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Evolutionary biology

Temperature, stress and spontaneous mutation in *Caenorhabditis briggsae* and *Caenorhabditis elegans*

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Mutation rate often increases with environmental temperature, but establishing causality is complicated. Asymmetry between physiological stress and deviation from the optimal temperature means that temperature and stress are often confounded. We allowed mutations to accumulate in two species of *Caenorhabditis* for approximately 100 generations at 18° and for approximately 165 generations at 26°; 26° is stressful for *Caenorhabditis elegans* but not for *Caenorhabditis briggsae*. We report mutation rates at a set of microsatellite loci and estimates of the per-generation decay of fitness (ΔM_w), the genomic mutation rate for fitness (U) and the average effect of a new mutation ($E[a]$), assayed at both temperatures. In *C. elegans*, the microsatellite mutation rate is significantly greater at 26° than at 18° whereas in *C. briggsae* there is only a slight, non-significant increase in mutation rate at 26°, consistent with stress-dependent mutation in *C. elegans*. The fitness data from both species qualitatively reinforce the microsatellite results. The fitness results of *C. elegans* are potentially complicated by selection, but also suggest temperature-dependent mutation; the difference between the two species suggests that physiological stress plays a significant role in the mutational process.

1. Introduction

The relationship between metabolic rate, mutation and molecular evolution has generated much interest [1–3]. Competing hypotheses attribute the relationship to (i) generation time or (ii) mutagenic by-products of cellular metabolism. However, other factors covary with generation time and metabolic rate, including body size, life history, population size and temperature, all of which potentially influence rate of evolution for reasons not causally related to metabolic rate or generation time. In particular, the mutagenic effects of high temperature are well-documented [4]. However, many studies that identify a relationship between temperature and mutation may confound temperature with physiological 'stress'. Several lines of evidence suggest that physiological stress is mutagenic [5–9], and that an upward deviation from an optimum temperature is often more stressful than an equivalent downward deviation [10]. Thus, temperature dependence of mutation rate may be an indirect effect of stress rather than be a direct effect or be an effect mediated by metabolism.

To begin to disentangle the direct effects of temperature from those of its correlates, we allowed mutations to accumulate under relaxed selection ('mutation accumulation', MA) in two species of nematodes, *Caenorhabditis briggsae* and *C. elegans*, at 18°C and 26°C, for 103 and approximately 165 generations, respectively. The different temperatures are differently stressful for the

two species; absolute fitness of *C. briggsae* at 18°C is about 60 per cent of that at 26°C, whereas absolute fitness of *C. elegans* at 26°C is about one-third of that at 18° (electronic supplementary material, table S3). Mutation rate was assessed directly by genotyping a set of microsatellite loci chosen for their predicted high mutation rate. The cumulative effects on fitness were assessed by comparing MA lines to the cryopreserved common ancestor(s) at both temperatures. This design controls for selection mediated by population size and body size, and generation times and times of divergence are known, as is the relative degree of physiological stress. If the sole effect of temperature on the evolutionary process is via mediation of generation time, the per-generation mutation rate should not differ between the two MA temperatures. Conversely, if temperature affects the mutation process in other ways, the per-generation rate may differ between the two MA temperatures. If physiological stress is important, the relationship of mutation with temperature should differ predictably between the two species, with *C. elegans* having the higher mutation rate at 26°C.

2. Material and methods

(a) Mutation accumulation and fitness assay

The MA protocol follows Baer *et al*. [11]; see electronic supplementary material, text S1. We initiated two sets of 192 replicate MA lines from the N2 strain of *C. elegans* and from the PB800 strain of *C. briggsae*; 96 lines were kept at 18°C and 96 at 26°C. Lines were maintained by transfer of a single hermaphrodite for 103 generations at 18°C for each strain and for 164 generations at 26°C in N2 and 171 generations in PB800.

Fitness assays also follow Baer *et al* [11]; see electronic supplementary material, text S2. Fitness was assayed in two blocks; 30 MA lines from each strain/MA temperature were randomly selected for each block, along with ancestral controls. Fifteen thawed worms were picked from each control and used to establish replicate control 'pseudolines'. From each line, seven replicates were assayed for lifetime reproduction at 26°C and five at 18°C.

