Mutation Rates and Intraspecific Divergence of the Mitochondrial Genome of *Pristionchus pacificus*

Ruxandra I. Molnar,[1] Gabi Bartelmes,[1] Iris Dinkelacker,[1] Hanh Witte,[1] and Ralf J. Sommer*[,1]

[1]Department for Evolutionary Biology, Max-Planck Institute for Developmental Biology, Tübingen, Germany

***Corresponding author:** E-mail: ralf.sommer@tuebingen.mpg.de.

Associate editor: Patricia Beldade

Abstract

Evolutionary reconstruction of the natural history of an organism ultimately requires knowledge about the development, population genetics, ecology, and phylogeny of the species. Such investigations would benefit from studies of mutational processes because mutations are the source of natural variation. The nematode *Pristionchus pacificus* has been developed as a model organism in evolutionary biology by comparing its development with *Caenorhabditis elegans*. *Pristionchus pacificus* and related species are associated with scarab beetles, and their ecology and phylogeny are well known. More than 200 *P. pacificus* isolates from all over the world are available for this cosmopolitan species. We generated mutation accumulation (MA) lines in *P. pacificus* to study spontaneous mutation rates in the mitochondrial genome and compared mutation rate estimates with natural variation between nine representative isolates of the species. The *P. pacificus* mitochondrial genome is 15,955 bp in length and is typical for nematodes. *Pristionchus pacificus* has all known mitochondrial genes and contains an unusual suppressor transfer RNA (tRNA) for the codon UAA. This has most likely influenced the spectrum of observable mutations because 6 of 12 mutations found in the 82 MA lines analyzed are nonsense mutations that can be suppressed by the suppressor tRNA. The overall mutation rate in *P. pacificus* is 7.6×10^{-8} per site per generation and is less than one order of magnitude different from estimates in *C. elegans* and *Drosophila*. Using this mutation rate estimate in a comparison of the mitochondrial genome of nine *P. pacificus* isolates, we calculate the minimum time to the most recent common ancestor at 10^5–10^6 generations. The combination of mutation rate analysis with intraspecific divergence provides a powerful tool for the reconstruction of the natural history of *P. pacificus*, and we discuss the ecological implication of these findings.

Key words: mutation accumulation lines, *Pristionchus pacificus*, mitochondrial DNA, divergence times, *Caenorhabditis elegans*.

Introduction

The appearance of new mutations and the analysis of mutation rates are crucial for any attempt to build a comprehensive understanding of the evolution of animal and plant species (Lynch 2007). In combination with phylogenetics, population genetics, and ecology, knowledge of mutational processes can provide the basis for estimating the rate of molecular evolution, the effective population size, and the natural history of a given species (Charlesworth 2009). Indirect estimates of mutational rates depend on levels of divergence or polymorphisms and assume that mutations are effectively neutral or need the quantification of diverse fitness traits in mutation accumulation (MA) line experiments (see e.g., Vassilieva and Lynch 1999; Baer et al. 2005). With MA line experiments, it is also possible to make direct estimates of minimum mutation rates (Lynch 2010). These experiments are limited to a small number of experimental model organisms, such as the rhabditid nematodes *Caenorhabditis elegans* (Denver et al. 2000, 2004) and *C. briggsae* (Baer et al. 2005; Howe et al. 2010), *Drosophila melanogaster* (Mukai 1964; Haag-Liautard et al. 2007, 2008), *Daphnia pulex* (Seyfert et al. 2008), *Arabidopsis thaliana* (Ossowski et al. 2010), *Escherichia coli* (Barrick et al. 2009), and *Saccharomyces cerevisiae* (Lynch et al. 2008). In a MA line experimental setup, the population suffers a drastic bottleneck for many generations, assuring that all but the most deleterious mutations accumulate in a nearly neutral fashion (Vassilieva and Lynch 1999). Herein, we have used a MA line experiment in the nematode *Pristionchus pacificus* and study mutations in the mitochondrial genome. We use the frequency of mitochondrial mutations obtained after 142 generations of inbreeding in 82 MA lines to interpret the natural variation of the corresponding mitochondrial sequences in nine *P. pacificus* wild isolates.

