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Molecular population genetics and phenotypic sensitivity to ethanol for a globally diverse sample of the nematode *Caenorhabditis briggsae*

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Abstract

New genomic resources and genetic tools of the past few years have advanced the nematode genus *Caenorhabditis* as a model for comparative biology. However, understanding of natural genetic variation at molecular and phenotypic levels remains rudimentary for most species in this genus, and for *C. briggsae* in particular. Here we characterize phenotypic variation in *C. briggsae*'s sensitivity to the potentially important and variable environmental toxin, ethanol, for globally diverse strains. We also quantify nucleotide variation in a new sample of 32 strains from four continents, including small islands, and for the closest-known relative of this species (*C.* sp. 9). We demonstrate that *C. briggsae* exhibits little heritable variation for the effects of ethanol on the norm of reaction for survival and reproduction. Moreover, *C. briggsae* does not differ significantly from *C. elegans* in our assays of its response to this substance that both species likely encounter regularly in habitats of rotting fruit and vegetation. However, we uncover drastically more molecular genetic variation than was known previously for this species, despite most strains, including all island strains, conforming to the broad biogeographic patterns described previously. Using patterns of sequence divergence between populations and between species, we estimate that the self-fertilizing mode of reproduction by hermaphrodites in *C. briggsae* likely evolved sometime between 0.9 and 10 million generations ago. These insights into *C. briggsae*'s natural history and natural genetic variation greatly expand the potential of this organism as an emerging model for studies in molecular and quantitative genetics, the evolution of development, and ecological genetics.

Keywords: breeding systems, Caenorhabditis, genetic variation, hermaphrodite, phylogeography

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Introduction

The nematode *Caenorhabditis elegans* has served as a model organism in biology for nearly four decades. As a simple multicellular organism, it has aided researchers in elucidating many of the most elementary biochemical, genetic and developmental processes in biology. However, the past few years have witnessed a surge in the literature devoted to the evolutionary genetics of *C. elegans* and related nematodes (Kammenga *et al.* 2008; Cutter *et al.* 2009). As part of this endeavour, it is necessary to characterize samples of *Caenorhabditis* species on a global scale in order to reveal the extent of segregating genetic variation, to evaluate the extent of phenotypic variation, and to infer the potential for natural selection to have shaped the variation. In particular, ecologically relevant phenotypic variation remains poorly understood for most species in this genus. We aim to fill this gap for the species

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C. briggsae, in part, by characterizing molecular genetic variation in a new collection of 32 wild isolates from around the world and by testing for heritable phenotypic variation in sensitivity to a potentially important and variable environmental toxin, ethanol.

Caenorhabditis briggsae, like its cousin *C. elegans*, is a bactivorous nematode with populations comprised primarily of self-fertilizing hermaphrodites (Kiontke & Sudhaus 2006). However, hermaphroditism evolved independently in the ancestors of these two species (Cho *et al.* 2004; Kiontke *et al.* 2004; Hill *et al.* 2006) and they are separated by millions of years of evolution (Coghlan & Wolfe 2002; Cutter 2008). Despite having similar species ranges (Kiontke & Sudhaus 2006; Sudhaus & Kiontke 2007), *C. briggsae* can tolerate higher temperatures than *C. elegans* (Fodor *et al.* 1983; Inoue *et al.* 2007). Intraspecific tolerance to temperature extremes is associated with biogeographically and genetically defined groups of *C. briggsae*: worm strains from tropical regions have higher fecundity at extreme high temperature and lower fecundity at extreme low temperature than do strains from temperate regions (A. Prasad, M. Croydon-Sugarman & A.D. Cutter, unpublished data). In contrast, genetic and geographic distances are largely independent in *C. elegans* (reviewed in Cutter *et al.* 2009). When reared at 20 °C, heritable variation in body size and fitness traits have been documented in *C. briggsae* (Dolgin *et al.* 2008; Howe & Denver 2008; Salomon *et al.* 2009) and strains of *C. briggsae* from different geographic regions also exhibit differences in lifespan (Joyner-Matos *et al.* 2009). However, it is unclear whether the previously observed temperate–tropical–equatorial designations ascribed to three latitudinally and genealogically segregated groups (Cutter *et al.* 2006; Dolgin *et al.* 2008; Howe & Denver 2008) is a general representation of genetic diversity in this species, or whether the global pattern of diversity might be more complex given greater sampling effort.

In addition to the importance of temperature on *Caenorhabditis* life history, variation in other ecologically important factors might underlie heritable differences among strains of *C. briggsae*. Because this species feeds on bacteria in rotting fruit and vegetation, one potentially critical environmental variable is the concentration of ethanol, which is released as a by-product of yeast metabolism. *Drosophila* larvae grow in analogous habitat and exhibit striking latitudinal clines in ethanol sensitivity (reviewed in Fry *et al.* 2008), which provides a biological rationale for testing for latitudinal differences in *C. briggsae*. Two main hypotheses have been proposed for greater ethanol tolerance in temperate latitudes: rotting fruit may contain higher ethanol concentrations in temperate regions, or, exploitation of rotting fruit with high levels of ethanol might be more advantageous in temperate regions (Eanes 1999; Fry *et al.* 2008). Although differences in ethanol sensitivity between two strains of *C. elegans* (one originally from Bristol, UK, the other from Hawaii, USA) could be traced to alternative alleles at *npr-1* (Davies *et al.* 2004), it now appears that these allelic differences do not represent variation originating in the wild, but the result of laboratory adaptation via new mutation (McGrath *et al.* 2009). Consequently, natural variation in the sensitivity to ethanol within and between species of *Caenorhabditis* remains largely unexplored.

