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Sampling from Natural Populations with RNAi Reveals High Outcrossing and Population Structure in *Caenorhabditis elegans*

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Summary

Despite a nearly worldwide distribution in nature, *Caenorhabditis elegans* exhibits low levels of genetic polymorphism [1–6], possibly as an indirect consequence of low levels of outcrossing. In the laboratory, *Caenorhabditis elegans* males are produced at low rates and are steadily eliminated from cultures [7, 8], so that reproduction happens largely through self-fertilization in hermaphrodites. *C. elegans* is increasingly the focus of evolutionary research [1–4, 7, 9–12]; however, natural outcrossing rates are difficult to measure because mating tests with laboratory strains are usually required to identify *C. elegans*. We sampled natural populations of *C. elegans* with an RNA interference (RNAi) assay. Heterozygosities and polymorphism patterns revealed surprisingly high levels of population structure and outcrossing (approximately 22% of individuals are estimated to be the result of outcrossing and not self-fertilization). The finding of strong local population structure, together with low levels of diversity on local and global scales, suggests a metapopulation model of frequent extinction and recolonization of local populations. The occurrence of substantial outcrossing suggests that the extinction of local populations is probably not driven by the accumulation of harmful mutations.

Results and Discussion

A total of 69 individual lines were established from ten populations in the Los Angeles area. Lines were genotyped for 15 microsatellite loci, all of which were variable among or within one or more populations. All but one population (LA in Table 1) showed multiple alleles at one or more loci. Estimates of the effective size of local populations were based on estimates of the population mutation rate ($\theta = 4N_e u$, where N_e is the effective population size and u is the mutation rate) and varied greatly in accordance with wide variation in numbers and frequencies of alleles. These estimates assume zero gene flow among populations, an assumption that may be approximately correct given the marked population structure observed (see below). The estimates also assume mutation-drift equilibrium, and they will be underestimates if populations have recently been formed. In the most variable populations, estimates of local effective population size are comparable to values estimated from a diverse collection of wild-type strains from the Caenorhabditis Genetics Center (CGC) [3]. Population structure on a local scale was very strong (R_{ST} = 0.826), suggesting little gene flow among local populations. There was no correlation between the genetic distances and geographic distances among samples (Mantel test; p = 0.45), further suggesting infrequent gene flow even among neighboring populations. These observations are very similar to findings within and among local French populations where F_{ST} values based on amplified fragment length polymorphism (AFLP) markers have similar high values [6].

Fourteen of the founder worms (~20%) were determined to have had nonzero heterozygosities. Ten of these were heterozygous at one locus (with the specific heterozygous locus varying among these ten lines), and the remaining were heterozygous at two, three, or four loci, indicating that a considerable degree of outcrossing has occurred in the recent history of these lines. Outcrossing rates were estimated from the difference between observed numbers of heterozygotes and the expected proportion of heterozygotes under random mating (Table 1). In some populations, the only variation was an observation of heterozygosity at one locus in one individual. Setting aside these cases (populations 2 and 7 in Table 1), which could possibly be due to recent mutation, the population-specific outcrossing rate, based upon the moment estimator $\hat{T}$, ranged from 0 to 1 (Table 1). The average estimated outcrossing rate (i.e., the fraction of individuals not produced by self-fertilization) among these populations was 0.22.

It is possible that some observations of heterozygosity are due to recent mutations and not to outcrossing. Under complete selfing, the proportion of the population that is heterozygous at a given locus is, to a first approximation, $H' = u - H/2$, where H' is the proportion in the next generation as a function of that in the current generation, H, and u is the mutation rate to new alleles per generation. At equilibrium, this suggests that the proportion of heterozygotes should be close to the mutation rate ($H_{eq} = 2u/3$). Given an estimated dinucleotide mutation rate in *C. elegans* of 1.78×10^{-4} per generation [13], only a small minority, if any, of the observed cases of heterozygosity could be due to mutation alone.