Animal mitochondrial genomes differ from nuclear DNA in many ways, with hypermutability being the most striking character (Lynch 2007). The mitochondrial DNA (mtDNA) evolves rapidly in population, and it is usually transmitted maternally without intermolecular recombination (but see Zouros et al. 1992; Howe et al. 2010), and therefore, it has been intensively used for phylogeographic studies (Avise 1998) for more than three decades. Usually, mitochondrial mutation rates are one order of magnitude higher than mutation rates in the nuclear genome. These high mutation rates resulted in the development of mtDNA as predominant marker for studies of biodiversity, phylogeny, and natural variation between populations. Metazoan mtDNA is usually a circular molecule between 14 and

Table 1. Mitochondrial Genome Size and Gene Content Overview of Representative Nematodes.

Nematode	Clade: Blaxter et al. (1998)	Clade: Megen et al. (2009)	Size (bp)	Noncoding Nucleotides (bp)[a]	Gene Content: PCGs	Gene Content: tRNAs	Gene Content: rRNAs	Reference
Pristionchus pacificus	V	9A	15,955	2,772	12	22 + $tRNA^{Sup}$	2	Present study
Caenorhabditis elegans	V	9A	13,794	575	12	22	2	Okimoto et al. (1992)
Ancylostoma duodenale	V	9B	13,721	513	12	22	2	Hu et al. (2002)
Necator americanus	V	9B	13,604	387	12	22	2	Hu et al. (2002)
Steinernema carpocapsae	IV	10A	13,925	696	12	22	2	Montiel et al. (2006)
Strongyloides stercoralis	IV	10B	13,758	575	12	22	2	Hu et al. (2003)
Meloidogyne javanica	IV	12B	20,565	7,000	12	22	2	Okimoto et al. (1991)
Radopholus similis[b]	IV	12B	16,791	3,904	12	22	2	Jacob et al. (2009)
Onchocerca volvulus	III	8B	13,747	357	12	22	2	Keddie et al. (1998)
Ascaris suum	III	8B	14,284	1,048	12	22	2	Okimoto et al. (1992)
Trichinella spiralis[c]	I	2A	21,000–24,000	8,098–10,098	13	22 + $tRNA^{Met2}$	2	Lavrov and Brown (2001)
Xiphinema americanum[c]	I	2C	12,626	305	12	17	2	He et al. (2005)

NOTE.—PCG, protein-coding genes.
[a] Sum of the bp included in the D-loop and other noncoding nucleotide regions longer than 10 bp.
[b] $UAA\text{-}tRNA^{STOP}$ to $UAA\text{-}tRNA^{Tyr}$ reassignment
[c] The mtDNA is transcribed bidirectional.

42 kb containing 36–37 genes, including 12–13 protein-coding genes for the various subunits of the enzyme complexes of the respiratory chain, two ribosomal RNA (rRNA) genes, and 22 transfer RNA (tRNA) genes (Wolstenholme 1992). Despite this generally conserved organization, there is substantial variation in size, which is attributable to differences in the noncoding regions. The mtDNA contains at least one large noncoding region called the D-loop, or control region, which contains the origin of replication of the DNA molecule. Nematode mitochondrial genomes are very compact and contain relatively short rRNA molecules and truncated tRNAs (table 1). The 45 complete nematode mtDNAs sequenced to date show a large proportion of variability: The gene *atp8* is absent from all the mtDNAs of sampled nematodes, except one species, *Trichinella spiralis* (Lavrov and Brown 2001); *Globodera pallida* presents a multipartite mitochondrial genome (Armstrong et al. 2000); pseudogenes are present in *C. briggsae* (Howe and Denver 2008); and repeated genes are present in the mitochondrial genome of *Romanomermis culicivorax*, which also exhibits an exceptionally large mtDNA of 26–32 kb (Hyman and Azevedo 1996).

