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Genetic Mapping of Quantitative Trait Loci Governing Longevity of *Caenorhabditis elegans* in Recombinant-Inbred Progeny of a Bergerac-BO × RC301 Interstrain Cross

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ABSTRACT

Recombinant-inbred populations, generated from a cross between *Caenorhabditis elegans* strains Bergerac-BO and RC301, were used to identify quantitative trait loci (QTL) affecting nematode longevity. Genotypes of young controls and longevity-selected worms (the last-surviving 1% from a synchronously aged population) were assessed at dimorphic transposon-specific markers by multiplex polymerase chain reaction. The power of genetic mapping was enhanced, in a novel experimental design, through map expansion by accrual of recombinations over several generations, internally controlled longevity selection from a genetically heterogeneous, homozygous population, and selective genotyping of extremely long-lived worms. Analysis of individual markers indicated seven life-span QTL, situated near markers on chromosomes I (*tcbn2*), III (*stP127*), IV (*stP13*), V (*stP6*, *stP23*, and *stP128*), and X (*stP41*). These loci were corroborated, and mapped with increased precision, by nonparametric interval mapping—which supported all loci implicated by single-marker analysis. In addition, a life-span QTL on chromosome II (*stP100-stP196*), was significant only by interval mapping. Congenic lines were constructed for the longevity QTL on chromosomes III and X, by backcrossing the Bergerac-BO QTL allele into an RC301 background with selection for flanking markers. Survival data for these lines demonstrated consistent and significant effects of each QTL on life span.

ESSENTIALLY all metazoa undergo a time-dependent loss of fitness, manifest at all levels of biological organization (Shmookler Reis 1976; Strehler 1977; Flanagan 1980; Shmookler Reis 1989; Finch 1990; Shmookler Reis and Ebert 1996). Both mean and maximal longevity are ultimately limited by this decline and are in large measure governed by multiple genetic factors (Johnson and Wood 1982; Yunis *et al.* 1984; Ebert *et al.* 1993, 1996). The heritability of longevity has been estimated in multiple species, generally falling in the range of 20–50% (Johnson and Wood 1982; Yunis *et al.* 1984; Rose and Service 1985; Hutchinson and Rose 1991; Ebert *et al.* 1993, 1996). In *Caenorhabditis elegans*, estimates of broad-sense heritability have clustered near the upper end of that range, at 39–52% (Johnson and Wood 1982; Ebert *et al.* 1993, 1996). A confluence of genetics, molecular biology, and the development of statistical tools for mapping quantitative trait loci (QTL) has only recently made possible the analysis of complex polygenic traits such as life span (Ebert *et al.* 1993, 1996; Nuzhdin *et al.* 1997; Leips and Mackay 2000; Vieira *et al.* 2000).

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Identification of single-gene mutations that influence *C. elegans* longevity [*daf-2* (Kimura *et al.* 1997), *age-1/daf-23* (Morris *et al.* 1996), *daf-16* (Ogg *et al.* 1997; Lin *et al.* 1997), and *clk-1* (Ewbank *et al.* 1997)] has led to the definition of two genetic pathways that strongly affect adult survival (Lakowski and Hekimi 1996; Tissenbaum and Ruvkun 1998). It remains unknown, however, to what extent natural polymorphism, in these and other genetic pathways, contributes to variation in longevity among strains or to evolutionary modulation of life span. *C. elegans* is an excellent model organism to study the genetics of aging, due to the absence of heterosis (Johnson and Hutchinson 1993) in conjunction with ease of handling, short generation time, and relatively brief life span. The genome of *C. elegans* contains at least six families of transposable elements (Moerman and Waterston 1984; Dreyfus and Emmons 1991), commonly termed *Tc* elements. The Tc1 family comprises 1.6-kbp elements bounded by conserved 54-bp inverted repeats (Moerman and Waterston 1984), inserted at 27–32 sites in the genomes of most *C. elegans* strains, but at >500 copies in strain Bergerac-BO (Egilmez *et al.* 1995). Tc1 elements have proven to be useful dimorphic markers in a number of genetic mapping analyses (Williams *et al.* 1992; Ebert *et al.* 1993, 1996; Shook *et al.* 1996; van Swinderen *et al.* 1997).

Interstrain crosses between Bergerac-BO (high Tc1 copy number) and Bristol-N2 (low copy number) have been employed to identify multiple chromosomal regions influencing the life span of *C. elegans* (Ebert *et al.* 1993, 1996; Shook *et al.* 1996; see also Johnson and Wood 1982). Only those genes that are dimorphic between the parents of a given cross will be susceptible to detection by genetic mapping. We therefore sought additional genes that determine longevity, by constructing a cross between Bergerac-BO and the RC301 strain. RC301 is quite far removed in strain evolution from both Bergerac-BO and Bristol-N2 (Egilmez *et al.* 1995). We thus identified seven highly significant QTL strongly affecting life span and one marginally significant locus, at least five of which were not observed in the previous cross.

MATERIALS AND METHODS

Strains: *C. elegans* strains Bergerac-BO and RC301 were obtained from the Caenorhabditis Genetics Center (St. Paul, Minnesota). Worms were grown at 20° on 100-mm plates of solidified agar containing nematode growth medium, seeded with a lawn of *Escherichia coli* strain OP50 (Brenner 1974).

Cross construction: Bergerac-BO and RC301 worms were crossed, which was initiated by placing one BO hermaphrodite and three RC301 males on each of 10 plates. The F_1 hybrids were allowed to mate at random and ~1300 L4 hermaphrodites (worms in their fourth larval stage of development) were picked from the F_2 progeny and carried to the F_7 generation by self-fertilization, while gradually expanding the population size. During these seven generations, eggs were recovered from hermaphrodites by alkaline hypochlorite lysis [5 min in 0.5 n NaOH and 1.05% hypochlorite (Emmons *et al.* 1979)], yielding ~10 eggs per worm. The eggs were rinsed in S-buffer (Brenner 1974) containing 0.1 m NaCl and 0.05 m potassium phosphate, pH 6.0.

Mass aging: Gravid F_6 worms were lysed as described above. F_7 eggs were hatched overnight in S-buffer, yielding ~10^6 L1 larvae (the first of four larval stages in *C. elegans* development), which were shaken at 20° in 500 ml liquid survival medium. Aging cohorts were grown *en masse* in the presence of 200 μm each of 5-fluoro 2′ deoxyuracil (FUdR; Sigma, St. Louis) and uridine monophosphate (UMP, 2′, 3′ mixed isomers; Sigma) to inhibit larval growth and development; all other culture conditions were as described previously (Ebert *et al.* 1993). For unselected controls, 175 worms were picked on day 5 and placed individually into 0.5-ml tubes containing single-worm lysis mix and proteinase K (Williams *et al.* 1992). On day 34, the last 1% of surviving worms were separated from carcasses by centrifugation on a step gradient of 60% sucrose overlaid with 40% Percoll (Sigma), as described by Fabian and Johnson (1994). The recovery of live worms by this method was 80–90%. Live, age-selected worms (a random sample of 175) were picked and lysed as described above.