Under the collecting protocol used, soil samples spent up to 11 days contained in plastic bags before worms were isolated by a liquid-extraction procedure. A further 8 to 10 days elapsed during the RNAi assay, between the removal of worms from the soil and the setting up of individual lines. Therefore, at the time of DNA extraction, the samples were about four or five generations removed from the field. It is possible that outcrossing rates during this period were lower or higher than those that occur in the field. To assess whether the outcrossing rate differed before and after the collection of soil samples, we applied a likelihood method that allows for estimation of the outcrossing rate in each of several generations prior to the time of sampling [14]. This method makes use of the distribu-

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Table 1. Sampled Populations and Polymorphism Summary

Population	Number of Lines	Number of Het. Lines	Number of Pol. Loci	N_e	H_{obs}	H_{exp}	$\hat{T}$
GP1	10	0	1	53	0.000	0.480	0.000
GP2	5	1	1	N/A	1.000	0.500	1.0
HG1	9	8	10	10200	0.176	0.393	0.256
HG2	1	1	2	N/A	1.000	0.500	1.0
HG3	7	0	3	160	0.000	0.490	0.000
HG4	9	2	5	5027	0.037	0.329	0.058
HG5	4	1	1	94	0.250	0.219	1.0
CT	10	1	4	178	0.036	0.300	0.250
DG	5	0	2	148	0.000	0.480	0.000
LA	9	0	0	0	0.000	0.000	

Sample sizes, number of lines heterozygous for at least one locus, effective population size estimates (N_e), observed (H_{obs}) and expected (H_{exp}) heterozygosities, and estimated outcrossing rates. Expected and observed heterozygosities for a population are averages across loci that were polymorphic within that population. $\hat{T}$ is a moment-based estimator of the outcrossing rate as explained in the text. Samples were collected from five localities: Griffith Park (GP), Huntington Botanical Gardens (HG), California Institute of Technology campus (CT), Descanso Gardens (DG), and Arboretum of Los Angeles County (LA). From GP and HG, more than one soil sample yielded *C. elegans*.

tion of the number of heterozygous loci among individuals. If some outcrossing had occurred, but only in the previous generation, then those individuals that resulted from outcrossing would be heterozygous at many loci. However, if outcrossing occurred prior to that, then the number of heterozygous loci in the sampled descendants would be fewer. With a model of two outcrossing rates—T_0 for the generation immediately before sampling and T_p for all generations prior to that—population samples HG1, HG4, and CT (i.e., those with sufficient numbers of individuals) yielded estimates of $T_0 = 0$ and nonzero estimates for generations prior to that ($T_p = 0.82$ for HG1; $T_p = 0.17$ for HG4; and $T_p = 0.11$ for CT). When a model was fitted with outcrossing set to zero in the generation prior to sampling ($T_0 = 0$) and with two rates thereafter ($T_{2,4}$ for generations two through four prior to sampling and T_p for all generations prior to that), these same populations all had nonzero estimates for both parameters, and in each case, the estimates of T_p were higher than for $T_{2,4}$. Although the joint likelihood surfaces for this model were fairly flat, reflecting the limited information available, these analyses suggest that the outcrossing rates were actually lower during the isolation procedure in relation to the rate that occurred in the natural habitat.

A second, more direct observation also supports the conclusion that outcrossing rates were lower during the isolation procedure. Half of all progeny resulting from outcrossing are males; this means that even a single outcrossed hermaphrodite would be expected to produce at least several dozen males, which would then provide the opportunity for further outcrossing in the next generation. Indeed, when males arise in lab populations, they are seen in large numbers for at least three to four generations before they begin to decline in numbers and are eventually eliminated from the population. Therefore, if there had been an elevated outcrossing rate in the samples after collection but prior to isolation, then a large number of males would be expected among the isolated worms that were assayed by the RNAi protocol. However, no males were observed at that stage, consistent with the outcrossing having occurred prior to the isolation procedure.

The finding of considerable outcrossing in natural populations indicates either that males arise at higher rates in the wild than they do in the laboratory, or that wild males have a survival or reproduction advantage that is not present in typical laboratory settings. A finding of moderate outcrossing is surprising, given previous reports of linkage disequilibrium (LD) in samples of wild strains [4, 5]. However, LD is also an expected by-product of strong population structure on local and regional scales. In the present study, populations were sampled on a very local scale (i.e., each population was sampled by collecting a garden spade of dirt, which was placed in a small plastic bag), and some populations in the same collecting location were separated by as little distance as 10 m (Figure 1). If populations are strongly structured on very local scales, then studies that pool collections over larger distances may perceive high LD and low effective outcrossing rate. The presence of strong local structure could also explain why polymorphism levels are highly variable when observed on local scales (Table 1) but are more consistent in samples pooled from multiple locations [3–5]. In this