Studies with the standard laboratory strain of *C. elegans* have demonstrated that ambient ethanol concentrations greater than 7% induce death after 6 h of exposure (Mitchell *et al.* 2007) or 24 h of exposure at 3% concentration (Dhawan *et al.* 1999). Much lower ambient concentrations (~0.4%) adversely impact reproductive rate, pharyngeal pumping and locomotor ability (Morgan & Sedensky 1995; Dhawan *et al.* 1999; Davis *et al.* 2008), although the anaesthetic effect of ethanol on pharyngeal pumping and locomotor ability reverses following removal from exposure (Davies *et al.* 2004; Mitchell *et al.* 2007). Like many organisms, *C. elegans* also exhibits evidence of physiological adaptation (i.e. behavioural tolerance) to ethanol (Davies *et al.* 2004). Moreover, ethanol has been demonstrated to play a role in basic neurologic circuitry in association with food in *C. elegans* (Davies *et al.* 2004). Laboratory mutant alleles of several genes confer resistance to the toxic effects of ethanol (Davies *et al.* 2003, 2004; Hong *et al.* 2008) and Kwon *et al.* (2004) identified 230 genes in *C. elegans* whose expression changes upon exposure of animals to ethanol, providing plausible genetic candidates to any heritable variation in ethanol sensitivity in *C. briggsae*.

To investigate how ethanol might exert differential selective pressure on populations of *C. briggsae* that inhabit different latitudes, here we quantify the effects of varying ethanol concentrations on the survival and reproduction of wild isolates of *C. briggsae*. We contrast these effects of ethanol with two strains of *C. elegans* to test for species-level differences in ethanol sensitivity. In addition, we assess nucleotide variation in a new collection of *C. briggsae* wild isolates from around the globe. We demonstrate that increasing ethanol concentrations have strongly detrimental effects on *C. briggsae* survival and reproduction. However, genetically distinct wild isolates of *C. briggsae* do not differ significantly in their sensitivity to ethanol, nor does *C. briggsae* differ from *C. elegans* in ethanol sensitivity in our assays. Newly isolated samples of *C. briggsae* reveal substantially greater molecular genetic variation in this species than was known previously, despite

most new strains conforming to previously described patterns of biogeography.

Materials and methods

Nematode strains

Thirty-two new isolates of *Caenorhabditis briggsae* from around the world are included in this study, most of which were collected by M.A. Felix or gifted to us by M. Ailion, E. Andersen and C. Rocheleau and have been deposited in the Felix lab nematode collection (Table 1; http://www.justbio.com/worms). Most of the new strains were isolated from rotting fruits (Table 1), whereas most previous collections of *C. briggsae* derive from compost. The stage of development at the time of sampling is unknown for the new strains. One strain (BW287) was acquired from the Caenorhabditis Genetics Center, and previously had its mitochondrial genome sequenced by Howe & Denver (2008). Strains used in phenotypic analysis are a subset of those described elsewhere (Cutter *et al.* 2006; Dolgin *et al.* 2008), containing several representatives from each of the three genotype clades that comprised temperate latitude, tropical latitude and Nairobi, Kenya samples (Table 2). We also generated an online interactive map of collection localities and strains for *C. briggsae* (http://www.google.com/maps/ms?msa=0&msid=117700919974655793194.00046c7ccc0afccc319b7) and other species in the genus (available upon request).

Molecular methods

The six gene fragments sequenced for this study (Table 3) correspond to the loci used in two previous global studies of *C. briggsae* nucleotide polymorphism

Table 1 New strains used for analysis of molecular variation and divergence

Strain	Latitude	Longitude	Locality	Date	Source
BW287	39°55′N	116°25′E	Beijing, China	1984–1989	Caenorhabditis Genetics Center
EG4360	40°36′S	111°48′W	Salt Lake City, Utah, USA	October 2007	Rotten plum
EG4365	40°46′N	111°49′W	Salt Lake City, Utah, USA	October 2007	Rotten acorn
EG5612	23°10′S	44°11′W	Dois Rios, Ilha Grande, Brazil	April 2009	Rotting fruit
EG5613	23°10′S	44°11′W	Dois Rios, Ilha Grande, Brazil	April 2009	Rotting fruit
EG5614	23°10′S	44°11′W	Dois Rios, Ilha Grande, Brazil	April 2009	Rotting fruit
JU1337	8°19′N	77°03′E	Poovar, Kerala, India	December 2007	Rotting coconut
JU1338	8°30′N	76°57′E	Trivandrum, Kerala, India	December 2007	Rotting palm fruit
JU1339	8°41′N	77°05′E	Ponmudi, Kerala, India	December 2007	Rotting wild figs
JU1340	8°45′N	77°05′E	Ponmudi, Kerala, India	December 2007	Leaf litter, rotting parasitic flower
JU1341	8°45′N	77°05′E	Ponmudi, Kerala, India	December 2007	Rotting fruits/bark
JU1342	8°45′N	77°05′E	Ponmudi, Kerala, India	December 2007	Rotting fruits
JU1343	8°45′N	77°05′E	Ponmudi, Kerala, India	December 2007	Rotting chestnut/dry mushrooms
JU1344	8°45′N	77°05′E	Ponmudi, Kerala, India	December 2007	Rotting fruits
JU1345	8°45′N	77°05′E	Ponmudi, Kerala, India	December 2007	Rotting fruits (?)
JU1346	9°34′N	76°49′E	Kanjirapalli, Kerala, India	December 2007	Fallen banana tree
JU1347	9°30′N	77°12′E	Periyar, Kerala, India	December 2007	Coffee grains, soil, elephant dung
JU1348	9°29′N	77°14′E	Periyar, Kerala, India	December 2007	Rotting fruit, leaf litter, bark, soil, flowers
JU1375	21°02′S	55°41′E	La Réunion, France	January 2008	Snail in rotting sapodilla
JU1376	21°02′S	55°41′E	La Réunion, France	January 2008	Mushrooms
JU1377	21°02′S	55°41′E	La Réunion, France	January 2008	Rotting torch ginger
JU1378	21°02′S	55°41′E	La Réunion, France	January 2008	Rotting velvet apple
JU1392	22°57′S	43°12′W	Rio de Janeiro, Brazil	January 2008	Fallen fruits under cannonball tree
JU1399	6°17′N	75°32′W	Medellin, Colombia	March 2008	Rotting orange fruits
JU1424	22°25′N	105°35′E	Ba Be lake, Vietnam	April 2008	Rotting fruit
JU1435	21°03′S	55°15′E	La Réunion, France	May 2008	Rotting fruit
JU1637	17°08′N	25°04′W	Santo Antao Island, Cape Verde	April 2009	Soil with cabbage leaves
JU1638	17°08′N	25°04′W	Santo Antao Island, Cape Verde	April 2009	Soil next to yam/taro plant
QR24	45°28′N	73°45′W	Montreal, Quebec, Canada	Spring 2008	Garden soil
QR25	45°28′N	73°45′W	Montreal, Quebec, Canada	Spring 2008	Garden soil
QX1410	13°48′N	61°02′W	St. Lucia	December 2008	Rotting flower
QX1414	38°53′N	92°53′W	Pilot Grove, MO, USA	September 2008	Rotten tomato and associated soil
JU1422	8°30′N	76°57′E	Trivandrum, Kerala, India	December 2007	Rotting flowers/leaves (inbred JU1325)