Analysis of genotypes: Single worms were placed in lysis mix and stored at −70°; they were later thawed and heated to 60° for 60 min, followed by 95° for 15 min (Williams *et al.* 1992). Young control worms and long-lived worms (the last-surviving 1%) were analyzed for genotypes by multiplex polymerase chain reaction using Tc1-specific and locus-specific primers (see Williams *et al.* 1992; Ebert *et al.* 1993). Strains Bergerac-BO and RC301 differ by >10-fold in copy number of the Tc1 transposon, allowing the parental strain of origin to be readily determined at Tc1 insertion sites across the genomes of recombinant-inbred F_7 worms. Individual worms were analyzed at 30 Tc1 polymorphic loci, which were divided into 6 multiplex PCR sets; in each reaction, five Tc1-flanking primers can pair with a common opposing primer specific to one end of the Tc1 sequence, to produce five locus-specific product bands. PCR thus generates a band of characteristic interprimer length, for each marker locus in a multiplex assay, if and only if the parental origin for the corresponding marker locus is BO.

Each multiplex reaction generated five informative DNA bands from Bergerac-BO worms (amplifying only one flank of each Tc1 insertion) and none from RC301. Five of the six multiplex sets also included a positive control for PCR, a primer specific to a Tc1 insertion site shared by both parental strains. This control was omitted from the sixth set due to comigration of the control band with a strain-specific band, but the reaction failure rate was sufficiently low (<3%) that this did not noticeably impair mapping. In any case, negative reactions were repeated at least once. After omission of eight incomplete genotypes, the final data set for identifying quantitative trait loci affecting life span consisted of 171 young and 171 age-selected individuals, each assessed at 30 site-specific Tc1 marker loci.

Multiplex PCR sets included five or six locus-specific primers and a single common Tc1-specific primer (the latter end-labeled by polynucleotide kinase with [γ-^{32}P]ATP). Reaction buffer contained 10 mm Tris pH 8.3, 50 mm KCl, 1.5 mm $MgCl_2$, 200 μm of each dNTP, and 0.5 μm of each primer. Amplification in a hot-air thermal cycler (Idaho Technology, Idaho Falls, ID) entailed 30 PCR cycles—each comprising 10 sec at 94°, 30 sec at 58°, and 30 sec at 72°—preceded by a 45-sec initial denaturation at 94° and followed by ~10 min final extension at 72°. Template for each PCR consisted of 3–6% of the DNA lysate from a single worm. Marker sets were selected so as to avoid primer-primer complementarity and to comprise bands distinguishable by size when electrophoresed on 5% polyacrylamide gels (Hoeffer, San Francisco) at 8 V/cm for 3 hr at ~22°.

Backcrossing QTL-spanning regions from BO into RC301: Chromosomal regions containing QTL on chromosomes III and X were introduced into the RC301 background by backcrossing for 20 generations. Initially, BO hermaphrodites were crossed to RC301 males, and F_1 hermaphrodites were then crossed to RC301 males to form generation backcross-1 (BC_1). Individual BC_1 progeny were picked at the last larval stage (L4) and isolated on 35-mm plates. After egg laying, single adults were lysed and their genotypes were analyzed using Tc1-specific and site-specific primers as described previously (Williams *et al.* 1992; Ebert *et al.* 1993). For the chromosome III QTL, *lsq3*, a BO-derived region spanning *stP127* and *stP17* was introduced into the RC301 background. Locus-specific primers thus corresponded to sequences adjoining Tc1 insertion sites *stP127* and *stP17*. Progeny of BC_n worms, retaining the QTL region from the Bergerac-BO parent, were crossed again to RC301 to yield BC_{n+1} progeny, etc. Backcrossed lines for the QTL on chromosome X, *lsqX*, were constructed and selected for retention of markers *stP40* and *stP41*, as described for *lsq3*.

Statistical genetics: Single-marker analysis and nonparametric interval mapping (Kruglyak and Lander 1995) were used to identify and position life-span QTL. The proportion of *Tc1+* alleles at each marker was determined separately for young unselected and age-selected subgroups, which were analyzed by PCR genotyping of individual worms. The PCR products were generated and analyzed in sets of 40–50 worms, for a total of 171 young and 171 age-selected worms. These sets did not differ significantly from one another with respect to

Tc1+ allele frequencies calculated for each marker. Allelotypes were reassessed for 80 assays judged to be ambiguous; even for this group, agreement between the assignments in replicate assays was >96%. For single-marker analysis, differences in allele frequency between longevity-selected and young-unselected data sets were assessed for significance by a χ^2-test, within Microsoft Excel. A genetic map was generated from the young control genotypes at all 30 markers, using MapMaker-EXP (Lander *et al.* 1987), and utilized for nonparametric interval mapping (Kruglyak and Lander 1995) in the MapMaker QTL program (Lander *et al.* 1987).

Because multilocus analysis involves multiple comparisons, false-positive thresholds (α-values) were determined for full genome scans (Kruglyak and Lander 1995). In single-marker analysis, an overall α-value of 0.05 (*i.e.*, a 5% chance of obtaining at least as strong an association of marker to trait, purely by chance, anywhere in the genome) corresponds to a false-positive threshold of ~0.002 at any marker, based on strict Bonferroni correction. This conservative criterion allows for 24 nonredundant linkage clusters in the marker set, treating closely linked markers (within a span corresponding to a recombinant fraction of $\leq$0.2) as one cluster. Single-marker thresholds were also estimated empirically (Churchill and Doerge 1994), by determining the χ^2-statistics for a comparison of age-selected to young control allele frequencies at each marker, over 1000 permutations of phenotype with respect to genotype. For nonparametric interval mapping, *Z*-score significance thresholds are based on simulations (Kruglyak and Lander 1995), while thresholds for Lander-Botstein interval mapping and composite interval mapping (Table 2) are based on 1000 permutations each, as described above.

RESULTS

To map QTL affecting life span, which are polymorphic between RC301 and Bergerac-BO, a cohort of 10^6 F_7 worms was synchronously aged, and 171 young unselected and 171 age-selected worms were analyzed for 30 markers detecting presence or absence of Tc1 insertions at specific sites.

