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Patterns of Molecular Evolution in Caenorhabditis Preclude Ancient Origins of Selfing

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ABSTRACT

The evolution of self-fertilization can mediate pronounced changes in genomes as a by-product of a drastic reduction in effective population size and the concomitant accumulation of slightly deleterious mutations by genetic drift. In the nematode genus Caenorhabditis, a highly selfing lifestyle has evolved twice independently, thus permitting an opportunity to test for the effects of mode of reproduction on patterns of molecular evolution on a genomic scale. Here we contrast rates of nucleotide substitution and codon usage bias among thousands of orthologous groups of genes in six species of Caenorhabditis, including the classic model organism *Caenorhabditis elegans*. Despite evidence that weak selection on synonymous codon usage is pervasive in the history of all species in this genus, we find little difference among species in the patterns of codon usage bias and in replacement-site substitution. Applying a model of relaxed selection on codon usage to the *C. elegans* and *C. briggsae* lineages suggests that self-fertilization is unlikely to have evolved more than ~4 million years ago, which is less than a quarter of the time since they shared a common ancestor with outcrossing species. We conclude that the profound changes in mating behavior, physiology, and developmental mechanisms that accompanied the transition from an obligately outcrossing to a primarily selfing mode of reproduction evolved in the not-too-distant past.

LONG-term reductions in effective population size (N_e) will result in pronounced shifts in molecular evolution across the genome. Specifically, a reduction in effective population size causes an increased role of genetic drift relative to selection, leading to the fixation of slightly deleterious mutations that would otherwise be eliminated, or kept at low frequency, by purifying selection (Kimura 1968). Provided that sufficient time has elapsed, the accumulation of such deleterious mutations should manifest in coding sequences as an elevated rate of replacement-site substitution (Popadin *et al.* 2007) and as a decline in codon usage bias (Akashi 1995; Kreitman and Antezana 1999) and eventually genome degeneration (Mira *et al.* 2001) or even extinction (Lynch and Gabriel 1990). Small population size might also facilitate intron evolution (Lynch and Conery 2003), and insertion/deletion mutational biases will more strongly influence intron size in small populations (Mira *et al.* 2001). Genomic features that are shaped by the weakest intensities of selection, such as the selection for translational efficiency and/or accuracy that results in biased usage of synonymous codons, should be most sensitive to changes in population size because they are the most susceptible to shifting into an effectively neutral state. However, because it is genetic drift due to relaxed selection that causes these phenomena, they will occur slowly, at a rate on the order of the mutation rate (Marais *et al.* 2004a). Here, we investigate the potential for differences in population size, mediated by reproductive mode (selfing *vs.* outcrossing), to have affected genomic patterns of molecular evolution throughout the nematode genus Caenorhabditis.

The onset of a self-fertilizing mode of reproduction is an example of an evolutionary transition that will cause a reduced effective population size compared to the obligately outcrossing ancestor and close relatives (Pollak 1987). This results as a consequence of the direct effects of homozygosity, induced by selfing, and indirectly through reduced effective recombination (Nordborg 2000; Charlesworth and Wright 2001). The strong linkage disequilibrium within selfing species will cause selective sweeps and purifying selection to reduce genetic diversity across large portions of the genome, rather than just at the targets of selection, which further reduces the effective population size (Maynard Smith and Haigh 1974; Charlesworth *et al.* 1993). Additional reductions in the effective size of selfing populations can occur if they tend to have greater metapopulation dynamics (Pannell and Charlesworth 1999; Pannell

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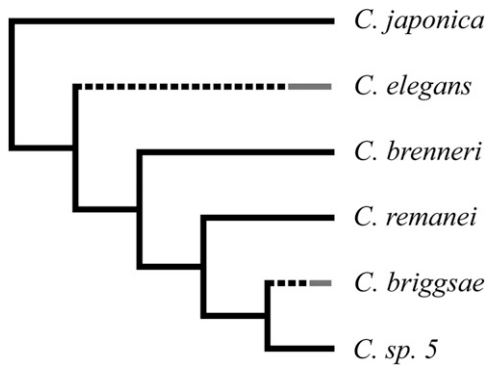


Figure 1.—Phylogenetic topology of Caenorhabditis that is assumed in calculations of lineage-specific divergence from Kiontke *et al.* (2004), Kiontke and Sudhaus (2006), and Kiontke *et al.* (2007). Dashed lines for *C. elegans* and *C. briggsae* indicate that self-fertilization (shaded tips) evolved at some point along these branches. Branch lengths are not to scale.

2003); more generally, the generation of population structure is facilitated by inbreeding and leads to lower effective population sizes locally (Charlesworth *et al.* 1997). The combined action of these forces can result in drastically depressed effective breeding sizes of selfing populations beyond the simple twofold reduction due to elevated homozygosity, with potentially dramatic consequences for genomic patterns of molecular evolution (Charlesworth and Wright 2001; Charlesworth 2003).

Self-fertilization has arisen independently twice within the nematode genus Caenorhabditis (Kiontke *et al.* 2004) (Figure 1), but it remains unknown as to how long this mode of reproduction has persisted in these lineages and whether sufficient time has elapsed for the molecular evolutionary consequences of self-fertilization to become apparent. Surveys of population genetic variation show that the selfing species of Caenorhabditis, *Caenorhabditis elegans* and *C. briggsae*, do have lower polymorphism levels and effective population sizes than the obligate outcrossing *C. remanei* (Graustein *et al.* 2002; Jovelin *et al.* 2003; Sivasundar and Hey 2003; Barrière and Félix 2005, 2007; Cutter 2006; Cutter *et al.* 2006a,b), and observed levels of linkage disequilibrium indicate that the effective selfing rate is exceedingly high (Koch *et al.* 2000; Barrière and Félix 2005; Haber *et al.* 2005; Cutter 2006; Cutter *et al.* 2006b). Studies of polymorphism also have determined that weak selection for preferred codons operates in contemporary populations of *C. remanei*, with the intensity of selection ($N_e s$) estimated to be ~0.1 on average (Cutter and Charlesworth 2006; Cutter 2008b). For *C. elegans*, genomic analyses have demonstrated that selection for translational efficiency and/or accuracy has shaped codon usage in its history (Stenico *et al.* 1994; Duret and Mouchiroud 1999; Duret 2000; Cutter *et al.* 2006c), although the ~20-fold smaller effective sizes of present-day *C. elegans* and *C. briggsae* populations imply that current selection for preferred codons will effectively be absent. The extent of codon usage bias, the conservation of preferred codon identity, and any association of codon usage bias with reproductive mode is presently uncharacterized in Caenorhabditis generally.

In this study, we test the prediction that selfing species will accumulate slightly deleterious mutations more rapidly than obligately outcrossing relatives in six species of Caenorhabditis. We obtain new coding sequences for three species of Caenorhabditis (*C. japonica*, *C. brenneri*, and *C.* sp. 5 strain JU727) through an expressed sequence tag (EST) effort, yielding 4760 unique nuclear-encoded loci. Using the number of EST hits as a measure of gene expression level, we show that codon bias is stronger in highly expressed genes throughout the genus, identify a set of preferred codons that is conserved across species, and find only modest differences among species in overall biases in codon usage patterns. On the basis of a subset of loci for which orthology could be inferred, we compute lineage-specific substitution rates, but find little evidence for differences in rates of protein evolution among lineages. Application of a model of codon bias evolution suggests that it is unlikely that more than ~4 million years have elapsed since the onset of relaxed selection on codon usage in *C. elegans* and *C. briggsae* and, by implication, the origin of a predominantly self-fertilizing mode of reproduction. We therefore conclude that the novel features involving hermaphroditism and self-fertilization in these species are not ancient in origin.