Table 2 Strains used for analysis of phenotypic variation

Species	Strain	Biogeographic region	Locality
C. elegans	CB4856	—	Hawaii, USA
C. elegans	N2	—	Bristol, UK
C. briggsae	ED3092	—	Nairobi, Kenya
C. briggsae	ED3101	—	Nairobi, Kenya
C. briggsae	EG4181	Temperate	Utah, USA
C. briggsae	HK104	Temperate	Okayama, Japan
C. briggsae	JU439	Temperate	Reykjavic, Iceland
C. briggsae	PB826	Temperate	Ohio, USA
C. briggsae	AF16	Tropical	Ahmedabad, India
C. briggsae	ED3083	Tropical	Johannesburg, South Africa
C. briggsae	VT847	Tropical	Hawaii, USA

(Cutter *et al.* 2006; Dolgin *et al.* 2008). These loci include relatively long intron regions (>500 bp) and short stretches of flanking coding sequence for the *C. briggsae* orthologues of six genes from *C. elegans*, the PCR primers for which were published previously (Cutter *et al.* 2006). Three of the loci reside on chromosome II and three loci are known, or presumed, to reside on the X chromosome (Cutter *et al.* 2006). We used these same primers to amplify and sequence the homologous regions from strain JU1422 of closely related, but as-yet undescribed, *C.* sp. 9. DNA for each strain was isolated using Qiagen DNeasy Blood and Tissue kits. Sequencing was performed by the University of Arizona UAGC sequencing facility and by the University of Toronto CAGEF sequencing facility. New sequence data have been deposited in GenBank with accession numbers GU215967–GU216164.

Sequence analysis

Sequencher 4.9 and BioEdit 7.0.9 were used in combination with manual editing to confirm sequence quality, to align sequences and to remove primers. Calculations of per-site diversity from pairwise differences (π) and the number of segregating sites (θ) were performed using DnaSP 5.0.0 (Rozas *et al.* 2003), with focus on silent sites (i.e. synonymous and intronic positions). Indel sites and incomplete data were excluded from the analysis. Detection of recombination with the 4-gamete test using R_{min} (Hudson & Kaplan 1985) also was performed with DnaSP. Neighbour-network trees were created using SplitsTree 4.10 from concatenated sequences (Huson & Bryant 2006). These neighbour-networks use genetic distance to cluster the multi-locus genotypes, but visually represent recombination among them as reticulation events in the resulting gene tree. This makes inference of potential recombination explicit, while recognizing that genetically effective recombination is relatively uncommon in the ancestry of these highly self-fertilizing organisms. To estimate divergence times for isolated *C. briggsae* lineages, we ran MCMCcoal (Rannala & Yang 2003) on intron regions of the six loci for the temperate (HK104), tropical (AF16) and Kerala (JU1341) samples. We applied Jukes-Cantor corrections to divergence between *C. briggsae* and *C.* sp. 9 to account for the potential of multiple mutational hits at a given site, as implemented in DnaSP (Rozas *et al.* 2003). For the resulting estimates of divergence time, we convert it to units of generations assuming a mutation rate in *C. briggsae* 5.4×10^{-9} mutations/generation, double the *C. elegans* rate (Baer *et al.* 2005; Denver *et al.* 2009; Phillips *et al.* 2009).

Table 3 Locus information on position, size and diversity

Primer*	Locus	Chromosome†	Position†	Silent sites	*S*	*H*	π_{si} (%)‡	θ_{si} (%)‡	STRPs§	Indels§
p09	CBG19635	II_random	1839170	467.7	10	4	0.102	0.402	2	6
p10	CBG03684 (*Cbr-toe-1*)	II	2251108	561.0	25	12	0.916	0.804	3	6
p11	CBG20775	II	10620410	383.3	12	6	0.310	0.581	0	7
p12	CBG14460 (*Cbr-lst-2*)	X	9514735	477.0	18	8	0.621	0.710	2	7
p13	CBG16368	X	981842	468.2	18	8	0.479	0.723	1	8
p14	CBG24509	ChrUn	2940902	589.5	18	9	0.215	0.574	0	0
	Average			491.1	16.8	7.8	0.441	0.632	1.3	5.7
	Concatenated			2951.7	101	25	0.450	0.637	8	34

S, number of segregating sites; *H*, number of haplotypes.
*From Cutter *et al.* (2006).
†From Wormbase WS204, note that not all genome contigs are perfectly assigned to chromosomes.
‡Per cent silent total-sample diversity per site.
§Short tandem repeat polymorphisms (STRPs) and longer insertion–deletion polymorphisms.

Phenotypic assays of ethanol sensitivity

We tested the influence of varying ethanol concentrations on the survival and reproduction of nine strains of *C. briggsae* and two strains of *C. elegans* (Table 2). We tested for an effect of genotype, species, treatment (ethanol concentration), and block in nested ANOVA models for each assay using JMP 5.0. Block effects were not significant, and were subsequently excluded from the analysis.

Survival. We assayed nematode survival in liquid using three different concentrations of ethanol in water (3%, 3.8% and 4.6% v/v) along with two controls (M9 buffer solution and water) in 24-well flat-bottom plates. Worms were reared to the fourth larval stage (L4) at 20 °C on NGM-lite nutrient agar with *Escherichia coli* OP50 as a food source. At L4, worms were transferred to an unspotted agar plate for 20 min, after which 10 worms were transferred to each well of the 24-well plates containing 2 mL of solution. After 24 h, animals that responded to gentle agitation with a pick were scored as alive. Eight replicates for each strain–treatment combination were conducted in a block design.