Estimating total recombination accumulated during the crosses: The total amount of recombination accumulated during the generations leading to the F_7 recombinant-inbred population was calculated for the young unselected worms. On average there was one crossover per 18.5 map units; from the total length of the *C. elegans* genetic map, this would indicate 2.7 recombinations per chromosome. This extrapolation is undoubtedly an underestimate, since the markers used are concentrated in the gene-rich chromosome centers, which have lower recombination than the more distal regions (*C. elegans* Sequencing Consortium 1998). The apparent genetic map was expanded roughly 4-fold (2.1- to 7.6-fold, for 24 intervals between adjoining markers) relative to a standard genetic map calculated from recombinants per meiosis, reflecting the accumulation of recombinations during multiple generations of crossing and inbreeding. Such map expansion—twice that seen in F_2 crosses—has been reported previously for recombinant inbred (RI) lines and populations (Dixon 1993; Ebert *et al.* 1993, 1996).

Reproductive fitness genes: In the absence of selection, the initial frequency of the Bergerac-BO (Tc1+) allele at each autosomal site, BO/[BO + RC301], would approximate 0.5 for the recombinant-inbred population. A higher allele ratio (0.67) is expected for markers on the X chromosome, because male infertility of the Bergerac-BO strain prevented us from performing reciprocal crosses. Thus, matings were always between BO hermaphrodites and RC301 males, skewing the contribution of X chromosomes to 2:1 (BO:RC301). Any significant deviation from 0.5 on autosomes, or from 0.67 on the X chromosome, in the initial allele frequencies observed prior to longevity selection (Table 1, "Young" column), suggests the presence of a polymorphic gene near that marker, either affecting Darwinian fitness or distorting segregation (*i.e.*, exhibiting "meiotic drive"). Over successive generations, even modest selection of either sort would cause the allele with greater mean fitness to increase in frequency within a population. We attempted to minimize Darwinian selection by collecting unlaid eggs, after alkaline-hypochlorite lysis of hermaphrodites, at every generation during the cross.

For 13 of the marker loci, the initial allele frequencies did not deviate significantly from the expected frequencies. However, the RC301 allele was significantly enriched for markers in linkage groups (chromosomes) I (*stP124*, *hP4*, *tcbn2*), III (*stP19*, *stP127*), V (*stP3*, *stP192*, *stP23*, and *bP1*), and X (all six markers), whereas the BO allele was favored in LG IV (*stP44* and *stP35*). Genes affecting reproductive fitness or segregation distortion can be tentatively localized to those markers with distinct local maxima or minima in the BO/RC301 allele frequency (see underlined loci in Table 1). Such fitness-conferring loci were thus mapped near marker *stP33* at −3 cM on the X chromosome and near *stP44* at +7 cM on chromosome IV.

Genes affecting longevity—single-marker analysis: Although the initial *Tc1*+ allele frequency at any marker can deviate from its expected value due to *intergeneration* selection, allele frequencies of young-control worms serve as the reference point for genotype-based selection on the *aging* population itself. Thus, genetic influences on longevity are indicated by shifts in allele frequency between the control and long-lived groups, at any given marker—with the greatest shifts indicating markers closest to loci affecting longevity. The significance of shifts associated with life-span selection (age-selected/young ratios ≠ 1; see "*A*/*Y*" column in Table 1) was determined by χ^2-tests. With stringent adjustment for multiple comparisons (see materials and methods) the probability of false positives should be <0.05 for the full genome provided that the single-marker threshold is set at $P < 0.002$. False-positive thresholds can also be determined empirically, by reassigning trait values randomly to genotypes over many permutations, as indicated in the P_{empir} column of Table 1. By either criterion, the RC301 allele was significantly enriched in the longest-lived subset of worms on chromosomes I

TABLE 1

Allele frequencies (*Tc1*+/total) for young and age-selected worms, and statistics derived from comparisons of these frequencies

		Allele frequencies				χ^2-significance	
Chromosome	Marker	Young (%)	Aged (%)	Δ ($Y - A$)	Ratio (A/Y)	P_Σ	P_{empir}
I	stP124	34	23	11	0.68	—	—
I	hP4	36	22	14	0.61	—	—
I	*tcbn2*	*38*	*21*	*17*	*0.55*	*0.01*	*0.025*
II	stP100	49	33	16	0.67	—	—
II	stP196	50	35	15	0.70	—	—
II	stP101	49	33	16	0.67	—	—
II	stP50	45	37	8	0.82	—	—
II	stP198	35	23	12	0.66	—	—
II	maP1	45	36	9	0.8	—	—
III	stP19	44	33	11	0.75	—	—
III	*stP127*	*44*	*28*	*16*	*0.64*	*0.05*	*0.06*
III	*stP17*	*54*	*38*	*16*	*0.70*	*0.05*	*0.06*
IV	*stP13*	*57*	*18*	*39*	*0.32*	$<10^{-11}$	*<0.005*
IV	*stP44*	*63*	*33*	*30*	*0.53*	$<10^{-6}$	*<0.005*
IV	*stP35*	*63*	*35*	*28*	*0.56*	10^{-5}	*<0.005*
V	stP3	29	23	6	0.80	—	—
V	stP192	23	21	2	0.91	—	—
V	*stP23*	*32*	*20*	*12*	*0.63*	*0.002*	*<0.005*
V	bP1	27	24	3	0.89	—	—
V	*stP6*	*45*	*21*	*24*	*0.47*	5×10^{-7}	*<0.005*
V	stP18	40	24	16	0.60	—	—
V	*stP108*	*35*	*23*	*12*	*0.66*	*0.007*	*0.025*
V	*stP105*	*43*	*22*	*21*	*0.51*	*0.001*	*<0.005*
V	*stP128*	*51*	*27*	*24*	*0.53*	10^{-4}	*<0.005*
X	*stP41*	*47*	*23*	*24*	*0.49*	6×10^{-5}	*<0.005*
X	*stP40*	*41*	*23*	*18*	*0.56*	5×10^{4}	*<0.005*
X	*stP33*	*27*	*13*	*14*	*0.48*	*0.003*	*0.025*
X	stP129	38	34	4	0.9	—	—
X	stP72	42	29	13	0.69	—	—
X	stP2	44	51	−7	1.16	—	—

Tc1-specific markers that showed significant associations to QTL by single-marker analysis are indicated in italics, whereas markers coupled to fitness QTL are indicated with double underlining. The columns (left to right) indicate chromosome or linkage group; marker name; young (Y) and age (A)-selected Tc1+ allele frequencies (as percentage of total); difference ($Y - A$) and ratio (A/Y) of age-selected to young allele frequencies; and χ^2-derived significance levels (P_Σ and P_{empir}) of the change in allele frequency. Significance of the difference at individual markers, based the on the χ^2-distribution, is given as P_Σ—the Bonferroni-corrected P value, $\{P_{single\ marker}/24\}$. P_{empir} is the empirical false-positive level, based on 1000 permutations of the trait category (Y or A) relative to genotype (Churchill and Doerge 1994).

(*tcbn2*), IV (*stP13, stP44,* and *stP35*), V (*stP23, stP6, stP108, stP105,* and *stP128*), and X (*stP41, stP40,* and *stP33*). On chromosome III, markers *stP127* and *stP17* were significantly affected by longevity on the basis of a genome-wide χ^2-criterion, but were not significantly altered on the basis of empirical thresholds (Table 1).