MATERIALS AND METHODS

Molecular methods: For each of *C. japonica* (strain DF5081), *C. brenneri* (strain CB5161), and *C.* sp. 5 (strain JU727), cDNA libraries were constructed with the BD Biosciences Clontech SMART cDNA library construction kit as per the manufacturer's instructions. This protocol enriches for full-length cDNAs with complete 5′ ends. Total RNA was extracted from populations of worms of mixed stage and mixed sex with a RNeasy mini kit (QIAGEN, Valencia, CA) from which mRNA was isolated with a QIAGEN Oligotex mRNA mini kit, following manufacturer instructions. The libraries were cloned into the λ-TriplEx2 phage vector and plated with *Escherichia coli* strain XL1-Blue for blue-white screening of recombinants. Recombinant plaques were picked into buffer, from which an aliquot was used for PCR amplification with primers TW5 (CTCGGGAAGCGCGCCATTGTGTTGGT) and TW3 (AGGCGGCCGACATGTTTTTTTTTTTT), followed by direct sequencing with primer TW5. Sequencing was performed on ABI 3730 automated sequencers by the University of Edinburgh School of Biological Sciences sequencing service and by the University of Arizona Genome and Technology Core sequencing service. EST sequencing was performed on a total of 42 96-well microtitre plates for *C.* sp. 5, 27 microtitre plates for *C. brenneri*, and 18 microtitre plates for *C. japonica*.

EST analysis: The PartiGene software system (Parkinson *et al.* 2004a) was used to process the raw EST sequence data

to trim low quality sequence, remove contaminating vector sequence, and cluster replicate sequences into a single sequence object. Hereafter, we refer to these clustered sequence objects as "genes," recognizing that in most cases they do not represent full-length coding sequences. The resulting genes were then passed through prot4EST (Wasmuth and Blaxter 2004), a computational pipeline that infers the most appropriate peptide translation, identifies and masks putative insertion/deletion sequencing errors, and identifies putative mitochondrial *vs.* nuclear-encoded loci. This procedure generated 2320 genes (3857 ESTs) for *C.* sp. 5, 1405 genes (2449 ESTs) for *C. brenneri*, and 1073 genes (1906 ESTs, including 218 already present in dbEST) for *C. japonica*, including mitochondrial loci. Of these, 4760 genes in total are putatively nuclear encoded. The resulting sequences have been deposited in GenBank (*C. japonica*, FD512256–FD513938; *C. brenneri*, FD509784–FD512255; and *C.* sp. 5, FD513939–FD517806) and added to Nembase (Parkinson *et al.* 2004b).

For the sequenced genomes, we obtained EST sequences from dbEST for *C. elegans* (346,064), *C. remanei* (28,205), and *C. briggsae* (2517). Using a custom Perl script, we associated these ESTs to corresponding predicted coding sequences from WormBase WS170. After applying the restriction that the BLAST alignment must match at least half the length of the EST with ≥90% identity, we associated 280,966 ESTs with 15,207 genes for *C. elegans*, 15,868 ESTs with 5603 genes for *C. remanei*, and 1798 ESTs with 765 genes for *C. briggsae*. Although predefined associations between EST and coding sequence are publicly available for *C. elegans*, we used the EST hit counts from this procedure to ensure uniform criteria for associating ESTs with genes among these three species.

Analysis of codon usage: We used the species-specific EST counts for each locus as a measure of the expression level for the corresponding locus in each species, for nuclear-encoded loci only (supplemental Data 1). Following Duret and Mouchiroud (1999) and Cutter *et al.* (2006c), we partitioned the 26,390 loci described above into two classes: those that had a single EST "hit" and those with EST "hits" numbering greater than or equal to the 90th percentile (n_{90}). Loci in these categories are taken to represent genes with low and high levels of expression. For *C. japonica*, *C. brenneri*, and *C.* sp. 5, $n_{90} = 3$. We also associated ESTs in dbEST with the predicted coding sequences of *C. elegans*, *C. remanei*, and *C. briggsae* (see above), and partitioned them into two categories as for the other species, with $n_{90} = 37$, 5, and 4, respectively. We then calculated the relative synonymous codon usage (RSCU) separately for the loci in the two expression categories, which for codon j of amino acid i is simply $\mathrm{RSCU}_{ij} = n_{ij}/(1/d_i)\sum_{k=1}^{d_i} n_{ik}$, where d is the degeneracy of the corresponding amino acid and n_{ij} is codon frequency.

To identify those codons that are disproportionately represented in highly expressed genes, termed preferred or optimal codons, we used *t*-tests to test for significant differences in RSCU between high- and low-expression loci (ΔRSCU) using JMP v.5.01. For this analysis, we restricted the data set to those loci with ≥100 codons. Codons experiencing significantly elevated RSCU in highly expressed genes (optimal codons) were subsequently used to calculate the frequency of optimal codons (F_{op}) codon usage bias statistic (Ikemura 1985) with a customized optimal codon table in CodonW (J. Peden, http://codonw.sourceforge.net). The effective number of codons (ENC) (Wright 1990) and base composition at fourfold degenerate sites (GC3s) were also calculated for each locus with CodonW and INCA 2.1 (Supek and Vlahovicek 2004). On the basis of these summary statistics, we constructed an ANOVA model to explain variation in codon bias (F_{op}) as a function of base composition (GC3s), length (log-transformed), and EST hit counts (log-transformed), and their first-order interactions using JMP. A significant positive effect of gene expression (EST hit counts) is consistent with selection having caused codon bias, although this analysis will underestimate the role of selection on codon bias because an association between codon bias and GC3s could result from selection rather than mutational effects. To obtain an overall index of codon usage bias for the species, we report the average of positive ΔRSCU values: $\overline{\Delta\mathrm{RSCU}_+}$ (Cutter *et al.* 2006c). This metric does not depend strongly on genomic base composition or on the identities of specific optimal codons and therefore represents a useful index for contrasting the strength of selection on codon usage among species (Cutter *et al.* 2006c), although it is not known how strongly this metric is affected by sample size (and therefore the 90th percentile cutoff level relative to the class of genes with a single EST hit).