Reproduction. We assayed the self-fertilizing reproductive output of individual hermaphrodites when they were subjected to continuous exposure to liquid, using three concentrations of ethanol in M9 buffer (0.5%, 1% and 2% v/v) and one control (M9 buffer solution). We also transferred 2 μL of concentrated, heat-killed *E. coli* OP50 to the 1 mL of solution in the 24-well plates. Nematodes were reared as for the survival assay, except that only a single L4 animal was transferred to each well. At 24 h intervals for 3 days, we counted the accumulated number of individuals in each well for each of eight strain–treatment replicates. For analysis, we constructed a multiple regression model in JMP with lifetime progeny described as a function of ethanol treatment, species, latitudinal clade nested within species, strain nested within clade, and one-way interactions of ethanol treatment on the other factors. Post-hoc comparisons of least-square means for significant factors were performed with Tukey HSD multiple-test correction.

Results

Nucleotide variation in a global sample of Caenorhabditis briggsae

Our analysis of 4 kb across six nuclear loci (primarily intron sequence) for the combined data of these 32 new strains and 83 strains from the literature supports the notion of distinct genetic entities in *C. briggsae*, with a strong component of phylogeographic differentiation associated with latitude (Graustein *et al.* 2002; Cutter *et al.* 2006; Dolgin *et al.* 2008). Among the 32 newly isolated strains of *C. briggsae*, 27 fall neatly within previously described phylogeographic groups ('tropical' or 'temperate'). We identified substantial numbers of fixed differences among the groups of strains but failed to identify any shared polymorphisms (Table 4), indicative of very little if any gene flow that is coupled with recombination. Thus, the distinct clusters of multi-locus haplotypes indicate that several lineages of *C. briggsae* have been evolving independently for a long period of time. However, we do find evidence of recombination among individuals within the tropical group of isolates (minimum number of recombination events $R_{min} = 2$) and confirm greater genetic variation for these tropical samples (Table 5).

However, five new strains define the existence of substantially greater sources of intraspecific genetic varia-

Table 4 Per-site silent divergence (D_{xy}, above diagonal) and number of fixed differences (below diagonal) between samples from different regions for the six nuclear loci combined

	Tropical	Temperate	Nairobi	Montreal	Kerala*
Tropical (n = 42)		0.00466	0.00564	0.00461	0.01620
Temperate (n = 60)	12		0.00682	0.00569	0.01692
Nairobi (n = 8)	19	22		0.00570	0.01523
Montreal (n = 2)	14	19	26		0.01462
Kerala* (n = 3)	50	56	56	50	

*Only the three highly differentiated isolates are designated 'Kerala', other Indian isolates are designated 'Tropical'.

Table 5 Sample and diversity summary for each *Caenorhabditis briggsae* multi-locus haplotype group

Group	n	H	S	π_{si} (%)	θ_{si} (%)
Tropical	42	15	24	0.128	0.165
Temperate	60	5	9	0.011	0.059
Nairobi	8	2	1	0.008	0.012
Montreal	2	1	0	0.000	0.000
Kerala*	3	2	4	0.081	0.081
Total	115	25	101	0.450	0.637

S, number of segregating sites; H, number of haplotypes.
*Only the three highly differentiated isolates are designated 'Kerala', other Indian isolates are designated 'Tropical'.