(b) Microsatellite genotyping

Sixteen $AG_{(n)}$ loci ≥ 9 repeats were selected from the upper 5 per cent of the length distribution in each species and matched for repeat number as closely as possible. DNA extraction, amplification and genotyping follow Phillips *et al*. [12]. All surviving MA lines and their ancestral controls were genotyped at all loci. We found no cases of putative heterozygotes in either ancestor. Homozygous genotypes different from wild-type were re-amplified and re-genotyped for confirmation. Details of locus choice, primer design and genotyping are given in the electronic supplementary material, text S3 and table S1.

(c) Data analysis

(i) Microsatellites

Mutation rate is calculated as $\mu = n/lt$, where n is the number of mutations, l is the number of MA lines and t is generations of MA [12]. Because number of generations differs between the two MA temperatures, comparisons must be of mutation rates rather than of numbers of mutations. Within species, mutation rates at 18°C and 26°C were compared by paired-sample Wilcoxon signed-rank test; each locus at the two temperatures provides the paired observations. Indel spectra between temperatures and species were compared via a 2×2 contingency table, pooling over loci.

(ii) Fitness

Relative fitness (w) is defined by the following equation

$$w = \sum_x e^{-r_0 x} l_x m_x,$$

where $l_x m_x$ is the product of survivorship and fecundity at day x and r_0 is the mean intrinsic rate of increase of the G0 control, calculated by solving the following equation

$$\bar{w}_0 = \sum_x e^{-r_0 x} \overline{l_x m_x} = 1,$$

using the average l_x and m_x values of all control lines in an assay block. The per-generation change in the trait mean, $\Delta M_w = U \times E[a]$, where U is the genome-wide mutation rate and $E[a]$ is the average effect of a mutation on the trait [13]. Ancestral relative fitness w_0 is defined equal to 1, so

$$\Delta M_w = \frac{w_{MA} - w_0}{w_0 t} = \frac{w_{MA} - 1}{t}.$$

We generated 1000 bootstrap replicates (resampling lines) to estimate ΔM_w for each strain/MA temperature/assay temperature group, maintaining block structure and averaging over blocks. ΔM_w is considered to differ significantly between groups if the empirical 95% confidence limits of the groups do not overlap. See electronic supplementary material, text S4 for details.

The ratio of (twice) the squared change in the trait mean (ΔM) to the per-generation increase in the among-line variance (the mutational variance, V_M) provides a downwardly biased estimate of the genomic mutation rate U and $V_M/2\Delta M$ provides an upwardly biased estimate of the average mutational effect, $E[a]$, the 'Bateman–Mukai' estimators U_{min} and $E[a]_{max}$ [13]. V_M, U_{min} and $E[a]_{max}$ were calculated from the resampled data described earlier; details and some caveats are provided in the electronic supplementary material, text S5 and S6. The limitations of the B–M method are well-appreciated [14].

3. Results

(a) Microsatellites

The complete data are in electronic supplementary material, table S2. In *C. briggsae*, the mutation rate per-generation does not differ significantly between MA18 and MA26 treatments (table 1) and is very similar to a previous estimate from the same set of loci from PB800 MA lines propagated at 20°C (2.13×10^{-4} per generation; [12]). In contrast, in *C. elegans*, the per-locus mutation rate in the MA26 lines is greater than in the MA18 lines (one-tailed $p < 0.006$). One *C. elegans* in MA26 line (line 421) had an atypically high number of mutations (9/15 loci; the next highest number of mutations per line is 3/15). With line 421 omitted, the difference between the two MA treatments is smaller (1.96 : 1 versus 2.50 : 1) but remains significant (one-tailed $p < 0.05$). Because the sets of loci are not orthologous in the two species and were not chosen at random, formal comparison between species is inappropriate. The approximately twofold greater mutation rate in *C. briggsae* than *C. elegans* in the MA18 treatment (2.65 : 1) is consistent with a previous estimate in which mutations accumulated at 20°C (2.27 : 1; [12]).