Divergence: The coding sequences resulting from the computational procedures applied to ESTs from *C. japonica*, *C. brenneri*, and *C.* sp. 5 were combined with gene predictions for *C. elegans*, *C. briggsae*, and *C. remanei* acquired from WormBase release WS170 to infer putative one-to-one orthologs with OrthoMCL using default parameters (Li *et al.* 2003). Multiple-sequence alignments of the resulting putative orthologs with ClustalW (Thompson *et al.* 1994) were followed by calculation of lineage-specific synonymous (d_S) and nonsynonymous (d_N) site substitution rates with PAML (Yang 1997) assuming the Caenorhabditis phylogenetic topology of Kiontke *et al.* (2004, 2007) and Kiontke and Sudhaus (2006) (Figure 1), implemented with custom Bioperl-based scripts. Alignments were based on canonical peptide translations and substitution rates used the Goldman–Yang codon-based maximum-likelihood method, permitting branch-specific d_N/d_S (*i.e.*, model = 1, codon model F3X4) (Yang *et al.* 1994). Because the EST collections do not fully represent the gene complement of their respective genomes, we eliminated sets of genes for which orthology designation was likely to be inappropriate; specifically, we excluded putative orthologous groups that exhibited excessively high substitution rates along any branch ($d_N > 0.5$ or $d_S > 5$; <3% of genes fit these criteria). This procedure resulted in 63 orthologous groups with representatives in all six species of Caenorhabditis and 1244 orthologous groups with other combinations of three to five species (supplemental Data 2). We exclude from consideration the 6398 orthologous groups specific to the three sequenced genomes (*C. elegans*, *C. briggsae*, and *C. remanei*), because in the six-species context, they provide only lineage-specific data to *C. remanei*. However, we also followed the above procedure to infer orthologs and calculate divergence for the three sequenced genomes only, excluding the EST-derived sequences, which provided lineage-specific divergence for *C. remanei* and *C. briggsae* (7846 orthologous groups) with respect to their common ancestor (using *C. elegans* as outgroup).

The substitution rate at neutral sites will occur at a rate equal to the mutation rate and independently of population size (Kimura 1968), and therefore can be used to standardize replacement-site substitutions for genomic heterogeneity in mutation rate. Divergence at synonymous sites (d_S) is typically used as a neutral reference. However, all of these Caenorhabditis species demonstrate correlations between d_S and codon bias (ENC) (Spearman's nonparametric correlations: *C. elegans* = 0.55, *C. brenneri* = 0.60, *C. remanei* = 0.50, *C. briggsae* = 0.58, *C.* sp. 5 = 0.51; all $P < 0.0001$), reflecting selection on codon usage (Sharp and Li 1987). To represent neutral substitution rates, we consequently adjusted the divergence values to correct for selection on codon bias by adding the residuals of least-squares regressions of branch-specific d_S on ENC to the value of d_S expected at ENC = 61 (where codon bias is not present). These adjusted synonymous-site sub-

TABLE 1

Codon usage bias (ENC, F_{op}, $\overline{\Delta RSCU_+}$) and base composition (GC, GC3s) summary statistics

Species	Loci[a]	Mean ENC[a]	Mean F_{op}[a]	Mean GC_{63}[b]	Mean $GC3s_{63}$[b]	Mean ENC_{63}[b]	Mean $F^{b}_{op_{63}}$	Corr[ENC, EST hit count]	$\overline{\Delta RSCU_+}$
C. japonica	1,055	47.37	0.503	0.519	0.592	40.59	0.6617	−0.350***	0.3723
C. elegans	15,207	50.23	0.387	0.497	0.521	38.57	0.6587	−0.219***	0.3351
C. brenneri	1,370	45.90	0.491	0.496	0.535	37.13	0.6854	−0.274***	0.4182
C. remanei	5,603	48.62	0.432	0.497	0.529	37.35	0.6813	−0.144***	0.3643
C. briggsae	765	46.96	0.516	0.510	0.567	37.55	0.6991	−0.089***	0.3484
C. sp. 5	2,295	46.27	0.508	0.512	0.583	36.70	0.7077	−0.338***	0.4977

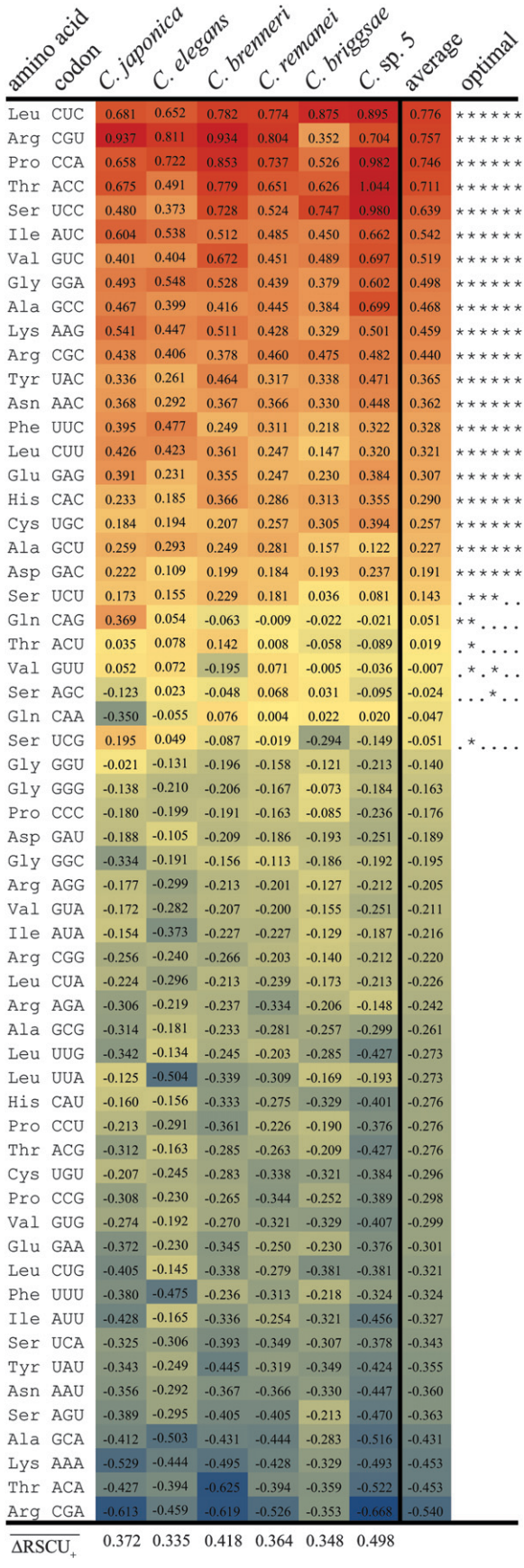
[a] Calculated for loci with ≥1 EST hits.
[b] Calculated for the 63 loci with orthologs in all six species; ***$P < 0.0001$.

FIGURE 2.—Fraction of variance in the frequency of optimal codons (F_{op}) explained by base composition, gene length, gene expression, and their interactions. Codon usage bias is more extreme in highly expressed genes ($F_{op} \times \log_{10}$[EST hit count] all $P < 0.0001$) and in shorter genes ($F_{op} \times \log_{10}$[length] $P < 0.0001$ for *C. elegans*, *C. brenneri*, *C. remanei*, and *C. briggsae*; $P > 0.05$ in other species).

stitution rates are referred to as d_S', and are used to standardize nonsynonymous substitution rates as d_N/d_S'. We restrict assessment of substitution rates to those sequences with a Caenorhabditis outgroup, and therefore exclude divergence for the basal *C. japonica* lineage from consideration (and for *C. elegans* for the sequenced-genome inference of orthologous groups). This restriction, and the exclusion of loci for which ENC could not be calculated, results in the different sample sizes of genes for each species.