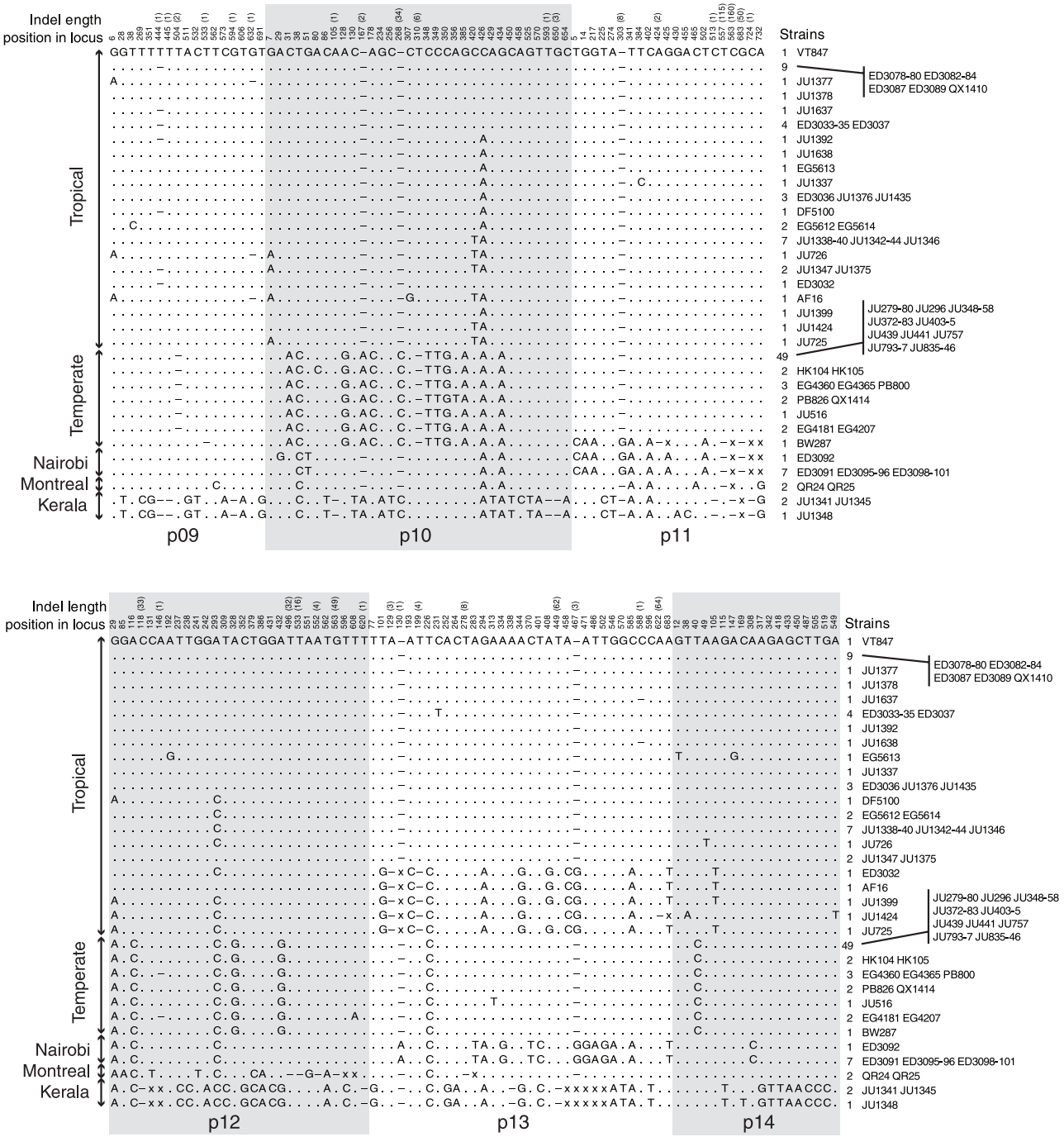

Fig. 1 Polymorphic sites and indels for haplotypes of a global sample of *Caenorhabditis briggsae* in six nuclear loci. Upper panel corresponds to the three loci linked to chromosome II; bottom panel contains the three loci predicted to be linked to the X chromosome. Differentiated multi-locus haplotype groups are indicated on the left axis as in Fig. 2. The numbers of strains per haplotype, and the strain names, are indicated along the right. Dots represent variants identical to the reference strain at top; dashes indicate the presence of microsatellite or indel polymorphisms; 'x' indicates that an indel overlaps with the position rendering site identity not applicable. Due to missing data for some samples, the numbers of segregating sites may differ from those used for analysis in the Tables. The length of microsatellite or indel polymorphisms and the positions of polymorphic sites within the sequence alignment are indicated along the top.

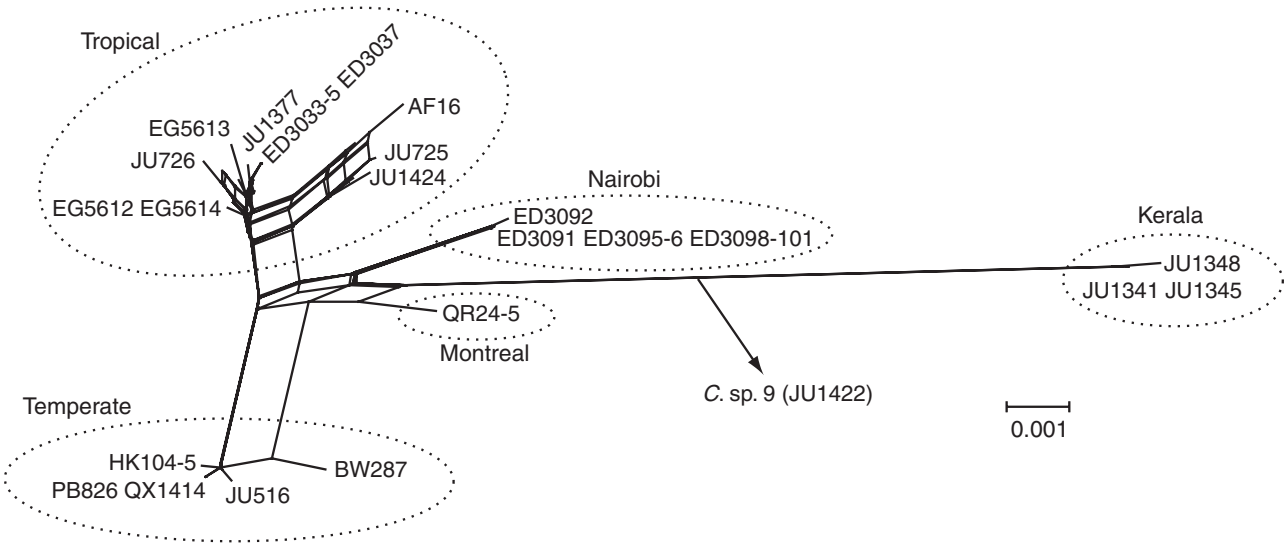

Fig. 2 Neighbour-network for global sample of *Caenorhabditis briggsae*, based on concatenated sequence for six nuclear loci. Nucleotide distance (Jukes-Cantor) excludes gaps; reticulation indicates potential recombination in the ancestry of the multi-locus genotypes. Major clades are enclosed within dotted circles with labels used in Fig. 1 and Table 2. A subset of strains are indicated on the network for reference, including the position leading to the outgroup strain JU1422 for *C.* sp. 9.

tion in *C. briggsae* than previously characterized. Three strains collected in Kerala, India, exhibit strikingly different haplotypes than all other isolates of *C. briggsae* for all loci (Figs 1 and 2), with an average divergence of ~1.5% at silent sites relative to other groups of haplotypes (cf. ~0.5% divergence between other pairs of haplotype groups; Table 3). Mating tests confirm that these genetically distinct strains of hermaphrodites are indeed conspecific with *C. briggsae*, so their coincident collection with several other strains from Kerala, India (Table 1), that cluster genetically with the 'tropical' samples raises the question of how they have retained their genetic distinctiveness.

Another two strains collected in Montreal, Canada, also harbour distinct multi-locus haplotypes (Figs 1 and 2), with little genetic affinity with other strains found in temperate latitudes. The strain BW287 collected from Beijing, China, presents a final interesting exception to the general phylogeographic patterns of other strains. The haplotype for locus p11 (on chromosome II) for BW287 is very similar to that of strains from Nairobi, Kenya, whereas the five other loci for this strain show clear affinity with other temperate-latitude strains (Fig. 1). This suggests that BW287 likely experienced recombination between these distinct haplotype groups in its history (Fig. 2). Similarly, discrepancy between nuclear and mitochondrial patterns suggests recombination between distinct phylogeographic groups for JU1347 collected in southern India (Raboin *et al.* in review). The cases of the Montreal (QR24, QR25) and Beijing (BW287) isolates suggest that successful migration of *C. briggsae* around the globe might be occurring more frequently than appreciated previously. Given the high differentiation and linkage disequilibrium among multi-locus haplotypes, we speculate that such migration might represent a relatively recent phenomenon, perhaps mediated by humans or other vectors of dispersal.