The diplogastrid nematode *P. pacificus* has been developed as a model system in evolutionary biology based on its short generation time, hermaphroditic, predominantly selfing mode of reproduction, easy laboratory culture, and other technical advantages, which allow a detailed comparison with *C. elegans* (Sommer et al. 1996; Hong and Sommer 2006). With the availability of forward and reverse genetics and DNA-mediated transformation (Schlager et al. 2009), functional studies of many biological processes are feasible. Original work concentrated on evolutionary developmental biology (evo-devo), more specifically on the signaling pathways involved in the development of the vulva (Zheng et al. 2005; Tian et al. 2008). More recent studies on *P. pacificus* extend to other areas in evolutionary biology with the aim to link evo-devo with population genetics and ecology (Sommer 2009). Phylogenetic studies have robustly inferred the position of *P. pacificus* within the family Diplogastridae (Mayer et al. 2009) and within the genus *Pristionchus* (Mayer et al. 2007).

Ecological studies in *Pristionchus* have shown that these nematodes live in close association with scarab beetles (Herrmann et al. 2006a). Strains of *P. pacificus* have been found on *Exomala orientalis* (Oriental beetle) in Japan and the United States (Herrmann et al. 2007) and, more recently, on *Oryctes borbonicus* (Rhinoceros beetle) and other scarab beetles on La Réunion in the Indian Ocean (Herrmann et al. 2010). A collection of more than 200 *P. pacificus* isolates from around the world is currently available for laboratory study. To provide a better account of the natural history of *P. pacificus*, we compared the mtDNA of nine *P. pacificus* strains and combined this knowledge of mitochondrial genetic variation with MA line–derived mutation rate estimates. We evaluate the mutation rate in the MA lines and derive dates of divergence by applying the internal molecular clock calibration to substitution rates inferred for the mitochondrial genome of the *P. pacificus* strains.

Materials and Methods

Strains

A list of all *P. pacificus* strains used in this study is provided in table 2.

MA Lines

MA lines were generated as depicted in figure 1, following the protocol of Vassilieva and Lynch (1999), from a single individual of the wild-type *P. pacificus* strain from California (PS312). Worms were cultured at 20 °C on nematode growth medium seeded with *E. coli* as a food source, as originally described for *P. pacificus* (Sommer et al. 1996). This line has been maintained in the lab for many generations

Table 2. Strains of *Pristionchus pacificus* Used in This Study.

	Sample Location	Year	Sample Type	Reference
PS312	Pasadena (California), USA	1988	Soil	Sommer et al. (1996)
RS5416	Saint Benoit, La Réunion	2009	*Maladera affinis*	Herrmann et al. (2010)
RS5211	Japan	2006	*Exomala orientalis*	Herrmann et al. (2010)
RS5282	Antalya, Turkey	2007	Soil	Herrmann et al. (2010)
JU150	Antananarivo, Madagascar	1999	Soil	Zauner and Sommer (2007)
RS5399	Trois Bassins, La Réunion	2009	*Oryctes borbonicus*	Herrmann et al. (2010)
RS5361	Nez du Boef, La Réunion	2010	Soil	Herrmann et al. (2010)
RS5275	Santa Cruz, Bolivia	2006	Scarab beetles	Hong et al. (2008)
RS5340	Basse Vallé, La Réunion	2008	*Adoretus* sp.	Herrmann et al. (2010)

before the experiment. We initiated the experiment by establishing 100 clonal lines from a single hermaphrodite that was inbred for three generations. Each MA line was propagated across 142 generations as a single randomly picked L3 stage worm from the middle of the reproductive period of the previous generation. Two previous generations for each line were kept at 15 °C in order to prevent accidental loss of the lines. From the original 100 lines, 82 survived until the 142nd generation. All the lines were frozen at generations 1, 27, 70, and 142.