RESULTS

Selection on codon usage: For all six species of Caenorhabditis, we find that codon usage bias is stronger in highly expressed genes (Table 1). In an ANOVA model that explains variation in codon bias (frequency of optimal codons, F_{op}) (IKEMURA 1985) as a function of gene expression (EST hit counts), base composition (GC3s), and sequence length, we show that expression level explains a significant amount of variation in codon bias in each species (Figure 2). These results indicate that selection for translational efficiency and/or accuracy has been a driving force shaping codon usage in the genomes of all Caenorhabditis. This general conclusion is further bolstered by the correlations observed between synonymous site divergence and measures of codon usage bias (see MATERIALS AND METHODS), such that loci with stronger codon bias exhibit slower evolution at synonymous sites. The significantly stronger codon bias in short genes that we observe for four of the Caenorhabditis species (Figure 2) is consistent with previous findings for *C. elegans* (DURET and MOUCHIROUD



amino acid	codon	*C. japonica*	*C. elegans*	*C. brenneri*	*C. remanei*	*C. briggsae*	*C.* sp. 5	average	optimal
Leu	CUC	0.681	0.652	0.782	0.774	0.875	0.895	0.776	******
Arg	CGU	0.937	0.811	0.934	0.804	0.352	0.704	0.757	******
Pro	CCA	0.658	0.722	0.853	0.737	0.526	0.982	0.746	******
Thr	ACC	0.675	0.491	0.779	0.651	0.626	1.044	0.711	******
Ser	UCC	0.480	0.373	0.728	0.524	0.747	0.980	0.639	******
Ile	AUC	0.604	0.538	0.512	0.485	0.450	0.662	0.542	******
Val	GUC	0.401	0.404	0.672	0.451	0.489	0.697	0.519	******
Gly	GGA	0.493	0.548	0.528	0.439	0.379	0.602	0.498	******
Ala	GCC	0.467	0.399	0.416	0.445	0.384	0.699	0.468	******
Lys	AAG	0.541	0.447	0.511	0.428	0.329	0.501	0.459	******
Arg	CGC	0.438	0.406	0.378	0.460	0.475	0.482	0.440	******
Tyr	UAC	0.336	0.261	0.464	0.317	0.338	0.471	0.365	******
Asn	AAC	0.368	0.292	0.367	0.366	0.330	0.448	0.362	******
Phe	UUC	0.395	0.477	0.249	0.311	0.218	0.322	0.328	******
Leu	CUU	0.426	0.423	0.361	0.247	0.147	0.320	0.321	******
Glu	GAG	0.391	0.231	0.355	0.247	0.230	0.384	0.307	******
His	CAC	0.233	0.185	0.366	0.286	0.313	0.355	0.290	******
Cys	UGC	0.184	0.194	0.207	0.257	0.305	0.394	0.257	******
Ala	GCU	0.259	0.293	0.249	0.281	0.157	0.122	0.227	******
Asp	GAC	0.222	0.109	0.199	0.184	0.193	0.237	0.191	******
Ser	UCU	0.173	0.155	0.229	0.181	0.036	0.081	0.143	.***..
Gln	CAG	0.369	0.054	-0.063	-0.009	-0.022	-0.021	0.051	**....
Thr	ACU	0.035	0.078	0.142	0.008	-0.058	-0.089	0.019	.*....
Val	GUU	0.052	0.072	-0.195	0.071	-0.005	-0.036	-0.007	.*.*..
Ser	AGC	-0.123	0.023	-0.048	0.068	0.031	-0.095	-0.024	...*..
Gln	CAA	-0.350	-0.055	0.076	0.004	0.022	0.020	-0.047	
Ser	UCG	0.195	0.049	-0.087	-0.019	-0.294	-0.149	-0.051	.*....
Gly	GGU	-0.021	-0.131	-0.196	-0.158	-0.121	-0.213	-0.140	
Gly	GGG	-0.138	-0.210	-0.206	-0.167	-0.073	-0.184	-0.163	
Pro	CCC	-0.180	-0.199	-0.191	-0.163	-0.085	-0.236	-0.176	
Asp	GAU	-0.188	-0.105	-0.209	-0.186	-0.193	-0.251	-0.189	
Gly	GGC	-0.334	-0.191	-0.156	-0.113	-0.186	-0.192	-0.195	
Arg	AGG	-0.177	-0.299	-0.213	-0.201	-0.127	-0.212	-0.205	
Val	GUA	-0.172	-0.282	-0.207	-0.200	-0.155	-0.251	-0.211	
Ile	AUA	-0.154	-0.373	-0.227	-0.227	-0.129	-0.187	-0.216	
Arg	CGG	-0.256	-0.240	-0.266	-0.203	-0.140	-0.212	-0.220	
Leu	CUA	-0.224	-0.296	-0.213	-0.239	-0.173	-0.213	-0.226	
Arg	AGA	-0.306	-0.219	-0.237	-0.334	-0.206	-0.148	-0.242	
Ala	GCG	-0.314	-0.181	-0.233	-0.281	-0.257	-0.299	-0.261	
Leu	UUG	-0.342	-0.134	-0.245	-0.203	-0.285	-0.427	-0.273	
Leu	UUA	-0.125	-0.504	-0.339	-0.309	-0.169	-0.193	-0.273	
His	CAU	-0.160	-0.156	-0.333	-0.275	-0.329	-0.401	-0.276	
Pro	CCU	-0.213	-0.291	-0.361	-0.226	-0.190	-0.376	-0.276	
Thr	ACG	-0.312	-0.163	-0.285	-0.263	-0.209	-0.427	-0.276	
Cys	UGU	-0.207	-0.245	-0.283	-0.338	-0.321	-0.384	-0.296	
Pro	CCG	-0.308	-0.230	-0.265	-0.344	-0.252	-0.389	-0.298	
Val	GUG	-0.274	-0.192	-0.270	-0.321	-0.329	-0.407	-0.299	
Glu	GAA	-0.372	-0.230	-0.345	-0.250	-0.230	-0.376	-0.301	
Leu	CUG	-0.405	-0.145	-0.338	-0.279	-0.381	-0.381	-0.321	
Phe	UUU	-0.380	-0.475	-0.236	-0.313	-0.218	-0.324	-0.324	
Ile	AUU	-0.428	-0.165	-0.336	-0.254	-0.321	-0.456	-0.327	
Ser	UCA	-0.325	-0.306	-0.393	-0.349	-0.307	-0.378	-0.343	
Tyr	UAU	-0.343	-0.249	-0.445	-0.319	-0.349	-0.424	-0.355	
Asn	AAU	-0.356	-0.292	-0.367	-0.366	-0.330	-0.447	-0.360	
Ser	AGU	-0.389	-0.295	-0.405	-0.405	-0.213	-0.470	-0.363	
Ala	GCA	-0.412	-0.503	-0.431	-0.444	-0.283	-0.516	-0.431	
Lys	AAA	-0.529	-0.444	-0.495	-0.428	-0.329	-0.493	-0.453	
Thr	ACA	-0.427	-0.394	-0.625	-0.394	-0.359	-0.522	-0.453	
Arg	CGA	-0.613	-0.459	-0.619	-0.526	-0.353	-0.668	-0.540	
$\overline{\Delta RSCU_+}$		0.372	0.335	0.418	0.364	0.348	0.498		

Figure 3.—Difference in RSCU between highly and lowly expressed genes (ΔRSCU) and inferred optimal codon identity in Caenorhabditis. Codons are sorted by mean ΔRSCU across species. Optimal codons are indicated along the right, with adjacent asterisks indicating statistical significance for different species ordered as for ΔRSCU columns (dots indicate lack of significance for the respective species). Yellow corresponds to ΔRSCU = 0, with shading toward red indicating ΔRSCU > 0 (maximum 1.04) and shading toward blue indicating ΔRSCU < 0 (minimum −0.67).