Sequences for homologous loci in the undescribed *C.* sp. 9, the closest known relative of *C. briggsae* (K. Kiontke, personal communication), indicate that these two taxa are substantially differentiated. Synonymous-site divergence for the short fragments of coding sequence across the six loci considered here yield average K_s =0.187 (Jukes-Cantor corrected; 789 coding nucleotides, of which 155.7 are synonymous). For three loci with alignable intron sequence (p11, p12, p14), we observe an average divergence at silent sites of K_{si} = 0.11 (Jukes-Cantor corrected; 1454.5 silent sites). We note, however, that intron regions of three other loci were largely un-alignable, possibly due to large indels, but also possibly indicating greater sequence divergence than implied by the alignable sites. Additionally, we have not adjusted the synonymous-site divergence for any potential effects of codon bias in this small sample of loci. In any case, this interspecific divergence is ~10 times less than observed between any other species pairs in this genus (Cutter 2008). Using *C.* sp. 9 as outgroup, we conclude that the strains corresponding to the new Kerala, India, set of *C. briggsae* multi-locus haplotypes likely hold a basal position within *C. briggsae* (Fig. 2).

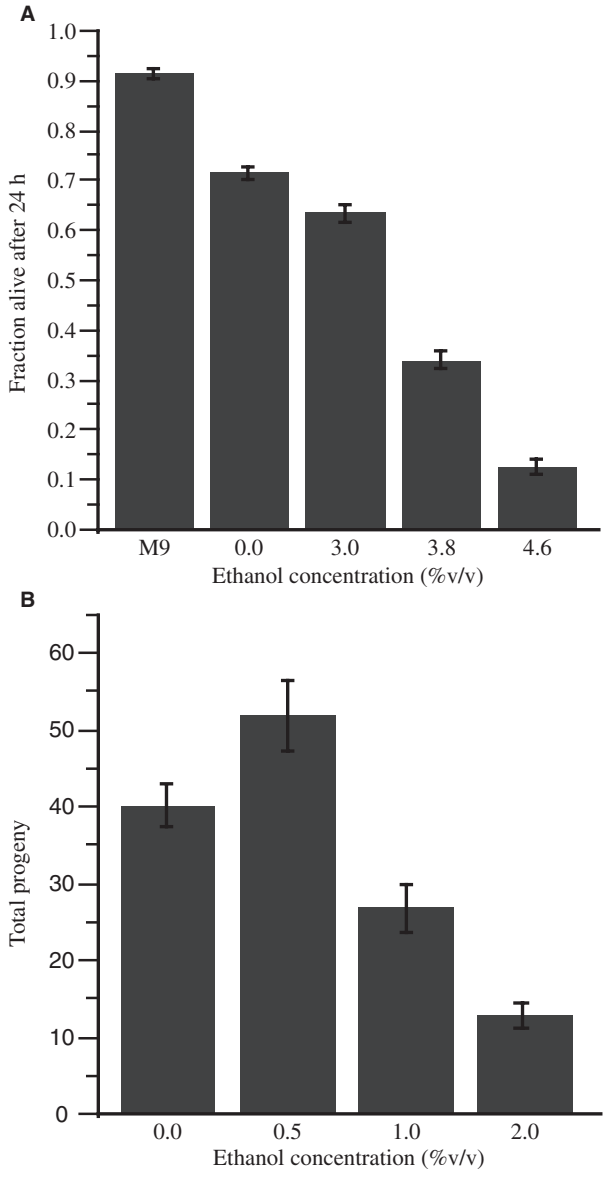

Fig. 3 Survival (A) and reproductive output (B) of *Caenorhabditis briggsae* in varying ethanol concentrations, averaged across nine strains of *C. briggsae*. In the survival assay ethanol diluted in water was used whereas in the fecundity assay ethanol diluted in M9 buffer was used. Error bars indicate ±1 SEM.

Phenotypic sensitivity of C. briggsae *to ethanol*

Across strains, survival declines significantly with increasing ethanol concentrations, with only 13% of animals surviving 24 h in 4.6% ethanol vs. 92% survival in M9 saline buffer (ANOVA $F_{54,381} = 29.3$, $P < 0.0001$, treatment effect-test $F_{4,431} = 222.2$, $P < 0.0001$; Fig. 3A). However, our ANOVA model failed to identify significant heterogeneity among genetic backgrounds in the effect of ethanol on survival (Fig. 4B). This lack of heritable differences extended to different strains within species ($P = 0.64$), different latitudinal groups of strains ($P = 0.66$), and different species ($P = 0.8$).

We also observed detrimental effects of high ethanol concentrations on reproductive output, such that worms produced nearly a third fewer progeny when exposed to 2% ethanol as in saline buffer over the course of 96 h (ANOVA $F_{43,303} = 5.26$, $P < 0.0001$, treatment effect-test $F_{3,343} = 22.7$, $P < 0.0001$; Fig. 3B). However, a low 0.5% concentration of ethanol generated a modest increase in progeny production relative to the saline buffer control (Tukey HSD treatment differences: 0.5% > 0% = 1% > 2%; Fig. 3B), similar to a trend seen for low ethanol concentrations in *C. elegans* (Dhawan *et al.* 1999). Strains differed significantly from one another in their interaction with ethanol concentration ($P = 0.012$), though we did not detect significant differences between species or among clades of *C. briggsae*. The only clear trend among the inter-strain variation in response to ethanol concentration is that temperate strain PB826 exhibits significantly higher fecundity than many other strains at low concentrations of ethanol (0.5%, 1%; Fig. 4B).