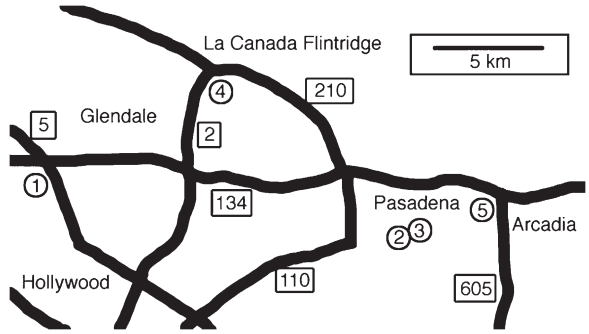

Figure 1. Collection Sites in the Los Angeles Area of California

Circled numbers indicate the following sample locations: (1) Griffith Park, Los Angeles; (2) Huntington Botanical Gardens, San Marino; (3) California Institute of Technology, Pasadena; (4) Descanso Gardens, La Cañada; and (5) Arboretum of Los Angeles County, Arcadia. Major highways are indicated by route numbers in boxes. Within locations, sampled populations were separated by distances between 10 m and 400 m.

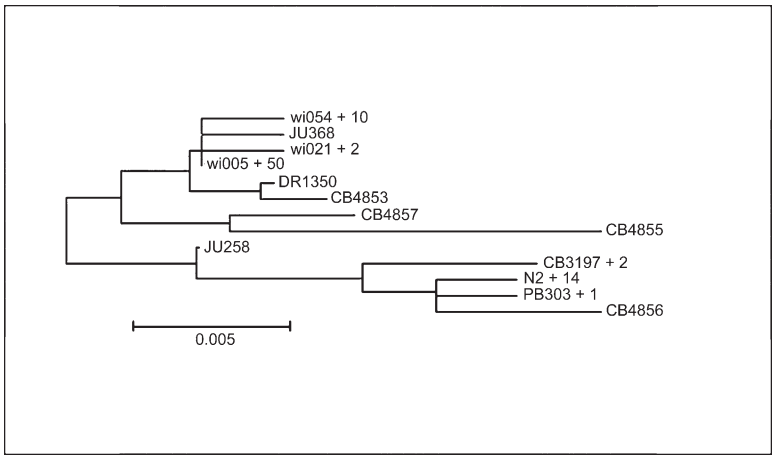

Figure 2. Minimum Evolution Tree for Wild Lines and CGC Strains

The gene tree was estimated with the Minimum Evolution method [42] for 380 bp of the ATPase subunit 6 mitochondrial gene, assuming the Kimura two-parameter model of nucleotide substitution. Where several lines or strains have the same haplotype, it is only shown once, and the number of additional lines or strains sharing that haplotype is indicated. For the wild lines, the label HG3.3, for example, refers to the third line from the population HG3. The tree is rooted by midpoint, rather than by a highly divergent outgroup sequence.

light, it is noteworthy that studies in Europe have reported significant LD within local collections [4, 6], suggesting that some populations have not had the moderate outcrossing levels reported here.

The evidence for considerable outcrossing undermines some explanations for the low level of genetic polymorphism that is found in natural populations of *C. elegans*. Under high selfing, there is a direct reduction in effective population size [15], as well as possibly an indirect effect via a reduced effective recombination rate, which leads in turn to a reduced efficiency of selection for beneficial alleles and for the removal of harmful mutations [16–18]. Local populations with high rates of self-fertilization might be prone to extinction and turnover as a result of the reduced effectiveness of natural selection, particularly if local populations are small [19–22]. These indirect effects generally require very high selfing rates, however, and so appear unlikely given the evidence presented here.

To examine the phylogenetic relationship of the sampled populations to other strains of *C. elegans*, we sequenced a segment of mitochondrial (mt) DNA in the wild lines as well as in a sample of CGC strains. The wild lines revealed only two polymorphic sites within 380 bp (with the nucleotide diversity, or mean number of differences between pairs of sequences, of 0.001 per bp), whereas there were 20 polymorphic sites among the CGC strains. The 69 lines are represented by three mitochondrial haplotypes. A phylogenetic tree of the mtDNA sequences (Figure 2) reveals that all the wild lines are very closely related to one another, probably descending from just a single lineage, with much less diversity than found among the CGC lines [2].

If the observed pattern of strong local population structure were to persist for a long period of time, divergence among populations would be expected to lead to high levels of variation on a broad geographic scale [23]. Because this is not observed, the observations of strong local structure and low variation suggest a metapopulation model in which local populations are subject to high rates of turnover [19, 22, 24–26]. Moreover, the local populations show very little variation at a mitochondrial locus known to be quite variable on a global scale. This contrast also supports a model in which local populations are going extinct at a rapid rate, such that variation cannot be maintained, even in the rapidly evolving mitochondrial genome [27]. Given the evidence of outcrossing, such turnover would likely be due to environmental effects rather than genetic effects of limited outcrossing.