1999) as well as more distantly related nematodes (Cutter *et al.* 2006c). Intragenic background selection (Loewe and Charlesworth 2007) or Hill–Robertson interference (Comeron and Guthrie 2005) could potentially explain this length effect.

Identity of optimal codons: We identified the optimal codon identities for each species on the basis of significant differences in RSCU between highly and lowly expressed loci. Lowly expressed loci are defined as those loci with only a single species-specific EST hit, whereas highly expressed loci have EST hits exceeding the species-specific 90th percentile. This approach identified 20 optimal codons from 17 amino acids that were common to all six species (Figure 3). An additional 6 codons (CAG, UCG, UCU, AGC, ACU, and GUU) from 4 amino acids (glutamine, serine, threonine, and valine) were identified as optimal in some but not all species. It is difficult to discern whether these few variable optimal codons (i) actually are preferred in all taxa, but the current sample is insufficient to define them in some species, (ii) represent gain/loss of optimal codons in some lineages, or (iii) reflect a spurious result. In the case of CAG (Gln) and UCG (Ser), species other than *C. japonica* and *C. elegans* show a trend in the opposite direction to what would be expected if this codon were preferred. Consequently, these codons might not truly represent preferred codons or could indicate true losses of preferred codons among the *C. brenneri–remanei–briggsae*–sp. 5 monophyletic group. Stenico *et al.* (1994) pointed out that the glutamine CAG codon is used more in highly than lowly expressed genes of *C. elegans*, despite being underrepresented relative to alternative codons across gene expression classes. Similarly, the threonine ACU codon is significant only for *C. elegans*, but a positive trend is evident in all species except *C. briggsae* and *C.* sp. 5. The valine GUU and serine AGC patterns show no obvious phylogenetic signal, and may be spurious. In contrast, UCU (Ser) is more abundant in highly expressed genes in all taxa, but only significantly so in *C. elegans*, *C. brenneri*, and *C. remanei*. Thus, UCU might represent a preferred codon in all Caenorhabditis, albeit preferred only weakly. tRNA gene copy numbers are characterized only in *C. elegans* and *C. briggsae* at present (*C. elegans* Sequencing Consortium 1998; Stein *et al.* 2003) (http://lowelab.ucsc.edu/GtRNAdb/); however, the cognate tRNAs do not differ dramatically in abundance between these two species. Because of the ambiguous nature of

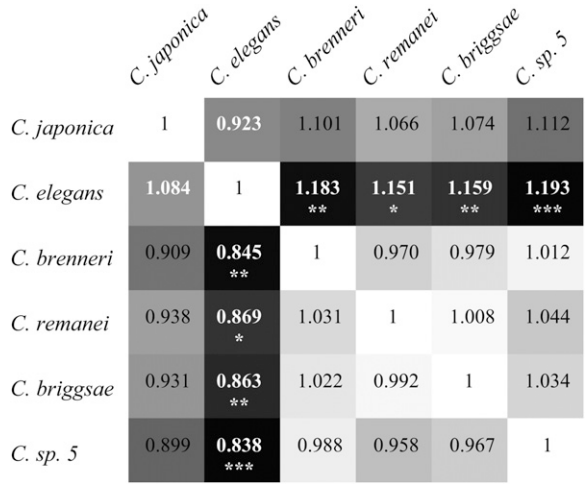


Figure 4.—Slopes from orthogonal regressions of the frequency of optimal codons (F_{op}) between pairs of species. Values below the diagonal correspond to species listed along the side as dependent variable; values above the diagonal correspond to species listed along the top as dependent variable. In all comparisons exhibiting a slope >1, the intercept is negative; all comparisons with slope <1 have a positive intercept. The combination of slopes <1 and intercepts >0 for *C. elegans* as independent variable (second column from left, white text) is indicative of reduced codon bias in this species. Asterisks indicate significant differences from a slope of 1 (*P = 0.017, **P < 0.01, ***P = 0.00023). Darker shades of gray represent slopes with greater deviation from 1.

these six codons and their generally weak effects (Figure 3), we calculate the F_{op} for individual loci (Ikemura 1985) solely on the basis of the 20 optimal codons that are conserved across Caenorhabditis.

Codon bias in selfers *vs.* outcrossers: We used three approaches to contrast overall codon usage bias among Caenorhabditis species. First, we calculated the average difference in relative synonymous codon usage between highly and lowly expressed genes ($\overline{\Delta RSCU_+}$) across all loci (Cutter *et al.* 2006c). Second, we calculated the average ENC (Wright 1990) and F_{op} for the set of 63 orthologous groups of loci that were common to all six taxa (values for the full data set are composed of different sets of loci for each species; Table 1). Because base composition is similar in this group of species (Table 1), it is permissible to contrast mean ENC and F_{op} across taxa. For all of these metrics, the outcrossing *C.* sp. 5 exhibits the most extreme value in the direction of strong codon bias (Table 1), although all species of Caenorhabditis demonstrate strong overall codon bias relative to many other nematodes, particularly parasites that have $\overline{\Delta RSCU_+}$ typically ~0.1 (Cutter *et al.* 2006c). However, average codon bias did not differ significantly among species (63 orthologous groups, Kruskal–Wallis $P > 0.2$ for both ENC and F_{op}). We also tested for a difference in codon bias levels in a pairwise contrast of the >800 orthologs shared between *C. briggsae* and *C.* sp. 5 (the two closest relatives). This analysis revealed that codon bias is significantly weaker in the selfing *C. briggase* (median F_{op} *C. briggsae* = 0.461, *C.* sp. 5 = 0.498, Wilcoxon's $P = 0.0022$; median ENC *C. briggsae* = 48.40, *C.* sp. 5 = 46.67, Wilcoxon's $P = 0.0003$). Finally, to investigate the correspondence of codon bias in matched orthologous sequences, we performed orthogonal regressions of the frequency of optimal codons for each pair of species for the 63 orthologous groups common to all six species (Figure 4). This third analysis revealed that *C. elegans* exhibits reduced codon bias relative to most other species of Caenorhabditis, despite the small magnitude of effect. In summary, we find evidence for weaker codon bias in both selfing species, although the magnitude of the reduction is relatively small.