Table 1. Microsatellite mutations. Locus ID, arbitrary identifier; *N* repeats, number of AG repeats; *N* lines, number of MA lines genotyped; Ins 18(26), number of insertions; Del 18(26), number of deletions; $\mu_{18}(26)$, per-generation mutation rate $\times 10^4$. Averages are means, s.e.m. in parentheses.

species	locus ID	*N* repeats	*N* lines, 18°C	Ins 18	Del 18	μ_{18} ($\times 10^4$)	*N* lines, 26°	Ins 26	Del 26	μ_{26} ($\times 10^4$)
Caenorhabditis	17/18	30.5	96	0	0	0	92	0	2	1.26
briggsae	35/36	14	96	2	6	8.09	93	10	13	1.43
	39/40	16.5	96	0	0	0	92	2	1	1.89
	47/48	19.5	92	1	1	2.11	92	2	0	1.26
	61/62	22	96	0	0	0	92	0	0	0
	63/64	19	96	0	0	0	93	0	0	0
	73/74	21	96	0	0	0	93	0	1	0.62
	77/78	22	96	0	0	0	92	0	1	0.63
	79/80	13	96	2	1	3.03	93	1	1	1.24
	89/90	21.5	95	1	4	5.11	92	3	12	9.43
	91/92	19	96	0	0	0	91	0	1	0.64
	97/98	27.5	95	2	3	5.11	92	5	4	5.66
	99/100	9	91	0	0	0	93	1	0	0.62
	113/114	17.5	96	0	0	0	88	0	0	0
	115/116	28.5	96	1	8	9.10	91	3	5	5.09
	117/118	15.5	95	0	3	3.07	92	0	2	1.26
	average	19.75	95.3			2.23 (0.78)	91.9			2.74 (1.01)
Caenorhabditis	16	19	93	0	0	0	77	1	1	1.58
elegans	17	21.5	94	1	0	1.03	76	3	0	2.41
	19	24	94	1	0	1.03	77	4	0	3.17
	20	25	93	0	1	1.04	77	5	2	5.54
	36	15.5	94	2	0	2.07	77	0	0	0
	37	18.5	81	0	0	0	65	0	0	0
	38	25	94	0	0	0	77	2	1	2.38
	39	26	94	2	0	2.07	77	1	2	2.38
	40	29.5	94	1	0	1.03	77	3	2	3.96
	64	18.5	92	0	0	0	74	1	0	0.82
	65	19.5	93	0	0	0	77	2	0	1.58
	67	23.5	93	1	0	1.04	75	1	0	0.81
	68	26	80	0	1	1.21	64	1	3	3.81
	69	23.5	93	1	1	2.09	75	1	1	1.63
	70	28	94	0	0	0	77	2	0	1.58
	average	23.1	91.7			0.84 (0.21)	74.8			2.11 (0.40)

In *C. briggsae*, deletions are more common than insertions, whereas in *C. elegans* insertions predominate (table 1). The difference between species in the direction of indel bias is similar at both temperatures, is highly significant (likelihood-ratio χ^2, $p < 0.001$) and is consistent with previous results [12].

(b) Fitness

Results are summarized in table 2; detailed results are presented in the electronic supplementary material, tables S3 and S4. There are three relevant two-way comparisons: between MA treatments within an assay temperature/species; between assay temperatures within a MA treatment/species; and between species within a MA treatment/assay temperature. The species evolve qualitatively differently: in *C. briggsae*, on average, MA26 lines decline in fitness (ΔM_w) about twice as fast as MA18 lines, and the result is consistent across assay temperatures. In contrast, in *C. elegans*, ΔM_w is about two times larger when fitness is assayed at 26°C than at 18°C. However, there is a substantial variation between blocks, and the differences between groups approach significance ($p \sim 0.05$) only between the *C. briggsae* MA18 and MA26 lines when assayed at 26°C.