DNA Extraction, Amplification, and Sequencing

For each MA line, we prepared genomic DNA from two plates of worms using worm lysis buffer (50 mM KCl, 10 mM Tris–HCl pH 8.3, 2.5 mM $MgCl_2$, 0.45% NP-40, 0.45% Tween-20, and 5 μg/ml proteinase K) and incubated the suspension for 2 h at 65 °C, followed by inactivation of the proteinase K at 95 °C for 10 min. The DNA was diluted to approximate 25 ng/μl for the polymerase chain reactions (PCRs). The entire mtDNA (except the D-loop) was amplified from total genomic DNA following two procedures: 1) small amplicons (500–1,000 bp) in 20 μl reactions using Taq (New England BioLabs) and 2) long amplicons (2,500–3,000 bp) using Expand Long Range dNTPack (Roche). A list of primers is available in supplementary table S1, Supplementary Material online. If necessary, additional primers were used to ensure reliable amplification of the entire mtDNA. The small amplicons were sequenced with the PCR primers using a ABI3730*xl* capillary platform. We used the software SeqMan from DNAStar Inc. to visualize the trace files, align the trace files, and assemble the mtDNA for each line independently. The long PCR products were visualized on 1.5% agarose gels to ensure amplification success and screen for large heteroplasmic deletion events. For the *P. pacificus* strains, we prepared DNA from ten worms in 20 μl of lysis buffer with 160 μl final volume of DNA, which was diluted 1:3 in the PCR reactions. We used PCR primers designed for the MA lines.

Sequence Analyses

We assembled all the generated sequences independently for each MA line and strain using SeqMan (DNASTAR, Inc.). All the trace files were visually inspected. Each single nucleotide polymorphism or indel in the MA lines was called if two independent PCR reactions verified the change. MA line base substitutions were marked as heteroplasmic if there was any evidence of the original progenitor base present in the chromatogram data. The frequencies of the mutant bases were estimated using the comparative peak height approach applied previously for the analysis of *D. melanogaster* MA line mtDNA divergence (Haag-Liautard et al. 2008). In all cases, the correction was carried out on both strands, and the frequencies averaged. All the assembled mitochondrial genomes were aligned using MUSCLE (Edgar 2004). The protein-coding genes of *P. pacificus* mtDNA were assigned according to the open-reading frames in SeqBuilder (DNASTAR, Inc.), set for the invertebrate mitochondrial codon table, and by multiple alignments using *C. elegans* and *Ascaris suum* mitochondrial genes for determining the initiation and termination codons. Nineteen of the 22 tRNA genes were identified by tRNAscan-SE 1.21 (http://lowelab.ucsc.edu/tRNAscan-SE/) (Lowe and Eddy 1997) using the nematode mitochondria as source, strict search mode, and cove score

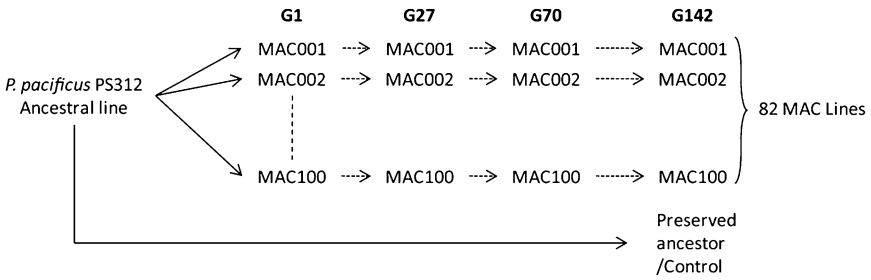


Fig. 1. Schematic representation of the experimental set up for the MA lines. 100 progeny from a single virgin hermaphrodite were used to start the experiment. At generations G1, G27, G70, and after the final generation G142, worms were frozen down to preserve the genomes of the mutated lines. A total of 82 lines survived the experiment.

cutoff of 20. With the cove score of 42.6, the $tRNA^{Tyr}$ has the highest cove score and the ones with the lowest scores are the $tRNA^{Arg}$ with 23.1 and $tRNA^{Sup}$ with 20.6. The secondary structures were determined in tRNAscan-SE. The remaining tRNA genes ($tRNA^{Ser(UCN)}$, $tRNA^{Ser(AGN)}$, and $tRNA^{Ile}$) were found based on alignments with the expected regions of the *C. elegans* mtDNA. The two rRNA genes were detected according to alignments with the *C. elegans* rRNA genes. The analyses of the pairwise comparison for the protein-coding genes in the intraspecific studies were performed using DnaSP v.5.10.01 (Librado and Rozas 2009), with the genetic code set to correspond to nematode mtDNA.