We estimated the standardized effect of the QTL associated with each marker, $2a/\sigma_P = s/i$, where s is the coefficient of selection and i is the intensity of selection in standard deviation units (Table 1). The coefficient of selection is the change in allele frequency between age-selected and young unselected worms (Δq) at each marker, which for homozygous individuals in a recombinant-inbred population is given directly by the genotypes, while the intensity of selection i is set by experimental design at 2.67, the mean Z value for the last-surviving 1% of the population (Falconer and Mackay 1996). Effects associated with peak markers (those of highest significance by χ^2) were determined as normalized differences between homozygotes of the two allelotypes. Estimated effects ranged from 0.25 (*lsq5a*, equivalent to ~1.4 days) to 1.0–3.2 (*lsq4*, ≥5.6 days) with

variation attributable in part to varying distance of markers from a QTL.

Genes affecting longevity—nonparametric interval mapping: To more precisely determine maximum-likelihood positions for quantitative trait loci established by single-marker analysis, interval mapping was performed using a nonparametric algorithm (Kruglyak and Lander 1995), as implemented within MapMaker QTL (Lander *et al.* 1987). The test statistic for nonparametric interval mapping is a Wilcoxin rank-sum, allowing analysis of traits without regard to their distribution, although with slightly reduced power (Kruglyak and Lander 1995). Results are plotted by chromosome in Figure 1, calculated from genotypes of long-lived and control F_7 worms from the present RC301 × BO analysis. For comparison, we have also plotted a similar reanalysis of our earlier data (Ebert *et al.* 1993) derived from F_{12} progeny of a Bristol-N2 × BO cross (Figure 2). QTL peaks on chromosomes I and X, and probably also the peak on chromosome IV, were coincident in the two crosses (compare Figures 1 and 2).

Genome-wide significance thresholds for Z scores, based on simulations varying both genome size and marker density (Kruglyak and Lander 1995), are shown as dashed horizontal lines. Horizontal double arrows in Figures 1 and 2 indicate 2-Z support intervals (peak width at 2 SD below a maximum), which nominally correspond to 95% confidence intervals for peak location (Lynch and Walsh 1998). Eight significant QTL affecting life span were identified by nonparametric interval mapping, of which seven [on chromosomes I, III, IV, V (3 peaks), and X] were also significant by single-marker χ^2-tests (Table 1). An interval-mapping peak on chromosome II (Figure 1) reached significance between markers, but not at individual markers by χ^2-test when adjusted for multiple measures (Table 1).

Interval mapping was also performed as described by Lander and Botstein (1989), using MapMaker QTL (Lander *et al.* 1987), and by composite interval mapping (Zeng 1994), using QTL Cartographer. Both of these procedures, although designed for trait variables with continuous, Gaussian distributions, agree remarkably well with the nonparametric analysis (see Table 2). Empirical false-positive thresholds were calculated for whole-genome scans using 1000 permutations for each of these algorithms, as indicated in Table 2. These procedures also generate estimates at QTL peaks of r^2, the fraction of variance explained (Table 2), which ranged from <0.06 (*lsq1*, *lsq2*, and *lsq5a*) to 0.11–0.14 (*lsq3*) and 0.22–0.24 (*lsq4*).

Confirmation of QTL effect on life span in backcrossed lines: We created nearly isogenic lines containing the QTL on chromosomes III and X by marker-based selection of progeny during 20 generations of backcrossing. RC301 × Bergerac-BO progeny were crossed into the RC301 strain, followed by self-fertilization and selection of homozygous BO-introgressed offspring. Three lines (diverging early in the backcross) were selected for retention of the BO allele on chromosome 3, and two lines retained the BO allele on X. Each line thus contained one selected segment of Bergerac-BO DNA, expected to extend ∼6 cM beyond either flanking marker, isolated in an RC301 background with <1 ppm of Bergerac-BO loci *unlinked* to the selected markers (Lynch and Walsh 1998). All five lines were examined for survival and had median longevities reduced by 1–3 days (5–14%) relative to the RC301 parental strain (*e.g.*, see Figure 3). These life-span differences did not differ between lines congenic for the same interval and were reproducible over multiple experiments ($P < 0.002$ and $P < 0.05$ for QTL on chromosomes III and X, respectively, by paired *t*-test). The 95% confidence intervals for decrease in median longevity were 1.1–2.7 days (chromosome III, 6 comparisons) and 1.0–3.4 days (X chromosome, 3 comparisons). Conversely, after 3 generations of backcrossing into the other parental strain, RC301, longevity was *increased* by 1–3 days relative to RC301 controls (data not shown).

Epistatic interactions: Multiple genes affecting a quantitative trait may exhibit epistasis, allele-specific interactions that influence the trait values. For independent loci, diallele frequencies arise as the product of the component single-allele frequencies ($f_{AB} = f_A \cdot f_B$; $f_{Ab} = f_A \cdot f_b$; etc.). Significant departures from multiplicative diallelic frequencies—as determined by χ^2-test or Fisher's exact test for 2 × 2 matrices—imply interallele associations, indicating that the null hypothesis of independence should be rejected. Thus, if pairwise combinations of some alleles are either over- or underrepresented in a subpopulation, this suggests synergistic or antagonistic interactions in selecting that population. We attempted to determine pairwise interactions among a panel of 10 markers, selected for even spacing to represent all chromosomal regions for which markers exist—taking care to include those showing peak associations to QTL. All 45 possible pairs of markers were tested for independence by Fisher's exact test, separately in the young-control population ("fitness" interactions) and the age-selected population (indicating longevity interactions, provided that similar interaction is not seen in the control group).

Significant interactions were observed in the young unselected group, among the markers tested on chromosome V (*stP23*, *stP6*, *stP108*, and *stP128*; each pairwise $P < 3 \times 10^{-7}$). The false-positive thresholds over all comparisons, calculated as 45 × the P value for any given interaction, were each $P_\Sigma < 2 \times 10^{-5}$. Two significant interactions are seen only in the longevity-selected group, between markers *tcbn2* (chromosome I) and *stP40* (X) [$P \approx 5 \times 10^{-4}$; $P_\Sigma < 0.025$] and between *stP196* (II) and *stP17* (III) [$P \approx 10^{-4}$; $P_\Sigma < 0.01$]. A third possible interaction affecting life span was suggested between *stP196* (II) and *stP128* (V) [$P < 0.005$; $P_\Sigma \approx 0.22$]. Markers at the two ends of chromosome V appear