Table 2. Mutational parameters. ΔM_w, per-generation change in relative fitness (95% confidence interval); V_M, per-generation mutational variance (95% confidence interval); U_{min}, Bateman–Mukai estimate of genomic mutation rate for relative fitness; $E[a]_{max}$, average effect of a new mutation on relative fitness. See §2 and electronic supplementary material text for details of calculations.

species	Trt	assay 18				assay 26			
		ΔM_w ($\times 10^3$)	V_M ($\times 10^4$)	U_{min}	$E[a]_{max}$	ΔM_w ($\times 10^3$)	V_M ($\times 10^4$)	U_{min}	$E[a]_{max}$
Caenorhabditis briggsae	MA18	−0.93 (−1.82, 0.06)	2.47 (0.81, 4.60)	0.007	−0.133	−1.10 (−1.69, −0.54)	0.83 (0.05, 1.78)	0.029	−0.038
	MA26	−1.83 (−2.48, −1.19)	2.89 (1.68, 4.29)	0.023	−0.079	−2.19 (−2.79, −1.64)	2.70 (1.79, 3.60)	0.035	−0.062
Caenorhabditis elegans	MA18	−0.97 (−2.39, 0.53)	2.44 (0.28, 5.02)	0.008	−0.126	−2.66 (−3.87, −1.27)	1.29 (0, 4.41)	0.110	−0.024
	MA26	−1.25 (−2.05, −0.33)	1.04 (0, 2.50)	0.030	−0.042	−2.44 (−3.05, −1.69)	0.23 (0, 1.18)	0.491	−0.005

Two broad patterns emerge from the B–M estimates. First, in both species at both assay temperatures, U_{min} is greater in MA26 than MA18, and second, in every case $E[a]_{max}$ is smaller when assayed at 26°C than at 18°C, although the differences between assay temperatures are trivial in the *C. briggsae* MA26 lines.

4. Discussion

Three features of the microsatellite results are consistent with previous findings: (i) at cool, non-stressful temperatures (20°C in Phillips *et al*. [12] and 18°C here), the per-locus mutation rate of *C. briggsae* is about twice that of *C. elegans*; (ii) those rates are similar in the two studies; and (iii) the two species differ consistently in the direction of indel bias. The significantly higher per-generation mutation rate in the *C. elegans* MA26 lines compared with the MA18, but the lack of a similar difference in *C. briggsae* has two implications. First, it strongly suggests that there is not a universal relationship between metabolic rate and mutation rate as implied by proponents of a 'global molecular clock' [2]. Second, it implicates physiological stress as a cause of elevated mutation rate. Agrawal and his co-workers [6,9] have convincingly demonstrated that *Drosophila melanogaster* in poor condition accumulate mutations more rapidly than flies in good condition, and that a likely cause is the preferential use of an error-prone DNA repair mechanism by individuals in poor condition. Moreover, Muller [4] observed an almost identical, twofold difference in lethal mutation rate between *D. melanogaster* maintained at 26.5°C—near the upper limit of *D. melanogaster*'s thermal tolerance—and flies maintained at 19°C.

Taken as a whole, the fitness data suggest that (i) mutations do accumulate at least a little faster at high temperature and (ii) more mutations with smaller effects contribute to the mutational decay of fitness at 26°C than at 18°C. Thus, there is evidence that high temperature *per se* is mutagenic to some extent. The pattern is much more pronounced in *C. elegans* than in *C. briggsae*, which further suggests that physiological stress either exacerbates the mutagenic effects of temperature or is itself mutagenic. These results are quite consistent with the microsatellite data.

Two additional observations suggest that some aspect of the mutational process in *C. elegans* is temperature-dependent. First, many more *C. elegans* MA26 lines were lost during the MA phase than in the other groups (19/96 versus $\leq$3/96). Second, N_e in the *C. elegans* MA26 lines was $\approx$ 2, whereas in the other three groups $N_e \approx 1$, the result of having to use backups more frequently due to much higher mortality (electronic supplementary material, text S1). These results suggest that the larger N_e in the MA26 lines leads to a class of mutations that are effectively neutral ($4N_e s < 1$) in the MA18 lines that are purged by selection in the MA26 lines. Mutations in this window ($12.5\% < s < 25\%$) contribute significantly to the mutational decay of fitness in *C. elegans* [15]; note that in both species, $E[a]_{max}$ in MA18 assayed at 18°C is approximately 13 per cent (table 2). Taken together, the evidence suggests that the mutation rate is much more strongly temperature-dependent in *C. elegans* than in *C. briggsae*, which in turn suggests a predominant role for physiological stress in the mutational process.

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