Mutation Rate Estimates

Mutation rates (per site per generation) were calculated as $\mu = m/(Lnt)$, and standard errors as $[\mu/(Lnt)]^{1/2}$, where m is the number of mutations, L is the number of lines, n is the number of base pairs analyzed per line, and t is the time in generations, as previously described (Denver et al. 2000). Heteroplasmy was inferred from the peak height and the frequencies calculated following equation (1) from Haag-Liautard et al. 2008. Taking this into account, we use equation (2) from Haag-Liautard et al. 2008 to estimate the overall mutation rate. We consider all mutations equally likely because the available number of mutations obtained in the MA line experiments does not yet justify to select any of the differential mutational models over any other model.

TMRCA Estimations

We used BEAST v1.6.1 (Drummond and Rambaut 2007) to estimate divergence times from the coding region of mtDNA data set for *P. pacificus* strains. We analyzed the data in three ways: 1) by third codon position, 2) partitioned according to codon position, and 3) by the entire coding region. The third codon data set consisted of 3,417 sites. For the partitioned data set, we created two alignments, one consisting of only the first and second codon positions (6,834 sites) and another one containing only the third codon position (3,417 sites). This is a proxy for the codon-based models that incorporate information about the genetic code (Shapiro et al. 2006). For the entire coding region, we used all 10,251 sites. For all alignments, we used the Bayesian Inference Criterion (Kass and Raftery 1995) to estimate the best-fitting model in jModelTest 0.1.1 (Guindon and Gascuel 2003; Posada 2008). All calculations were made assuming a strict molecular clock on all branches.

The taxon sets and implicitly the nodes for the divergence calculations were selected based on previously available information from two mitochondrial genes *nad6* and *nad4L* (Herrmann et al. 2010). For the divergence estimations, we allowed BEAST to infer topology of the trees. We included two speciation process models, birth–death (Gernhard 2008) and pure Yule process. We used Bayes factor as implemented in Tracer v1.5 (Drummond and Rambaut 2007) to select the best-fitting model under smoothed marginal likelihood estimate and with 100 bootstrap replicates (Suchard et al. 2001). In all cases, the birth–death was preferred by the Bayes factor analysis. Runs were initiated on random starting trees. A total of five independent runs of 10 million generations each were performed locally, sampling every 1000th generation, for each of the data sets. Convergence was assessed with Tracer v1.5. After discarding the first 1,000 samples as burn-in, the trees and parameter estimates from the five runs were combined using LogCombiner v1.6.1. The results were considered reliable once the effective sampling size of all parameters was above 100. Using TreeAnnotator v1.6.1 (Drummond and Rambaut 2007), the samples from the posterior were summarized on the maximum credibility tree, with the posterior probability limit set to 0.5 and summarizing mean node heights. These were visualized using FigTree v1.3.1 (Drummond and Rambaut 2007). Means and 95% higher posterior densities of age estimates were obtained from the combined outputs using Tracer v1.5.

Results

MA Lines

To provide the basis for mutation rate and divergence time estimates in *P. pacificus*, we generated MA lines in the strain PS312 from Pasadena (California, USA) (Sommer et al. 1996). This strain has been selected because it has been characterized in great detail and has been used as "wild-type" strain for all the developmental biology work carried out in the lab (Zheng et al. 2005; Tian et al. 2008). In addition, *P. pacificus* PS312 was sequenced with 9× coverage, and transcriptome and proteome date are available (Dieterich et al. 2008; Borchert et al. 2010). We initiated 100 MA lines of which 82 survived the 142 generations of single progeny propagation, giving a survival rate of 87.3% per 100 generations (fig. 1). In *C. elegans*, 87.8% of the MA lines survived for 100 generations, indicating a similar survival rate as observed in *P. pacificus* (Denver et al. 2000). From now on we refer to the MA lines of *P. pacificus* California as mutation accumulation lines California (MAC lines).