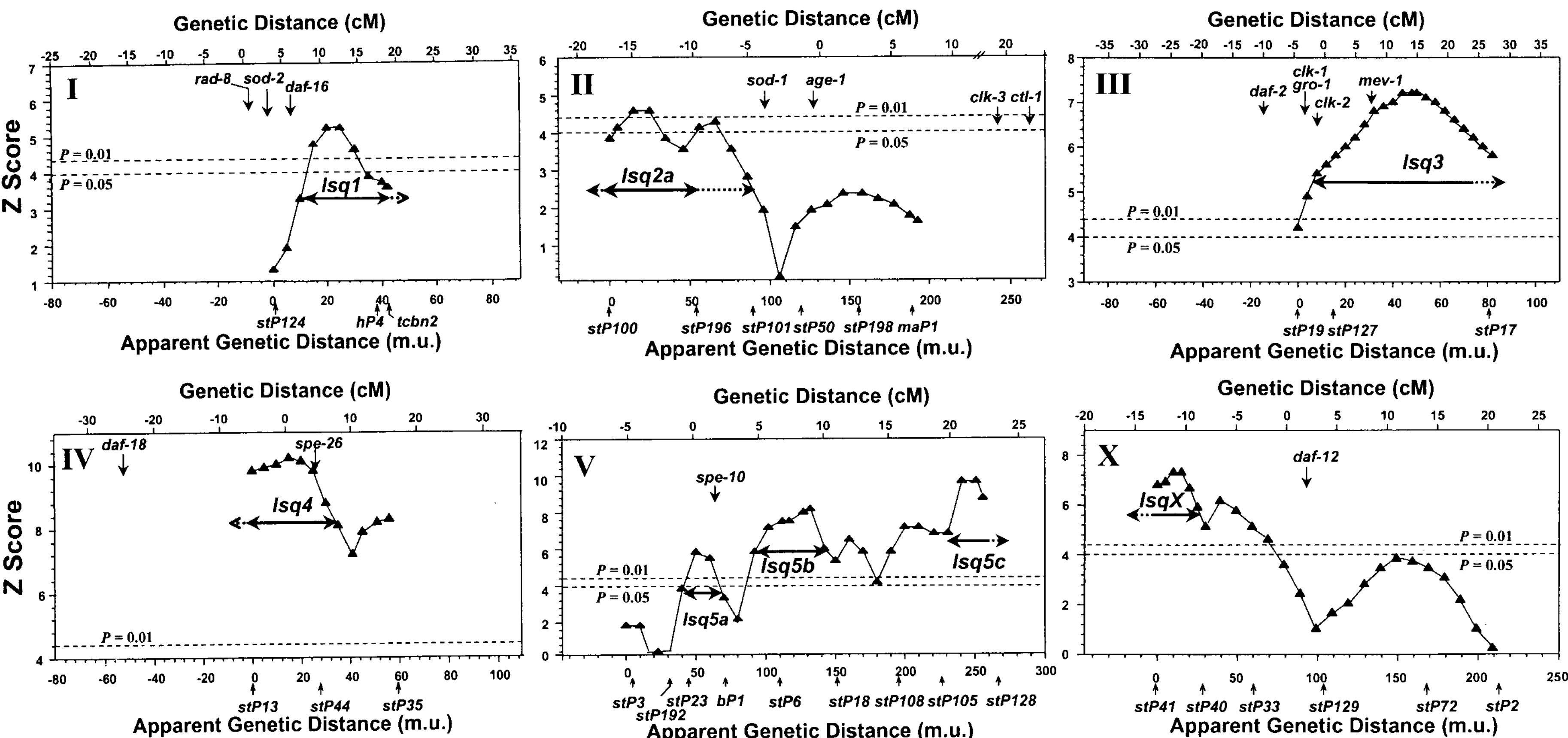

FIGURE 1.—Genetic map for longevity QTL, based on nonparametric interval mapping analysis (KRUGLYAK and LANDER 1995) of long-lived and control samples from a recombinant-inbred population derived from an RC301 × Bergerac-BO interstrain cross. Outputs are shown as Z scores, standardized normal deviation units; note *nonzero origin* on ordinates of some graphs. Significance thresholds are indicated by dashed horizontal lines at $Z = 4.03$ (genome-wide $P < 0.05$) and $Z = 4.4$ (genome-wide $P < 0.01$), as determined by simulations (KRUGLYAK and LANDER 1995) with adjustment for the use of recombinant-inbred lines. Genotype markers used for mapping are indicated at the bottom. Apparent Genetic Distance refers to the expanded map (in map units, m.u.) determined at generation F_7 or F_{12} without correction for recombination accrual over multiple generations, while Genetic Distance at the top (cM) is corrected to correspond to the standard F_2-derived genetic map. Horizontal double arrows indicate the $2 \cdot Z$ support intervals (peak width, 2 SD below peak maximum) for life-span QTL *lsq1*–*lsqX* and represent nominal 95% confidence intervals for locations of maxima (LYNCH and WALSH 1998).