Modeling changes in codon bias: Selection for codon bias is very weak, with the selection intensity ($\gamma = 4N_e s$) estimated to be only ~0.4 in Caenorhabditis (Cutter and Charlesworth 2006; Cutter 2008b). Consequently, a sharp reduction in effective population size, like that brought on by the evolution of self-fertilization, will result in relaxed selection on codon usage such that its evolution will be governed solely by the neutral processes of mutation and genetic drift. Applying this logic, we draw on the results of Sueoka (1962) and Marais *et al.* (2004a) to infer the degree of codon bias at time t, p_t, following a complete relaxation of selection on codon usage. We begin with the Li–Bulmer (Li 1987; Bulmer 1991) equation for the expected equilibrium frequency of preferred codons in the ancestor that experienced selection for codon bias $p^* = 1/(1 + k \exp[-\gamma])$, where k is the ratio of the mutation rate from (u) and to (v) preferred codons and γ is the selection intensity ($4N_e s$). The equilibrium frequency of preferred codons in the absence of selection is simply $p^{**} = v/(u + v)$. Analogous to previous results for the evolution of base composition (Sueoka 1962; Marais *et al.* 2004a), we find that change in codon bias over time following relaxation of selection can be described by the relation

$$p_t - p^{**} = (p^* - p^{**})\exp[-(u + v)t]. \qquad (1)$$

Assuming that *C. elegans* codon bias has degenerated following the origin of selfing, we can calculate the time over which this process has led to the reduced level of codon bias observed today. We shall focus on the frequency of preferred codons among those 63 highly codon-biased genes that have putative orthologs in all six species of Caenorhabditis, because the change in codon bias should be most apparent for genes with initially high levels of bias. In this case, we shall assume that the ancestral codon bias $p^* = 0.7$ and has degenerated to $p_t = 0.658$ in contemporary *C. elegans* as a consequence of mutation accumulation (see F_{op} in Table 1). We take the median frequency of preferred codons in genes with low expression (0.336) as our

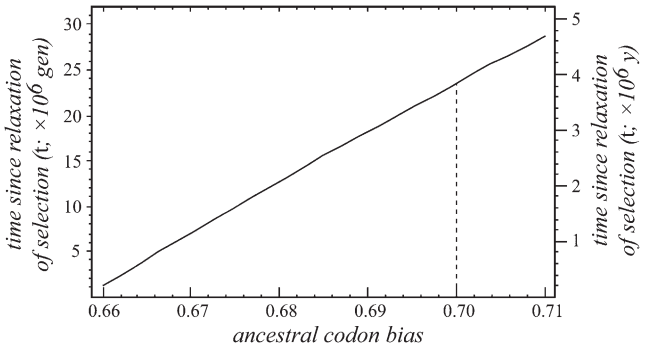


FIGURE 5.—The predicted time since the onset of relaxed selection on codon usage to yield 65.8% optimal codons, as observed for 63 *C. elegans* genes that are conserved across the genus, as a function of the ancestral level of codon bias. Provided that the evolution of selfing initiated the relaxed selection, this time is equivalent to the duration of selfing in *C. elegans*. Timescale in years assumes a 60-day generation time in nature; dashed line demarcates the value for ancestral codon bias assumed in point estimation of *t*.

estimate of p^{**}, which is very close to the value expected under uniform codon usage ($20/59 = 0.339$) (STENICO *et al.* 1994). The total per-site single nucleotide mutation rate is estimated to be 9×10^{-9} per generation in *C. elegans* (DENVER *et al.* 2004). Given that 58.5% of synonymous mutations would result in a change from a preferred to unpreferred codon (or vice versa; so, $u + v = 5.26 \times 10^{-9}$ per site per generation), from our equation describing p^{**}, we can infer that $v = 1.89 \times 10^{-9}$ and $u = 3.37 \times 10^{-9}$. This also assumes that mutational biases have not changed along the lineage. Inserting these values into Equation 1 and numerically solving for *t*, the model predicts that relaxation of selection upon the origin of selfing began 23.3×10^6 generations ago. Assuming conservatively that *C. elegans* has a generation time of 60 days in nature, by virtue of spending most of its life in the dauer stage (BARRIÈRE and FÉLIX 2005), then this translates to ~3.8 million years. It is plausible that the generation time in nature is faster than we have assumed, which would imply that relaxation of selection, and selfing, originated more recently (assuming a 14- to 90-day generation time, $8.9 \times 10^5 < t < 5.7 \times 10^6$). Likewise, a lower ancestral codon bias would imply a more recent origin than suggested by these calculations (assuming $F_{op} = p^*$ range 0.66–0.71, $1.9 \times 10^5 < t < 4.7 \times 10^6$; Figure 5).

Divergence: We observe no significant differences in median values of replacement-site divergence (d_N/d_S') (Table 2) for all genes in all lineages considered together (Kruskal–Wallis $P > 0.86$) and only for the subset of orthologous groups common to all six species of Caenorhabditis (Kruskal–Wallis $P > 0.35$). These tests do not correct for common ancestry, which would serve to further reduce the likelihood of inferring heterogeneity in substitution rates. When we restrict analysis to a contrast of orthologs of *C. briggsae* (selfing) and *C.* sp. 5 (outcrossing), the two closest relatives, again we find no differences in either d_N (*C. briggsae* median = 0.0254, *C.* sp. 5 median = 0.0213; Wilcoxon's $P = 0.075$) or d_N/d_S' (Wilcoxon's $P > 0.73$; Table 2). These results generally accord with the small data set that contrasted substitution rates between inbreeding *C. briggsae* and outbreeding *C. remanei* (CUTTER and PAYSEUR 2003). We also find that divergence at replacement sites correlates with codon bias (Table 2), such that loci with stronger purifying selection on amino acids also exhibit stronger selection to maintain biased usage of codons, as also reported for Drosophila (BETANCOURT and PRESGRAVES 2002; MARAIS *et al.* 2004b; BIERNE and EYRE-WALKER 2006).

For the much larger set of orthologous groups of genes shared between *C. elegans–C. remanei–C. briggsae*, we also calculated lineage-specific rates of divergence. For these data, the *C. briggsae* lineage had a significantly higher rate of nonsynonymous site substitution than *C. remanei* (Wilcoxon's $P < 0.0001$ for both d_N/d_S and d_N/d_S'; Table 3). Despite sharing a common ancestor at the same point in the past, the *C. briggsae* lineage also exhibits a higher rate of synonymous site substitution than *C. remanei*, potentially reflecting more generations per unit time in *C. briggsae* or possibly a higher mutation rate per generation (BAER *et al.* 2005) (Table 3). In addition, d_N is positively correlated with both d_S (Spearman's

TABLE 2

Lineage-specific divergence for orthologous groups among six Caenorhabditis species, with *C. japonica* as outgroup

	All orthologous groups			Six-species orthologous groups	
Species	Loci	Median d_N/d_S'	Corr[d_N, ENC]	Loci	Median d_N/d_S'
C. elegans	356	0.0284	0.449***	63	0.0196
C. brenneri	430	0.0272	0.373***	63	0.0169
C. remanei	1263	0.0291	0.348***	63	0.0152
C. briggsae	822	0.0316	0.401***	63	0.0145
C. sp. 5	819	0.0292	0.363***	63	0.0153

***$P < 0.0001$.

TABLE 3

Lineage-specific divergence for *C. elegans–remanei–briggsae* orthologs, with *C. elegans* as outgroup