Mitochondrial Genome of *P. pacificus*

The mitochondrial genome of *P. pacificus* is 15,954 bp in size and is similar to other known nematode mitochondrial genomes (table 1 and fig. 2). It contains 12 protein-coding genes, two rRNAs, and 22 predicted tRNAs, ranging from 53 bp ($tRNA^{Ser(UCN)}$) to 63 bp ($tRNA^{Lys}$) in length. All genes are transcribed unidirectionally from the coding strand, which has an asymmetrical nucleotide composition of 44.0% T, 32.2% A, 14.9% G, and 8.9% C. The high AT composition is also reflected at the level of the individual genes, where *nad2* is 82.8% AT, whereas *cox1* is only 70.2%, these being the extremes encountered in the mtDNA of *P. pacificus*. Almost one-sixth of the genome is noncoding, comprising two large regions, the D-loop of 2,222 bp (76.33% AT) and a second region of 425 bp (75.76% AT), situated between $tRNA^{Asp}$ and $tRNA^{Gly}$. Other small intergenic regions are found, the biggest being the 66 bp region,

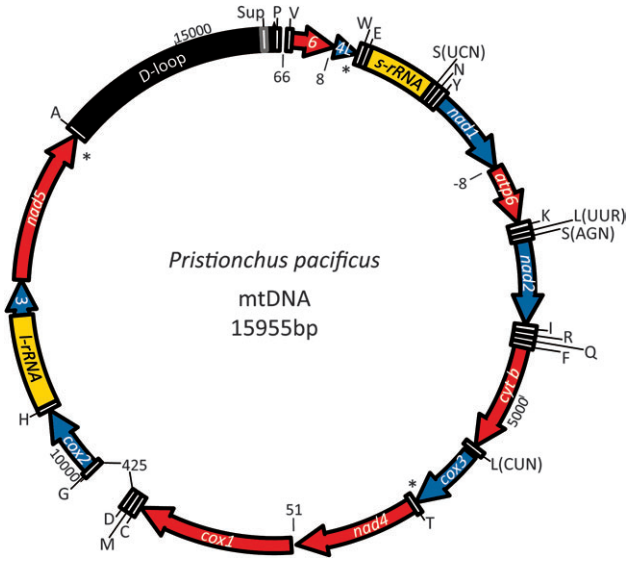


FIG. 2. Overview of the organization of the circular mtDNA of *Pristionchus pacificus*. The genes are colored according to the reading frame with red, green, and blue representing the first, second, and third reading frame, respectively, and s-rRNA and l-rRNA are represented in yellow. The tRNA genes, called by their amino acid symbol (Sup: Suppressor tRNA), are represented with white rectangles. Numbers at the inner periphery of the ring represent noncoding or overlapping nucleotides between neighboring genes. Numbers at the outer periphery indicate the length in base pairs. The incomplete termination codons are symbolized by the asterisk (*); 6, 4L, and 3 represent the genes *nad6*, *nad4L*, and *nad3*, respectively.

between $tRNA^{Pro}$ and $tRNA^{Val}$ and a 51 bp region between *nad4* and *cox1* (fig. 2). In *P. pacificus*, only 17.9% of the D-loop sequence consists of repeated sequences, contrary to *C. elegans*, where 55.4% of the D-loop sequence consists of different types of repeats (Okimoto et al. 1992). Specifically, in the case of *P. pacificus*, there is a run of 20 AT dinucleotides repeated in tandem. In addition, there are two repeats consisting each of 68, 27, and 12 nt, respectively, and two inverted repeats of 22 and 15 nt, respectively.