Discussion

Most strains of *Caenorhabditis briggsae* found around the globe conform well to a simple latitudinal pattern of biogeographic segregation: individuals from temperate latitudes or from tropical latitudes (on either side of the equator) each share similar genealogical histories. However, here we demonstrate that the extent of molecular diversity in *C. briggsae* is substantially greater than previously recognized. Curiously, none of the samples from small islands is the source of the greater genetic novelty, despite over one-third of the new samples coming from islands. Instead, we recover three isolates from Kerala, India, with multi-locus haplotypes that differ substantially from all previously described individuals of this species (roughly 1.5% silent-site divergence with other haplotype groups), yet they were found in comparable habitat and localities as some other individuals with haplotypes that conform to the tropical-latitude group. An additional two strains from Montreal, Canada, also exhibit novel multi-locus haplotypes that ally more closely to strains from equatorial Nairobi, Kenya, and the divergent Kerala strains than they do to other temperate samples. Given this additional phylogeographic diversity and complexity, we refer to distinct haplotype groups represented by unique locations of origin by the location name (e.g. Nairobi, rather than 'equatorial' as in Dolgin *et al.* 2008), and reserve general names (e.g. 'temperate' and 'tropical') for those haplotype groups with isolates from a diversity of sampling locations. We uncover extensive differentiation

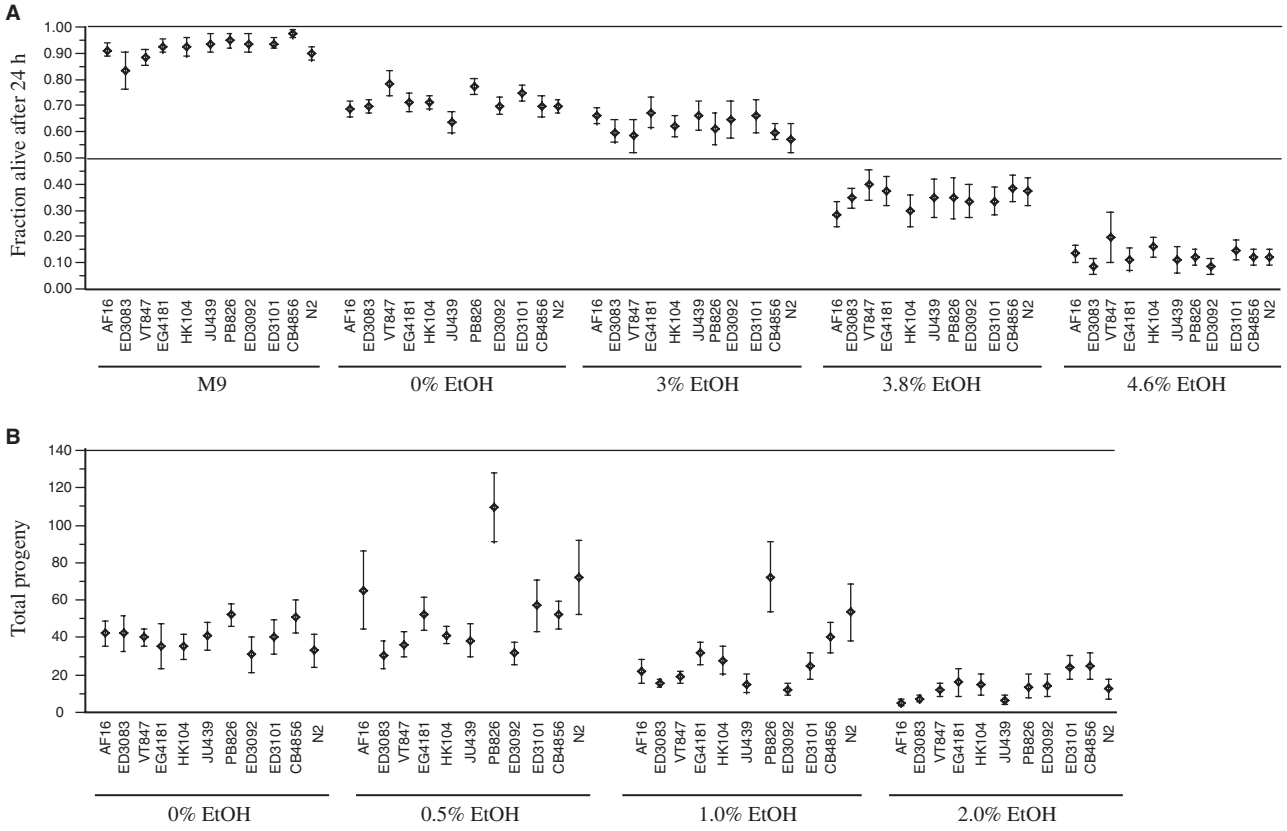

Fig. 4 Effects of different ethanol concentrations on the survival (A) and reproduction (B) of each strain tests for *Caenorhabditis elegans* (N2, CB4856) and *C. briggsae* (other strains).

and no shared polymorphism among the major groups of multi-locus haplotypes, and only a single potential instance of recombination between nuclear loci of divergent lineages. Thus, the strains comprising the major multi-locus haplotype groups appear to represent lineages that have evolved independently for a substantial period of time. Indeed, the close physical proximity of 'tropical' strains and the divergent strains from Kerala, India, beg the question of how they have maintained their genetic distinctness, particularly given that some recombination is evident within the 'tropical' strains. We note that mitochondrial molecular data from a separate study suggest that strain JU1347 from southern India might represent a recombinant genome between the tropical and Kerala multi-locus haplotypes reported here (Raboin *et al.* in review). Further work is required to determine whether local adaptation or partial reproductive isolation might represent underlying causes, or whether the strong inter-chromosomal linkage disequilibrium simply reflects the highly selfing mode of reproduction (Cutter *et al.* 2006).