Species	Loci	Median d_N	Median d_N/d_S'	Median d_S	Median d_S'
C. remanei	7846	0.0330	0.0350	0.635	0.904
C. briggsae	7846	0.0446	0.0416	0.759	1.059

rank correlations: *C. remanei* = 0.50, $P < 0.0001$; *C. briggsae* = 0.53, $P < 0.0001$) and d_S' (Spearman's rank correlations: *C. remanei* = 0.43, $P < 0.0001$; *C. briggsae* = 0.45, $P < 0.0001$). Consequently, it becomes difficult to conclude confidently that the difference in replacement-site divergence between the *C. remanei* and *C. briggsae* lineages is best explained by a difference in N_e between these species due to selfing. It is plausible that the *C. briggsae* lineage experiences an elevated mutation rate (causing higher d_S and d_S') that is incompletely accounted for in this data set (due to synonymous site saturation), leading to higher replacement-site divergence in the *C. briggsae* lineage simply as a by-product. Thus, an unambiguous signature of deleterious mutation accumulation in peptide sequences for the selfing *C. briggsae* lineage also is not found for this larger set of sequences.

DISCUSSION

Molecular evolutionary implications for the evolution of selfing: These results demonstrate that the demographic history of all Caenorhabditis species has enabled their genomes to respond to even very weak natural selection. This is evidenced by the strong patterns of codon usage bias in highly expressed genes that are indicative of selection for translational efficiency and/or accuracy; such selection is very weak, with a selection intensity ($N_e s$) estimated to be ~0.1 in *C. remanei* (Cutter and Charlesworth 2006; Cutter 2008b). Therefore, selection on codon usage is expected to be relaxed completely in the present day for those species that reproduce by self-fertilization, by virtue of their ~20-fold lower effective population sizes (Graustein *et al.* 2002; Jovelin *et al.* 2003; Cutter *et al.* 2006a). However, we report that the overall levels of codon bias are not much reduced in the selfing species, implying that little time has elapsed to permit the accumulation of slightly deleterious mutations in the form of unpreferred codons.

Consistent with the conclusion that the selfing mode of reproduction is not ancient, we also find little evidence for differences in rates of substitution at replacement sites within coding sequences for orthologous groups of genes in these six species of Caenorhabditis. In the large data set of orthologs defined for the three sequenced genomes (*C. elegans*, *C. remanei*, and *C. briggsae*), we do observe an elevation in the rate of replacement substitutions in the selfing *C. briggsae* lineage. However, the synonymous substitution rate is also higher, perhaps as a consequence of a more rapid turnover of generations in *C. briggsae* and its recent ancestors or to a higher mutation rate per generation (Baer *et al.* 2005). Due to the high absolute levels of synonymous site divergence (saturation is observed in pairwise comparisons) and the strong correlation between synonymous and replacement divergence, it is possible that the observed differences in d_N and d_N/d_S' are due to a more rapid pace of mutation per year rather than to a change in population size coincident with the origin of selfing in *C. briggsae*'s ancestry. The contrast of *C. briggsae* with the more closely related *C.* sp. 5 is the more informative comparison, for which no significant difference in replacement-site divergence was detected. It is worth noting that if selection coefficients on replacement sites are sufficiently large, then even a significant reduction in effective population size due to selfing might not lead them to be effectively neutral. However, given a broad distribution of selection coefficients, as observed in Drosophila (Piganeau and Eyre-Walker 2003; Loewe and Charlesworth 2006; Loewe *et al.* 2006), we would nonetheless expect a reasonable fraction of replacement sites to become effectively neutral with a 20-fold smaller effective population size.

Using a model that describes the change in codon bias over time due to relaxed selection, we estimate that selfing likely evolved in the *C. elegans* lineage within the last ~4 million years, assuming coincident onset of relaxed selection with the evolution of selfing. If a divergence time of ~18 million years separating *C. elegans* from its closest relatives is roughly correct (Cutter 2008a), then this implies that the lineage leading to *C. elegans* spent <25% of the time since the common ancestor in a selfing state. These calculations could overestimate the date for the origin of selfing for two reasons. First, if codon bias were not as strong in the outcrossing ancestor of *C. elegans* as we assume, which is plausible because the outgroup *C. japonica* also exhibits nominally weaker codon bias than the monophyletic group that is sister to *C. elegans* (Table 1; Figure 1), then less time would be required to account for the smaller resultant change in codon bias to the present day. For example, taking the level of codon bias in *C. japonica* as the ancestral state for *C. elegans*, we would infer an origin of selfing of only $\sim 2.1 \times 10^6$ generations or ~347 thousand years ago (given an average 60-day generation time in nature). Second, if *C. elegans*'s generation time is more rapid than the conservative rate used in our calculations, then a more recent origin of selfing would be inferred. The lack of difference in nonsynonymous substitution rates among species also is consistent with the conclusion that low population sizes in the selfing

taxa have not persisted for very long. *C. briggsae* demonstrates very little reduction in codon bias relative to its sister species, *C.* sp. 5, suggesting that selfing might have evolved even more recently in *C. briggsae*'s ancestry than for *C. elegans*. These findings imply that the evolution of hermaphrodite mating behavior (Chasnov and Chow 2002; Garcia *et al.* 2007; Kleemann and Basolo 2007), chemo-attraction (Chasnov *et al.* 2007), sperm production (Cutter 2004; Geldziler *et al.* 2006), and developmental mechanisms (Hill *et al.* 2006) likely do not have ancient origins.

Additional circumstantial support for the notion of a recent origin of selfing in Caenorhabditis comes from the lack of hermaphrodite-only clades comprising multiple species (Kiontke *et al.* 2004, 2007), although further sampling of biodiversity in this genus could refute this line of reasoning. Selfing hermaphroditism appears to arise regularly in Rhabditid nematodes, but occurs primarily among tip-taxa (Kiontke and Fitch 2005), suggesting that extinction of hermaphroditic lineages likely outpaces speciation. In addition, to the extent that relaxed selection on male function and hermaphrodite attractiveness to males is occurring in *C. elegans* and *C. briggsae*, or active selection against these traits, insufficient time has elapsed for the complete loss of a functional male phenotype. According to the model of male maintenance by Chasnov and Chow (2002) (Equation 13), it would seem that too many male-specific genes occur in the *C. elegans* genome to permit male maintenance via a balance between degeneration and mating (see also Cutter *et al.* 2003). Such a balance could maintain males if there were fewer than ~62 male genes, given an average per gene deleterious mutation rate each generation of $0.48/20{,}000 = 2.4 \times 10^{-5}$ (Denver *et al.* 2004), an average X chromosome nondisjunction rate of 0.0033 per generation (Teotonio *et al.* 2006), and an average male mating-ability parameter of 0.18 (Teotonio *et al.* 2006)—yet there are likely >100 male genes in the genome (Kim *et al.* 2001; Reinke *et al.* 2004; Cutter and Ward 2005). Consequently, this suggests that male genes are expected to degenerate, which is consistent with phenotypic observations of strains in nature with genetically sterile males (Hodgkin and Doniach 1997; Lints and Emmons 2002) and generally poor male *C. elegans* mating ability relative to gonochoristic males (Chasnov and Chow 2002). The lack of significantly elevated rates of sequence evolution in male genes (Cutter and Ward 2005) therefore is consistent with a relatively recent origin of the extreme form of selfing currently inferred for *C. elegans*.