The mtDNA of *P. pacificus* has two characteristic features. First, the intergenic region situated between $tRNA^{Pro}$ and $tRNA^{Val}$, where Blast searches do not identify any homologous sequences. It is formed by a series of T:A and A:T homopolymeric stretches with a maximum of ten repeated nucleotides, but no microsatellite-like (di-, tri-, and tetranucleotide repeat) sequences have been identified. Second, the presence of a suppressor tRNA for the codon UAA, situated within the D-loop (fig. 2). The predicted folding of this suppressor tRNA, based on the tRNAscan-SE 1.21 software (Lowe and Eddy 1997), suggests this tRNA to have the typical truncated shape of nematode tRNAs (supplementary fig. S1, Supplementary Material online), although Blast search fails to identify any homologous sequence. The origin of this tRNA is unknown, but part of its sequence can be explained by two tandemly repeated sequences of 14 nt followed by two incomplete repeats of the same kind. UAA encodes a STOP codon in all the protein-coding genes in the *P. pacificus* mitochondrial genome, except for those genes that finish with an incomplete codon of a single U (*nad4L* or *nad5*) or a UA dinucleotide (*nad2* and *cox3*), that are possibly completed after polyadenylation (Ojala et al. 1981). As initiation codons, four of the six known possible alternatives are used in *P. pacificus*: AUU (*nad4L*, *nad1*, *atp6*, *cox3*, *nad3*, and *nad5*), AUA (*nad6*, *cyt b*, and *cox1*), UUG (*nad2* and *nad4*), and AUC (*cox2*).

The coding region of the mtDNA of *P. pacificus* consists of 3,417 codons, 45.3% are composed only of the nucleotides T and A, and in 73.9% of the codons contain at least one T or A. The most frequently used codon is UUU (11.7%), whereas the most abundant amino acid is leucine (14.5%). Of all the codons specifying leucine, 70% are UUA, the only leucine codon to contain only U and A. The least frequently used amino acid is arginine (0.9%). In general, the analysis of the relative synonymous codon usage shows that within each family, the codons with the highest content of A and U are preferred in the *P. pacificus* mitochondrial genome. Three codons are not used at all: GCG (alanine), CUC (leucine), and CGC (arginine), which are the most CG-rich codons of the families.

Mutation Rates and Spectrum

We made a direct estimate of the mutation rate in the mitochondrial genome in *P. pacificus* MA lines by sequencing 13,500 bp in 82 MA lines at 142nd generation. Among the 1,107,000 bp sequenced, we observed 12 mutations (table 3) resulting in a total mutation rate $\mu_{total} = 7.6 \times 10^{-8}$ ($\pm 2.2 \times 10^{-8}$) per site per generation.

Seven of these mutations were substitutions (table 3A), yielding a direct estimate of the mitochondrial mutation rate for base substitutions $\mu_{bs} = 4.5 \times 10^{-8}$ ($\pm 1.7 \times 10^{-8}$) per site per generation. This rate is lower than (but not significantly different from) the equivalent data for *C. elegans* of 9.7×10^{-8} per site per generation (Denver et al. 2000), *C. briggsae* (7.2×10^{-8} or 1.1×10^{-7} per site per generation depending of the strain used in the experiment) (Howe et al. 2010), and the mutation rate reported for *D. melanogaster* (mean mutation rate 6.2×10^{-8} per site per generation) (Haag-Liautard et al. 2008). The seven base substitutions found in the MA lines of *P. pacificus* occurred at different positions in the genome and in seven different lines, which suggests that there is no mutational hot spot in the nucleotides analyzed and that none of the lines is particularly predisposed to mitochondrial mutations. The mutation rate calculated for all mutations except the ones associated with repetitive sequence is 3.2×10^{-8} per site per generation (table 3A and B), which gives a mean number of mutations per line of 0.0609. Assuming a Poisson distribution, the expected number of lines with 0, 1, or 2 mutations is 77, 5, and 0, respectively. These values are almost identical to the observed numbers, where the number of lines with 0, 1, or 2 mutations are 71, 10, and 1, respectively, supporting the fact that there are no mutational hot spots, nor contamination. We found five transitions and two transversions (table 3), a ratio that agrees with mtDNA evolution, as observed at the intra- and interspecies level and characterized by a strong bias toward transitions (A:T to G:C and C:G to T:A) (Denver et al. 2000).