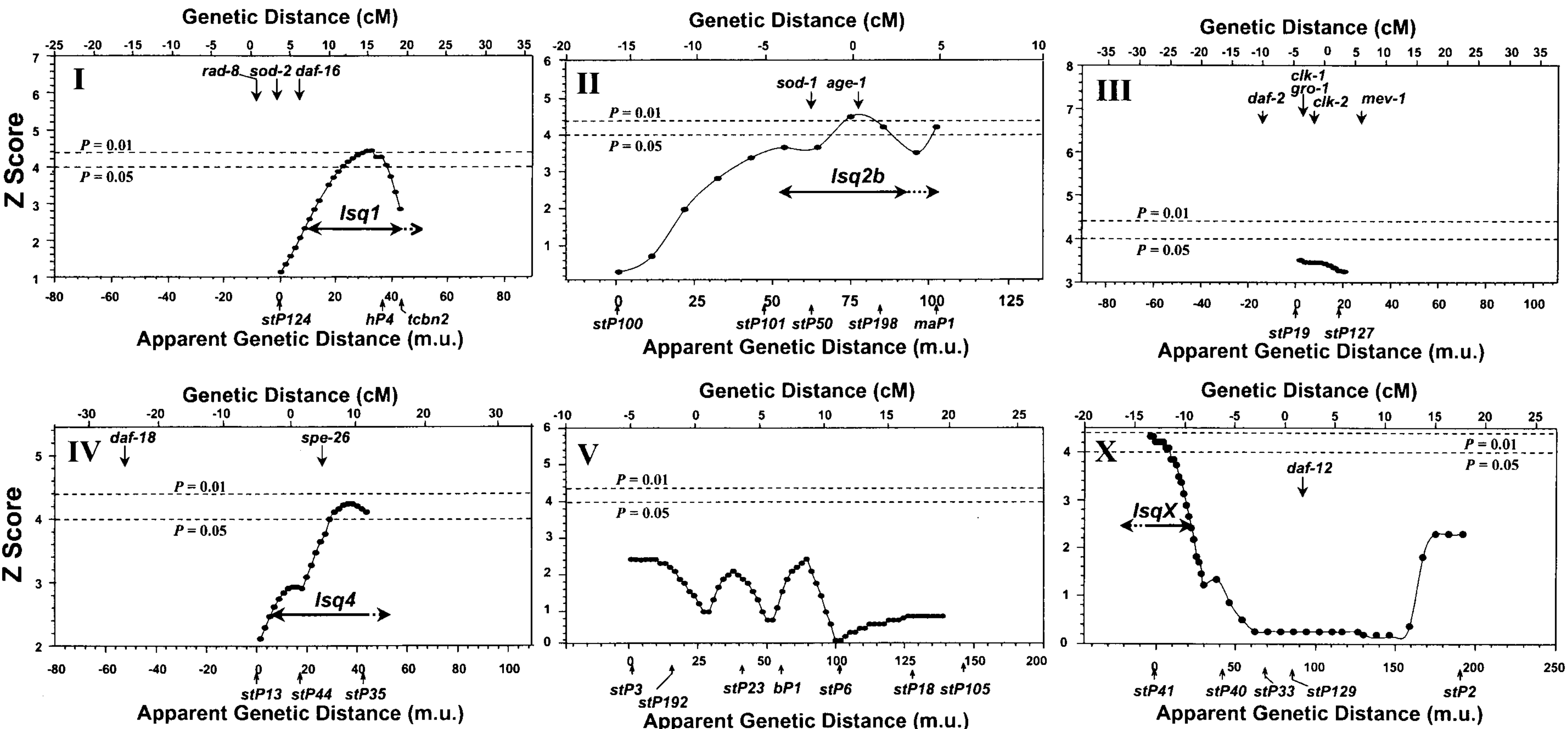

FIGURE 2.—Genetic map for longevity QTL, based on nonparametric interval mapping analysis (KRUGLYAK and LANDER 1995) of long-lived and control samples from a recombinant-inbred population derived from a Bristol-N2 × Bergerac-BO interstrain cross (raw data are from EBERT *et al.* 1993, "mass aging"). Outputs are shown as Z scores, standardized normal deviation units; note *nonzero origin* on ordinates of some graphs. Significance thresholds are indicated by dashed horizontal lines at $Z = 4.05$ (genome-wide $P < 0.05$) and $Z = 4.45$ (genome-wide $P < 0.01$), as determined by simulations (KRUGLYAK and LANDER 1995) with adjustment for the use of recombinant-inbred lines. Genotype markers used for mapping are indicated at the bottom. Apparent Genetic Distance refers to the expanded map (in map units, m.u.) determined at generation F_7 or F_{12} without correction for recombination accrual over multiple generations, while Genetic Distance at the top (cM) is corrected to correspond to the standard F_2-derived genetic map. Horizontal double arrows indicate the $2 \cdot Z$ support intervals (peak width, 2 SD below peak maximum) for life-span QTL *lsq1–lsqX*, and represent nominal 95% confidence intervals for locations of maxima (LYNCH and WALSH 1998).

TABLE 2

Life span QTL peak locations and relative effects

Chromosome	QTL	Peak position (cM)	Maximal scores			Standardized effect of QTL ($2a/\sigma_p$)	r^2 (fraction of variance explained)	
			LOD_{CIM}	LOD_{L-B}	Z		CIM	L-B
I	*lsq1*	12	1.2	2.3	5.0***	0.37	0.025	0.045
II	*lsq2*	−14	0.9	2.6*	4.6***	0.27	<0.01	0.06
III	*lsq3*	14	>5.3**	>3.5*	7.4****	0.26–0.37	0.14	0.11
IV	*lsq4*	~1	>5.9**	>10.0**	10.4****	1.0–3.2	0.22	0.24
V	*lsq5a*	~0	—	3.5*	5.14***	0.25	0.04	0.06
V	*lsq5b*	7–8	2.1	6.1**	6.7***	0.64	0.04	0.12
V	*lsq5c*	20–22	4.3**	4.9**	7.5****	0.24–0.71	0.08	0.10
X	*lsqX*	−12	4.2**	6.8**	6.4***	0.34–0.66	0.07	0.15

Peak positions are given with respect to the standard *C. elegans* map, in centimorgans (top axis scales in Figures 1 and 2). LOD_{CIM} is the peak LOD score (the base-10 logarithm of likelihood ratio) determined by composite interval mapping (Zeng 1994) with seven background markers and window size 15. LOD_{L-B} is the peak LOD score determined by Lander-Botstein interval mapping (Lander and Botstein 1989). Z is the peak Z-score obtained by nonparametric interval mapping (Kruglyak and Lander 1995). Standardized QTL effect associated with each marker is calculated from the change in allele frequency due to selection (Falconer and Mackay 1996), in units of survival standard deviations; for this F_7 population, $\sigma = 5.6$ days. The fraction of trait variance explained was calculated by each parametric interval mapping procedure within QTL cartographer (CIM and L-B) as r^2, where r is the correlation coefficient between life span and genotype at highest-LOD position for which the maximum-likelihood function converged. *Genome-wide $P_{empir} < 0.05$; **genome-wide $P_{empir} < 0.01$ (empirical thresholds based on 1000 permutations of phenotype with respect to genotype); ***genome-wide $P_Z < 0.01$; ****genome-wide $P_Z < 0.001$ (Z significance thresholds derived from Kruglyak and Lander 1995).

to interact for longevity, in the direction opposite to that of their fitness interactions; that is, aberrant diallele ratios in the young group were reversed in the age-selected group. The large differences in χ^2-values for young *vs.* age-selected allele ratios ($P_{age\text{-}selected}/P_{young} > 10^3$) suggest that *stP23* (*lsq5a*) interacts with both *stP108 (lsq5c)* and *stP128* (*lsq5c*), while *stp128* (*lsq5c*) also interacts with *stP6* (*lsq5b*).

DISCUSSION

We began genetic mapping by construction of a cross between the *C. elegans* strains RC301 and Bergerac-BO and tested for QTL associations with life span after seven generations of inbreeding. Through the combined use of recombinant-inbred (and hence homozygous) worms, map expansion during inbreeding, and selective genotyping of phenotypic extremes in a population, we have generated data sets with improved power for the discovery and resolution of multiple QTL affecting life span. This gain in sensitivity and reliability entailed a somewhat unconventional experimental design (Ebert *et al.* 1993), not accommodated by existing interval-mapping tools. Results are quite consistent, however, between a statistical test appropriate to categorical trait data (χ^2-analysis at individual markers) and an interval mapping procedure designed to position QTL with higher resolution, based on nonparametric quantitative data.