The identification of more divergent genetic lineages of *C. briggsae* indicates that the evolution of self-fertilizing hermaphroditism originated longer ago than implied by previous population data. Applying a molecular clock to the divergence among lineages of *C. briggsae*, with a mutation rate in *C. briggsae* twice that of *C. elegans* (Baer *et al.* 2005; Denver *et al.* 2009; Phillips *et al.* 2009), we estimate using MCMCcoal (Rannala & Yang 2003) that the temperate, tropical and Kerala samples shared a common ancestor roughly 8.92×10^5 generations ago (95% confidence interval 6.06×10^5–1.22×10^6 generations ago; ~89 200 years ago, assuming 10 generations per year). Because all of the *C. briggsae* strains share the same derived mode of reproduction (Kiontke & Fitch 2005), this implies that selfing hermaphroditism arose in this species at least $\sim 8.92 \times 10^5$ generations ago. Temperate latitude strains may have expanded in this part of the world only very recently (Cutter *et al.* 2006), so a potentially longer generation time experienced in temperate regions should not greatly impact the conversion of time units from generations to years. In addition, divergence of *C. briggsae* from obligately outbreeding species *C.* sp. 9 allows us to estimate an upper-bound on the origin of selfing hermaphroditism. Assuming that these species share the same mutation rate (K_{si} = 2 μT; silent-site divergence K_{si} = 0.11, neutral mutation rate per site per

generation $\mu = 5.4 \times 10^{-9}$, divergence time T), then sequence divergence at silent sites between them suggests that they share a common ancestor in the order of 10.2×10^6 generations ago (~1 million years ago, assuming 10 generations per year). These estimates of the duration of selfing hermaphroditism, while with broad confidence limits, are comparable to estimates for *Arabidopsis thaliana* (Shimizu *et al.* 2004; Tang *et al.* 2007) but much longer ago than for *Capsella rubella* (Foxe *et al.* 2009). Previous attempts to infer the age of hermaphroditism in *C. elegans* and *C. briggsae*, based on rates of decay in codon usage bias, were substantially coarser (Cutter *et al.* 2008). Finally, we point out that the modest level of sequence differentiation we observe between *C. briggsae* and *C.* sp. 9 implies that the methods of divergence population genetics may be applied successfully to these taxa – permitting a range of molecular tests of evolution previously unavailable in this genus, due to the high sequence divergence (saturation at silent sites) among other known species (Cutter *et al.* 2009).

Compared to obligately outbreeding relatives, natural phenotypic variation with a heritable basis in *C. elegans* is limited (Jovelin *et al.* 2003), and here we demonstrate that the highly selfing *C. briggsae* also exhibits limited heritable phenotypic variation in sensitivity to ethanol. Moreover, we find little evidence for differentiation between these two species in their ethanol sensitivity, at least in terms of its gross effects on survival and reproduction. Thus, if environmental concentrations of ethanol in the habitats typically experienced by *C. briggsae* (and *C. elegans*) varies in a consistent manner with geography, it does not appear to have yielded a differential evolutionary response for nematodes from different regions of the world. Ethanol concentrations in rotting fruits vary over a fairly broad range (Omura & Honda 2003), so it is conceivable that this could contribute to the range of rotting habitats available for exploitation by *C. elegans* and *C. briggsae*. Given the similarity in responses between these species, we suspect that assays would prove comparable with the newly identified strains with divergent haplotype groups within *C. briggsae*, although future work should compare representatives from all of these major haplotype groups. Here we focused on the gross effects that ethanol exerts on survival and reproduction; it is possible that more subtle phenotypes, such as motility and taxis behaviour, might reveal heritable differences among strains in their sensitivity to ethanol that, in nature, would allow the animals to avoid experiencing the negative effects of this toxin on survival and reproduction.

Despite the lack of a strong signal of phenotypic differentiation among strains or among genealogically separated groups of strains, we quantify for the first time the reaction norm for survival and fecundity in response to ethanol for *C. briggsae*. Higher concentrations of ethanol induced greater mortality and reduced production of progeny. One exception to this general, and expected, trend is that low ethanol concentrations induce hermaphrodites to produce more offspring than control conditions that lack ethanol. Previous experiments with *C. elegans* showed a similar trend for low ethanol concentrations on fecundity, although the difference was not significant (Dhawan *et al.* 1999). This effect could be a physiological by-product, if low levels of ethanol adversely affect the nervous system to make 'errors of judgement' in reproduction. Alternatively, in nature, low levels of ethanol might act as a cue for a high quality habitat that contains a proliferating microbial food resource, which might trigger increased reproductive output in the nematodes. Low concentrations of ethanol induce hyperactivity in *C. elegans* (Morgan & Sedensky 1995; Dhawan *et al.* 1999). However, it remains to be determined in an ecological context whether hyperactivity might represent an escape response or attraction, although as a volatile, ethanol is only weakly attractive (Bargmann *et al.* 1993). Moreover, low concentrations of ethanol also exert a beneficial effect on species of *Drosophila* (Parsons *et al.* 1979), suggesting that this may be a widespread pattern among animals that live within rotting fruit habitats.

Conclusions

By identifying substantially greater genetic differentiation in the wild than previously recognized, as well as testing for heritable phenotypic variation, we have expanded the potential of *Caenorhabditis briggsae* as an emerging model for studies in molecular population genetics, quantitative genetics, the evolution of development and ecological genetics (Wang *et al.* 2004; Baird *et al.* 2005; Baird & Chamberlin 2006; Hill *et al.* 2006; Hillier *et al.* 2007; Dolgin *et al.* 2008). Although we do not detect strong patterns of heritable phenotypic variation in the sensitivity of *C. briggsae* to a potentially ecologically important toxin – ethanol – our quantification of the norms of reaction in survival and reproduction nevertheless provides important new data about fitness-related traits in this organism. However, our molecular analysis of divergent lineages within *C. briggsae*, coupled with comparison to a newly discovered close relative that reproduces via obligate outcrossing of males and females (*C.* sp. 9), allowed us to narrow dramatically the estimated time since selfing hermaphroditism evolved (Cutter *et al.* 2008). These insights into the natural history of *C. briggsae*, in combination with its experimental tractability and genomic resources, make this organism excep-

tionally attractive for expanding studies of a variety of problems in ecology and evolution.

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Research in Asher Cutter's laboratory addresses genome evolution and the evolution of breeding systems, reproductive isolation, and adaptation in nematodes, focusing on population genetic and molecular evolutionary approaches.

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