Contrasts with other taxa: How does codon usage bias in Caenorhabditis compare with other eukaryotes? Differences in background base composition make it infeasible to use average ENC or F_{op} as a general comparative tool, whereas the $\overline{\Delta RSCU_+}$ statistic (the average difference in RSCU between highly and lowly expressed genes) provides a useful index for comparison among species with very different GC content (Cutter *et al.* 2006c). The consistently strong selection-mediated codon bias among Caenorhabditis species is consistent with other free-living nematodes and contrasts with the limited role for selection causing codon bias in parasitic nematodes (Cutter *et al.* 2006c). For more distant relatives, a reanalysis of Duret and Mouchiroud's (1999) data to calculate $\overline{\Delta RSCU_+}$ for short proteins indicates that differential selection on codon usage among genes with high *vs.* low expression has been substantially stronger in *C. elegans* history ($\overline{\Delta RSCU_+} = 0.515$) than for *Drosophila melanogaster* ($\overline{\Delta RSCU_+} = 0.287$) or *Arabidopsis thaliana* ($\overline{\Delta RSCU_+} = 0.141$). The different value for *C. elegans* $\overline{\Delta RSCU_+}$ than reported here (0.335; Table 1) stems from their data set containing only short proteins, which experience stronger codon bias and a different definition of highly and lowly expressed genes. Kanaya *et al.* (2001) also showed that *C. elegans* exhibits stronger selection on codon usage than *D. melanogaster*, *Xenopus laevis*, and human. The substantially higher average frequency of optimal codons observed in *D. melanogaster* than *C. elegans* (Duret and Mouchiroud 1999) likely reflects the greater overall GC content of the fly genome relative to the worm, rather than differences in selection on codon usage, because optimal codons tend to be GC rich. However, polymorphism-based inference of codon bias suggests stronger selection intensities for species of Drosophila than Caenorhabditis (reviewed in Cutter and Charlesworth 2006), indicating that further work is necessary to elucidate the conflicting implications of population polymorphism and genomic trends in codon usage for these groups.

Breeding system variation analogous to that in Caenorhabditis is found within the plant genus Arabidopsis. Like the results reported here, no differences in substitution rates or codon bias have been observed between the inbreeding *A. thaliana* and outbreeding *A. lyrata* (Wright *et al.* 2002). Overall codon usage bias is much weaker in Arabidopsis than in Caenorhabditis (see above), so an effect of selfing would be more difficult to detect in Arabidopsis because codon usage will be closer to the no-selection equilibrium levels. Furthermore, a recent origin of selfing in *A. thaliana* (Charlesworth and Wright 2001) or population bottlenecks in *A. lyrata* might also obscure a detectable effect of selfing on the expected patterns of molecular evolution (Wright *et al.* 2002, 2003). Analyses of the self-incompatibility locus in Arabidopsis on the basis of patterns of diversity (Shimizu *et al.* 2004) and linkage disequilibrium (Tang *et al.* 2007) implicate an origin of selfing of ≤1 million generations ago. This contrasts with the broader plausible range for *C. elegans* of 0.32×10^6–23.3×10^6 generations ago, on the basis of patterns of nucleotide diversity (Cutter 2006) or the decay of codon bias.

Effects of selfing on other genomic features: The most straightforward prediction of the smaller effective

population sizes induced by selfing is that nucleotide diversity will be reduced genomewide. This prediction is clearly met in Caenorhabditis (Graustein *et al.* 2002; Jovelin *et al.* 2003; Cutter *et al.* 2006a).

We have focused on the potential for accumulation of slightly deleterious mutations in orthologous coding sequences. However, relaxed selection might also impact multigene families disproportionately in selfing lineages, through patterns of divergence and pseudogene production. Such an effect would be important for understanding whether accelerated rates of protein evolution in some gene families result from positive selection or relaxed selection (Stewart *et al.* 2005; Thomas *et al.* 2005), making sure to account for past selection on synonymous sites when standardizing by synonymous site divergence.

In addition, the rapid generation of homozygosity produced by self-fertilization might facilitate more rapid fixation of genomic rearrangements, if heterozygous fitness is most strongly affected (Charlesworth 1992). It will be important to determine whether the extensive intrachromosomal rearrangements observed between *C. elegans* and *C. briggsae* (Coghlan and Wolfe 2002; Hillier *et al.* 2007) is a general feature of the genus or whether the rates of translocation, inversion, and transposition are elevated specifically in the selfing lineages.

Models of transposable element dynamics in selfers *vs.* outcrossers depend critically on the relative importance of deleterious insertion *vs.* ectopic exchange (unequal crossing-over caused by repetitive elements) as selective agents against transposable element proliferation (Nuzhdin 1999; Morgan 2001). A dominant effect of deleterious insertion could cause mobile elements to be eliminated completely from highly selfing lineages. Consequently, if it is generally true that outcrossing Caenorhabditis have higher mobile element loads than the selfing species, as appears to be the case for retroelements in *C. remanei* (Z. Bao and N. Jiang, personal communication), then it might support a primary role for the deleterious effects of element insertion in controlling copy numbers, in contrast to the prevailing view for Drosophila (Petrov *et al.* 2003). The greater abundance of transposable elements in the genome of *C. briggsae* than in *C. elegans* (Stein *et al.* 2003) therefore might be a consequence of a more recent origin of selfing in *C. briggsae*. It is also important to note that differences among species in the effectiveness of an RNA-interference response to limit transposition could play a fundamental role in transposable element activity and genomic copy number (Sijen and Plasterk 2003).

Conclusions: Transitions in breeding system from obligately outcrossing to predominantly self-fertilizing will shift genomic patterns of molecular evolution, through the accumulation of slightly deleterious mutations by genetic drift due to the concomitant reduction in effective population size. However, the extent of change resulting from relaxed selection depends on the mutation rate and the time since the origin of selfing and will be more apparent for genomic features that are more susceptible to becoming selectively neutral, such as codon usage bias. We have shown that Caenorhabditis genomes have been shaped by weak selection in their history, yet selfing species do not differ greatly from outcrossing ones in terms of the magnitude of codon bias or replacement-site substitution rate. As a result, a model of relaxed selection suggests that it is unlikely that selfing evolved much longer than ~4 million years ago. We therefore conclude that evolution over a modest timescale resulted in the novel features of hermaphrodites relative to females, in such traits as mate searching and avoidance (Lipton *et al.* 2004; Kleemann and Basolo 2007), activity during mating (Garcia *et al.* 2007), loss of pheromone attraction (Chasnov *et al.* 2007), self-sperm production, activation, and numerical optimization (Cutter 2004; Geldziler *et al.* 2006), and the sex-determination pathway (Hill *et al.* 2006). Additional consequences of selfing for other aspects of genome architecture, such as chromosomal rearrangements, intron size, and gene family and transposable element dynamics, will be much informed by comparisons among the ongoing genome sequencing projects in this group.

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