By using recombinant-inbred populations, rather than recombinant-inbred lines, we defined a subgroup with an extreme-longevity phenotype among $\sim 10^6$ worms, representing ~2600 genotypes. This cohort was tested for life span in the same survival rather than several thousand survivals—thus facilitating the simultaneous comparison of longevity among many homozygous genotypes. The distribution of longevities is approximately normal, with a mean of ~20 days (data not shown, and Ebert *et al.* 1993), providing a robust internal control for assignment of an extreme-longevity class. Selective genotyping at phenotypic extremes enhances the power of QTL analysis per genotype assessed; *i.e.*, genotyping 171 long-lived worms and a like number of controls produced QTL mapping power equivalent to complete genotyping of a 2000-worm population (Lander and Botstein 1989; Lynch and Walsh 1998). Replicate survivals, even in varied environments, yield consistent results by this method (Ebert *et al.* 1993; S. Ayyadevara, unpublished data), and further corroboration is implied by our recurrent discovery of many loci in more than one cross (Figures 1 and 2 and additional data not shown). Mapping results should nevertheless be viewed with caution until confirmed (Tanksley 1993).

Loci affecting fitness or segregation of alleles in young-control worms: Initial (control) allele frequencies could deviate from their expected values at some marker loci, due either to the cumulative effect of fitness selection over five generations of inbreeding or to dis-

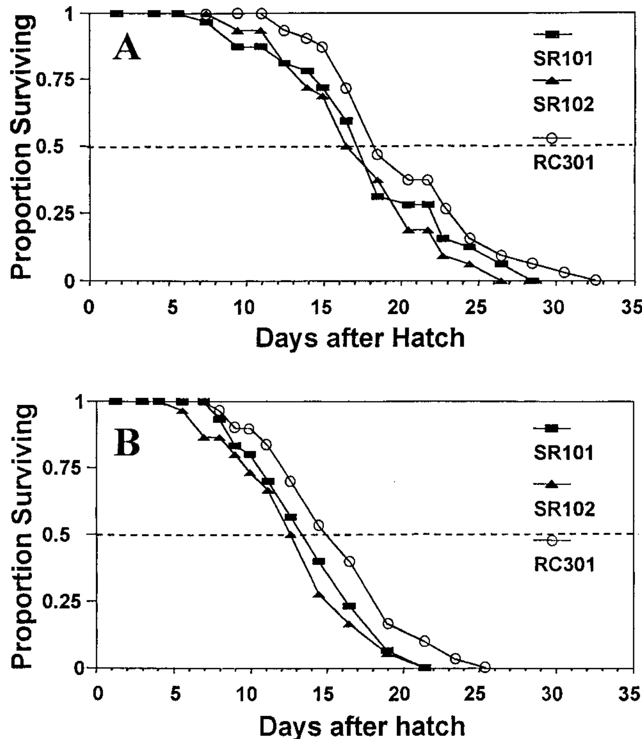

Figure 3.—Effect of QTL on chromosomes III and X on life span of *C. elegans* at 20°. To assay the effects of QTL *lsq3* and *lsqX* on life span, several near-isogenic lines containing each QTL were constructed by backcrossing the BO QTL into RC301 for 20 generations (see materials and methods). SR101 (solid rectangles) containing *lsq3* and SR102 (solid triangles) containing *lsqX* show decreased mean and maximum life spans relative to the RC301 parental strain (open circles). A and B show results from two independent survival experiments.

tortion of segregating ratios earlier in the cross. The mechanism of segregation bias is unknown, but may involve competition among gametes for preferential fertilization (Lyttle 1991). Several segregation-distortion loci have been mapped in Drosophila (Lyttle 1991), maize (Ahn *et al.* 1993), barley (Garner *et al.* 1991), and rice (Causse *et al.* 1994; Xu *et al.* 1997). The preponderance of selection favoring RC301 (13 markers, *vs.* 2 favoring BO, out of 30 assessed; see Table 1) may be attributed to BO alleles responsible for reduced male fertility (Liao *et al.* 1983) and to BO embryonic-lethal mutations of low penetrance (*e.g.*, *zyg-9*). Indeed, Bergerac-BO is a mutator strain with active germline transposition of Tc1 elements (Moerman and Waterston 1984; Mori *et al.* 1988) and thus could have accumulated many such mildly deleterious mutations, resulting in lower-than-expected initial *Tc1*+ frequencies. If this were so, however, then BO alleles should be underrepresented at the *same* loci in all crosses between BO and other strains. In fact, our data for this and several other crosses (Ebert *et al.* 1993, 1996; S. Ayyadevara, R. Ayyadevara, J. J. Thaden and R. J. Shmookler Reis, unpublished results) are not consistent with this scenario. Many markers show initial allele frequencies close to expectation, while others display both lower- and higher-than-expected *Tc1*+ allele frequencies in similar numbers, as might be expected for markers linked to genes affecting reproductive or gametic fitness.

Loci affecting nematode longevity: Nonparametric interval mapping located eight significant loci (genome-wide $P_\Sigma < 0.01$), of which seven had also been implicated by single-marker analysis after adjustment for multiple comparisons (Table 1; genome-wide $P_\Sigma < 0.05$). These seven loci, with standardized effects ranging from 0.25 to >1.0 (in *Z* units), accounted individually for 2.5–24% of the total population variance in longevity (Table 2). It should be noted that r^2 values, although widely understood to reflect the portion of variance explained, tend toward upward bias and are not additive unless corrected for covariance among loci. Thus, the appearance that we have here accounted for the majority of total life-span variance [a total of 87.7% as estimated by Lander-Botstein interval mapping, or >61.5% by composite interval mapping (CIM)] may be misleading.

Single-marker analyses, based on χ^2-tests of marker: longevity association, provide the primary statistical basis for inferring the presence of QTL (Kachigan 1986). Fourteen markers, defining seven putative life-span QTL, achieved unadjusted values of $P_{\text{single marker}} \leq 0.002$, equivalent to a genome-wide false-positive level $P_\Sigma <$ 0.05 (where $P_\Sigma = P_{\text{single marker}} \times 24$; see Table 1). Similar results were obtained using empirical false-positive thresholds—determined for the entire genome scan by permutation of trait with respect to genotype (Table 1, P_{empir} column), except that two markers on chromosome III narrowly miss significance.

We then used interval mapping to define maximum-likelihood positions of QTL between markers. The available procedures were not intended for use with our experimental design, in which longevity is defined categorically rather than quantitatively. Although several methods have been proposed for interval mapping of categorical traits (Visscher *et al.* 1996; Xu and Atchley 1996; Xu *et al.* 1998), their application to recombinant inbred populations is currently under development (S. Ayyadevara, R. Ayyadevara, A. Galecki, J. J. Thaden and R. J. Shmookler Reis, unpublished data). For the present analyses, nonparametric interval mapping (Kruglyak and Lander 1995) was conducted within MapMaker QTL. The likelihood maxima generated by this algorithm are entirely consistent with the single-marker χ^2-analysis (compare Table 1 and Figure 1)—a surprising result given that the mapping procedure relies on rank-order regression, which offers little power for binary trait values. Moreover, maps derived from two interval mapping algorithms that assume Gaussian continuous trait values and utilize either likelihood maximization (Lander and Botstein 1989) or multivariate linear regression (Zeng 1994) were also consistent with single-marker χ^2-results—supporting seven or four of the eight peaks, respectively. From a comparison of these results (Table 2), it is clear that QTL can be de-

tected and positioned reliably even by statistically inappropriate procedures, provided that the experimental design provides sufficient power and the significance threshold is determined empirically, by permutation of genotypes with respect to trait values (Churchill and Doerge 1994).