Other recent studies also report evidence for outcrossing, although at lower levels. A study of variation in laboratory strains and wild-caught strains from Germany concluded that outcrossing had probably occurred on the basis of phylogenetic incongruency of different types of loci [4]. A more direct measure based on observed heterozygosity at two microsatellite loci in populations from France found a small number of heterozygotes, consistent with outcrossing rates of about 0.02 [6], approximately an order of magnitude lower than the estimates from the California collections. The difference may be due to natural variation in outcrossing rates. The possibility that outcrossing in the California samples increased during the isolation protocol seems unlikely given the apparent absence of males, the distribution of heterozygosities, and the estimates of outcrossing at multiple time points [14], but it cannot be ruled out. Given that local populations in California and in Europe [4, 6] typically reveal multiple genotypes, even low-to moderate outcrossing can play a strong role in generating new genotypes and allowing natural selection for favorable mutations, and against harmful mutations, to proceed. These findings suggest that other factors, in addition to inbreeding, need to be invoked to account for the low variation levels that are seen in *C. elegans* on continental and global scales. The possibility that mutation rates are low does not help explain the observations, given the high mutation-rate estimates for several types of markers [10, 13, 27]. It remains possible that a metapopulation structure, with high levels of population turnover, could explain the low levels of variation. However, environmental factors are more likely to be the cause of such turnover than genetic factors related to inbreeding.

Experimental Procedures

Collections

Soil samples were collected from parks and gardens around the Los Angeles area in southern California (Figure 1). Nematodes were

isolated from the samples, and *C. elegans* were identified by an assay based on RNA interference (RNAi) and described below. From each soil sample that contained *C. elegans*, several lines were established, each starting with a single unmated L4 larval worm. DNA was extracted from a pooled sample of over 200 offspring of each founder worm, ensuring sufficient DNA for genotyping and sequencing and that the genotype of the founder worm could be determined. Because genotyping of each founder was done with a large pooled sample of immediate offspring produced by self-fertilization, homozygosity of the founder is revealed by a single allele, and heterozygosity by two alleles, in the pooled offspring DNA, just as if the founder had been genotyped directly. A total of 69 lines were established from ten soil samples collected at five locations in California (see Figure 1 and Table 1 for details). Within locations, soil-sample sites were separated by distances ranging from about 10 m to about 400 m. The lines established from a single soil sample are referred to collectively as a population.

Care was taken to avoid crosscontamination between soil samples, and all containers used in the extraction procedure were autoclaved after each sample was processed. When isolated, each founder was washed and placed on a plate which was sealed with Parafilm to prevent desiccation as well as preclude contamination between plates.

RNAi Assay for Species Identification

In collections from nature, *C. elegans* co-occurs with other species that are not readily distinguishable from each other on the basis of morphology, and test crosses with laboratory strains of *C. elegans* are usually required to determine species status. To have a more rapid protocol that permits identification of heterozygosity in individual *C. elegans* candidates, we developed a new species-identification technique based on the phenomenon of RNA-mediated interference (RNAi). RNAi is a form of posttranscriptional gene silencing (PTGS) wherein a gene is silenced when double stranded (ds) RNA that is cognate to its mRNA is introduced into the cell. The resultant phenotype is similar to the null mutant phenotype for the gene involved. RNAi is sequence specific in its effects [28, 29], so that dsRNA corresponding to a region of a *C. elegans* gene is unlikely to cause PTGS in another species. If a culture containing a diversity of nematode species, including *C. elegans*, is treated by this method, then the phenotype should be observed in *C. elegans* to the exclusion of all other species. The *C. elegans* gene *unc-22* encodes a muscle protein, twitchin, implicated in the regulation of myosin activity [30]. The null phenotype is a movement defect characterized by strong twitching. Because this is a nonlethal, nonsterile phenotype and is observable relatively quickly, this gene was chosen for the RNAi assay. *Escherichia coli* containing the pLT61 plasmid [31] expressing dsRNA corresponding to a portion of the *unc-22* gene were fed to worms following the protocol of Kamath et al. [32].

