive success, although differences in hermaphrodite reproductive success cannot be excluded (see Chasnov and Chow 2002). Certainly, other natural strains will present a range of outcrossing rates in addition to those that we found.

The maintenance of genetic variation for male production, male mating ability, and outcrossing rates is difficult to predict from evolutionary theory, at least until we have knowledge about the actual genes determining phenotypic variation, under the relevant ecological conditions for these wild isolates. Although it can be maintained by natural selection (Stewart and Phillips 2002), some have suggested that genetic variation, at least in male population frequencies, is simply maintained by neutral processes (Chasnov and Chow 2002; for a rebuttal see Cutter et al. 2003). Studies based on molecular diversity among natural isolates have found evidence for low but substantial outcrossing in the wild. For example, some studies have reported a lack of similarity in the topology of nuclear and mitochondrial genealogies (Graustein et al. 2002; Denver et al. 2003), which is interpreted as resulting from the interbreeding of several genotypic isolates (rather than mutation), since many of the same polymorphisms are shared among isolates. Other studies have shown that levels of gene diversity and linkage disequilibrium in local populations are all compatible with models in which outcrossing occurs at rates of approximately 1% (Jovelin et al. 2003; Barrière and Félix 2005; but see Sivasundar and Hey 2005). Nevertheless, these studies continue to support the idea that *C. elegans* populations are highly structured in the wild, with much local differentiation, probably reflecting the colonizing life cycle of this species (Fitch 2005). Our data adds to growing evidence that despite a predominantly selfing breeding mode, *C. elegans* harbors genetic variation for characters related to outcrossing.

TABLE 2. Repeated-measures analysis of variance for male proportion (angular-transformed) during 11 generations of laboratory maintenance, contrasted among four isogenic strains. Mean squares are based on type III sum-of-squares.

Source	df[1]	MS	*F*	*P*
Strain	3, 84	0.667351	848.19	<0.001
Generation	7, 84	0.139047	176.73	<0.001
Strain × generation	21, 84	0.006398	8.13	<0.001
Replicate	12, 84	0.000441	0.56	0.867
Error	84	0.000787		

[1] Degrees of freedom for the numerator, denominator.

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Literature Cited

Barrière, A. and M. Félix. 2005. High local genetic diversity and low outcrossing rate in *Caenorhabditis elegans* natural populations. Curr. Biol. 15:1176–1184.

Byers, D. L., and D. M. Waller. 1999. Do plant populations purge their genetic load? Effects of population size and mating history on inbreeding depression. Annu. Rev. Ecol. Syst. 30:479–513.

Chasnov, J. R., and K. L. Chow. 2002. Why are there males in the hermaphroditic species *Caenorhabditis elegans*? Genetics 160: 983–994.

Cutter, A. D. 2005. Mutation and the experimental evolution of outcrossing in *Caenorhabditis elegans*. J. Evol. Biol. 18:27–34.

Cutter, A. D., L. Avilés, and S. Ward. 2003. The proximal determinants of sex ratio in *C. elegans* populations. Genet. Res. Camb. 91:91–102.

Denver, D. R., K. Morris, and W. K. Thomas. 2003. Phylogenetics in *Caenorhabditis elegans*: an analysis of divergence and outcrossing. Mol. Biol. Evol. 20:393–400.

Fitch, D. H. A. 2005. Evolution: an ecological context for *C. elegans*. Curr. Biol. 15:R655–R658.

Graustein, A., J. M. Gaspar, J. R. Walters, and M. F. Palopoli. 2002. Levels of DNA polymorphim vary with mating system in the nematode genus *Caenorhabditis*. Genetics 161:99–107.

Haber, M., M. Schüngel, A. Putz, S. Müller, B. Hasert, and H. Schulenburg. 2005. Evolutionary history of *Caenorhabditis elegans* inferred from microsatellites: evidence for spatial and temporal genetic differentiation and the occurrence of outbreeding. Mol. Biol. Evol. 22:160–173.

Hodgkin, J. 1983. Male phenotypes and mating efficiency in *Caenorhabditis elegans*. Genetics 103:43–64.

———. 2002. Exploring the envelope: systematic alteration in the sex-determination systems of the nematode *Caenorhabditis elegans*. Genetics 162:767–780.

Hodgkin, J., and T. Doniach. 1997. Natural variation and copulatory plug formation in *Caenorhabditis elegans*. Genetics 146: 149–164.

Jarne, P., and D. Charlesworth. 1993. The evolution of the selfing rate in functionally hermaphrodite plants and animals. Annu. Rev. Ecol. Syst. 24:441–466.

Jovelin, R. M., B. C. Ajie, and P. C. Phillips. 2003. Molecular evolution and quantitative variation for chemosensory behaviour in the nematode genus *Caenorhabditis*. Mol. Ecol. 12: 1325–1337.

Kiontke, K., N. P. Gavin, Y. Raynes, C. Roehrig, F. Piano, and D. H. A. Fitch. 2004. *Caenorhabditis* phylogeny predicts convergence of hermaphroditism and extensive intron loss. Proc. Natl. Acad. Sci. 101:9003–9008.

Koch, R., H. G. A. M. van Luenen, M. van der Horst, K. L. Thijssen, and R. H. A. Plasterk. 2000. Single nucleotide polymorphisms in wild isolates of *Caenorhabditis elegans*. Genome Res. 10: 1690–1696.

LaMunyon, C. W., and S. Ward. 2002. Evolution of larger sperm in response to experimentally increased sperm competition in *Caenorhabditis elegans*. Proc. R. Soc. Lond. B 269:1125–1128.

Pannell, J. R. 2002. The evolution and maintenance of androdioecy. Annu. Rev. Ecol. Syst. 33:397–425.

Nayak, S., J. Goree, and T. Schedl. 2005. *fog-2* and the evolution of self-fertile hermaphroditism in *Caenorhabditis*. PloS Biol. 3: 57–71.

Sivasundar, A., and J. Hey. 2005. Sampling from natural populations with RNAi reveals high outcrossing and population structure in *Caenorhabditis elegans*. Curr. Biol. 15:1598–1602.

Sokal, R. R., and F. J. Rohlf. 1995. Biometry, 3rd ed. Freeman, New York.

Stewart, A. D., and P. C. Phillips. 2002. Selection and maintenance of androdioecy in *Caenorhabditis elegans*. Genetics 160: 975–982.

Stiernagle, T. 1999. Maintenance of *C. elegans*. Pp. 51–67 *in* I. Hope. ed. *C. elegans*: a practical approach. Oxford Univ. Press, Oxford, U.K.

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