Comparison to previous genetic mapping of longevity QTL: Previous mapping experiments, using N2 × BO recombinant-inbred populations (Ebert *et al.* 1993, 1996) or lines (Shook *et al.* 1996), identified multiple chromosomal regions affecting life span. We initially observed five chromosomal regions associated with longevity, on chromosomes I, II, IV, V, and X (Ebert *et al.* 1993 and Figure 2). Several QTL observed in the present cross—*lsq1*, *lsqX*, and probably *lsq4*—coincide with QTL identified in the N2 × BO cross. The uncertainty regarding *lsq4* reflects a shift in peak position in the two crosses (see Figures 1 and 2), although additional data (not shown) suggest that this difference is artifactual. Interval mapping generates likelihood-ratio maxima, which provide rather imprecise guides to QTL location, with an expected error inversely proportional to peak LOD value (Roberts *et al.* 1999).

Among long-lived worms in the N2 × BO cross, the BO allele was favored for QTL on chromosomes II and IV, whereas the N2 allele was favored on chromosomes I and X. Comparison of these crosses allows a rough ordering of allele effects at each locus with respect to longevity; *i.e.*, (RC ≈ N2) > BO for *lsq1* and *lsqX*, RC > (N2 = BO) for *lsq2a* and *lsq3*,(RC = BO) > N2 for *lsq2b*, RC ≫ BO > N2 for *lsq4*, and RC > (N2 ≥ BO) for *lsq5a–c*. Several known genes mapping to these regions, which may be functional candidate genes for determinants of nematode longevity, are also indicated in Figure 1. These should be interpreted with caution, since each QTL interval contains many dozens of other genes, mostly of unknown function.

Estimation of the total number of life-span QTL in *C. elegans*: From the numbers and locations of the QTL mapped using different interstrain crosses, we can estimate the total number of QTL that influence the nematode's life span to a similar degree. A total of 8 QTL were identified in the RC301 × BO cross (Table 1 and Figure 1), and 5 QTL were in the N2 × BO cross (Ebert *et al.* 1993 and Figure 2). The total number of life-span QTL (n') may be estimated by recapture statistics (Feller 1968) as

$$n' = n_1 \cdot n_2 \, / \, k,$$

where n_1 is the number of QTL identified in a given cross, n_2 is the number of QTL identified in a second cross, and k is the number of QTL common to both crosses. Taking three QTL—on chromosomes I, IV, and X—as coincident in RC301 and N2 crosses, $n' = (4 \times 8)/3 \approx 11$, whereas excluding the QTL on chromosome IV would increase n' to 16. Thus, the number of QTL of comparable significance that govern the life span of *C. elegans* should be 11–16. However, incomplete map coverage (as on chromosomes I, III, and IV) and failure to resolve closely linked QTL (as on chromosome V) may lead to underestimation, whereas variability of QTL strength may cause overestimation, of total QTL number. The actual number could be as small as 10 (the total we have observed in these two studies), but is unlikely to exceed 30.

Epistatic interactions: Gene-gene interactions for fertility or Darwinian fitness were implied by our observation of significant departure from independence between markers at opposite ends of chromosome V. Although linkage could account for some degree of interlocus association, *stP6–stP128* and *stP23–stP128* span apparent genetic distances of >150 and 220 cM, respectively—corresponding to recombinant fractions >48% by Kosambi's mapping function (Lynch and Walsh 1998)—indicating that these distal loci are effectively unlinked. In addition, two interactions were seen only in the longevity-selected group, between markers on chromosomes I and X (Bonferroni-adjusted $P < 0.025$) and chromosomes II and III (Bonferroni-adjusted $P < 0.01$). Additional longevity interactions were suggested by diallele frequencies involving chromosome II (*lsq2a*) and the right end of V (*lsq5c*) and between the two ends of chromosome V. *Lsq2a* may thus interact with both *lsq3* and *lsq5c*, while *lsq5c* shows possible interaction with both *lsq2a* and *lsq5a*. Although epistasis among three or more loci can also be evaluated by χ^2-tests on larger matrices, the power and reliability of such tests drop precipitously as the data set is subdivided.

The observation of these oligogenic interactions is all the more remarkable, given that epistasis tends to be severely underestimated in QTL analyses of two-strain cross progeny. Only those QTL that are dimorphic between parental strains are identified in a mapping experiment, and detection of their interactive partners requires that these also be dimorphic between the same two parents. It is thus likely that we have glimpsed no more than a small portion of the intergenic network. Interaction between *lsq2a* and *lsq3* may detract from the apparent significance of the associated markers when they are analyzed individually by single-marker tests.

Confirmation of QTL effects on longevity: Confirmation and precise localization of longevity QTL depend on the construction and fine-map analysis of near-isogenic lines created by repeated backcrossing to one of the parental strains. We have created homozygous congenic lines for two QTL (*lsq3* and *lsqX*) and measured their effects. Presence of the BO allele spanning just the QTL interval reduced median life span by 1.8 days (∼10%) for *lsq3* and 2.3 days (14%) for *lsqX*, relative to RC301 controls. These values are within the effect ranges predicted from single-marker allele ratios (Table 2), 1.5–2.1 days for *lsq3* and 1.9–3.7 days for *lsqX*. (Predicted effects are based on the observed standard de-

viation for population survival, 5.6 days.) Conversely, backcrossing the RC301 allele of each QTL into a Bergerac-BO background produced longevity increases of 1–3 days relative to BO (data not shown). Two further QTL, apparently coincident with *lsq 4* and *lsq5a* reported here, were defined in a different interstrain cross and characterized after extensive backcrossing (A. Vertino, S. Ayyadevara and R. J. Shmookler Reis, unpublished results). Overall, four longevity QTL have now been isolated in an isogenic background and confirmed with respect to both location and phenotype.

A fine-mapping method recently developed in our lab (Ayyadevara *et al.* 2000), allowing most of the 500 differential Tc1 elements to be used as markers, is being employed to accurately demarcate the QTL regions in redundant sets of congenic (near-isogenic) lines. Upon assessment of life span for several such lines per QTL, the boundaries of a longevity-affecting locus can be precisely defined by the overlap of introgressed regions. Each QTL should thus be reducible to an absolute (rather than stochastic) span encompassing <100 genes. Selection of recombinants over diminishing intervals will enable us to zero in on the gene responsible for a QTL's effects.

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