The species specificity of this RNAi assay was tested with *C. briggsae* and *C. remanei*, as well as some other species of nematodes previously isolated from soil and confirmed not to be *C. elegans* (by mating tests with lab strains). It was first verified that this RNAi assay was ineffective in *C. briggsae* and *C. remanei*, which are the closest know relatives to *C. elegans* [33]. Next, several individuals of *C. elegans*, *C. briggsae*, *C. remanei*, and other nematodes of unknown species from wild isolates were mixed on a plate, and random subsamples were taken from this pooled sample of worms and subjected to the RNAi method. Although these species are quite divergent at the DNA level, they are morphologically very similar and are difficult to distinguish under a dissecting microscope. At the end of the incubation period, all worms that displayed the phenotype and, hence, are thought to be *C. elegans* were removed to individual plates and test crossed with laboratory strains. All worms displaying the phenotype proved to be *C. elegans*. Ten *C. elegans* strains from diverse worldwide locations were tested to examine intraspecific variability in the effectiveness of this RNAi assay. For each of the ten strains, over 90% of the worms displayed the phenotype. A similar assay of species-wide RNAi sensitivity found that all strains except one were susceptible to RNAi by feeding [34]. The resistant exception is the strain CB4856 from Hawaii, which is also a distant outlier from all other strains in terms of DNA sequence divergence [2].

Nematodes were extracted with a modified Baermann funnel technique. A plastic container with the bottom cut out was placed inside a larger plastic container with a lid. The bottom of the smaller container was lined with plastic mesh, a double layer of cheesecloth, and a layer of facial tissue. The soil sample was spread over this, and the larger (outer) container was filled with tap water until the soil sample was covered by a thin film of water. The container was covered loosely and allowed to stand for 3 days. At the end of this period, the water was collected into 50 ml plastic tubes and allowed to stand for a few hours. Most of the water was then decanted, and nematodes were recovered from the remaining water by transferring it onto an agar plate containing a lawn of *E. coli* strain OP50 and allowing the water to evaporate, leaving nematodes and other fauna that were present in the sample. Worms were then transferred onto plates containing bacteria that express the *unc-22* dsRNA. After the incubation period, several worms displaying the characteristic twitching phenotype were transferred onto individual plates. Worms isolated by this technique were confirmed to be *C. elegans* through mating tests with lab strains, verifying the accuracy of the assay.

Markers

On the basis of a list of known microsatellites in the *C. elegans* genome [35], dinucleotide loci were chosen and amplified in all the wild lines. PCR products were separated on a 7% polyacrylamide gel with a Licor 4200 automated DNA sequencer and genotyped with SAGA-GT software (Licor, Inc.). A highly variable region of the ATPase subunit 6 mitochondrial gene [2] was sequenced in the wild lines as well as 22 CGC strains. Sequences from a further 15 CGC strains were obtained from GenBank (accession numbers AY171148–AY171162). Individuals were genotyped multiple times to avoid errors.

Data Analyses

R_{ST} as well as F-statistics, including F_{IS} [36], were calculated from the microsatellite data with GENEPOP [37] and Fstat [38]. The outcrossing rate, T, was estimated with $F_{IS} = S / (2 - S)$ [37, 39, 40], where S is the selfing rate and $T = 1 - S$. $\hat{T}$, a simple moment estimator, is the average across loci found by solving the expression $T = 1 - 2F_{IS} / (1 - F_{IS})$ for each of the loci that showed multiple alleles in that population. For each locus $F_{IS} = 1 - (H_{obs} / H_{exp})$, where H_{obs} and H_{exp} are, respectively, the observed proportion of heterozygous individuals at that locus and the expected proportion of heterozygous individuals under random mating. By chance, H_{obs} may be higher than H_{exp}, in which case the corresponding negative F_{IS} value was set to zero.

The population mutation rate, $\theta = 4N_e u$, was estimated for each locus under the assumption of a stepwise mutation model as the mean squared pairwise difference in allele sizes expressed as the number of repeat units [41]. Differences between alleles from the same individual were not included, in order to avoid the effects of nonindependence due to selfing. Differences between all pairs of alleles from separate individuals were averaged to estimate θ for each locus. The effective population size N_e was estimated with direct estimates of the mutation rate, u, for microsatellite loci [13]. Mutation rates for microsatellites vary depending on the number of repeat units. The rate used here is that estimated for a locus with 26 repeat units, similar to the average (=24) for the loci in this study.

Supplemental Data

Supplemental Data include two supplemental tables and are available with this article online at http://www.current-biology.com/cgi/content/full/15/17/1598/DC1/.

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