Table 3. Mutations in the Mitochondrial Genomes of the Mutation Accumulation Lines.

Line	Position	Mutation	Gene	Effect
A: Base substitution mutations				
MAC55[a]	1,631	A:T → G:C	*s-rRNA*	
MAC62[a]	2,834	C:G → T:A	*atp6*	Pro → Leu
MAC04	3,781	T:A → A:T	*nad2*	Leu → STOP
MAC30[a]	3,840	C:G → T:A	*nad2*	Silent
MAC24	4,587	A:T → G:C	*cyt b*	Silent
MAC06	11,636	G:C → T:A	*l-rRNA*	
MAC16	12,993	T:A → C:G	*nad5*	Silent
B: Indels				
MAC27	5,199	+1 T:A	*cyt b*	Trp → Cys + premature STOP
C: Homopolymer mutations				
MAC19	263	$(T:A)_8 \rightarrow (T:A)_7$	*nad6*	Leu → Trp + premature STOP
MAC78	6,336	$(T:A)_6 \rightarrow (T:A)_5$	*cox3*	Phe → Leu + premature STOP
MAC74	7,009	$(T:A)_6 \rightarrow (T:A)_5$	*nad4*	Phe → Leu + premature STOP
MAC16	12,351	$(T:A)_5 \rightarrow (T:A)_6$	*nad5*	Val → Cys + premature STOP

NOTE.—Positions denoted are with respect to the progenitor mtDNA sequence. MAC, MA lines California.
[a] Heteroplasmic mutations.

In addition to the seven base substitutions, we found five insertion–deletion (indel) mutations, yielding the indel mutation rate at $\mu_{indel} = 3.2 \times 10^{-8}$ ($\pm 1.4 \times 10^{-8}$), and four of them were associated with T:A homopolymeric nucleotide runs. Surprisingly, the longest homopolymers (9–11 bp T:A homopolymers) seem to be very stable in all the MA lines, regardless of their position in the mtDNA. The longest homopolymeric stretch mutated in *P. pacificus* is $(T:A)_8 \rightarrow (T:A)_7$, situated in the *nad6* gene. All the indel mutations are predicted to change the coding function of the respective genes (table 3B and C). Specifically, a single base pair insertion in a stretch of five T:A residues resulted in a premature STOP codon (one amino acid upstream the mutation) and reduced the NADH dehydrogenase subunit 5–87 amino acids lacking the conserved domain. Single base pair deletions occurred in a stretch of eight residues in the *nad6* gene and in a stretch of six residues each in *cox3* and *nad4*, all three of which resulted in premature STOP codon. One single base pair insertion occurred in a region without any type of repeat in the *cyt b* gene, resulting in an amino acid change and a premature STOP codon. In addition to these five mutations, one of the base substitution mutations (T:A to A:T in the line MAC04) resulted in a direct STOP mutation (table 3) in the *nad2* gene. Thus, 6 of the 12 mutations resulted in STOP codon mutations. The amount of viable mutations leading to premature STOP codons in *P. pacificus* MA lines is substantially higher than in *C. elegans* and *Drosophila* but similar than in *C. briggsae*. We speculate that the presence of the suppressor tRNA has influenced the spectrum of viable mutations in this organism (see Discussion).

The segregation of mitochondrial genomes allows for heteroplasmy, a phenomenon observed in multiple species. In *P. pacificus*, heteroplasmic sites were detected at three positions, all of them being single base pairs sites (table 3A). This differs from *C. briggsae*, where large heteroplasmic deletions have been found (Howe et al. 2010). The frequency of the heteroplasmic sites in *P. pacificus* were 0.4 for the MAC55 and MAC62, respectively and 0.3 for MAC30. Using these corrections, the total mutation rate is 6.4×10^{-8} per site per generation, a value that is in the error interval of our calculations.

Strains

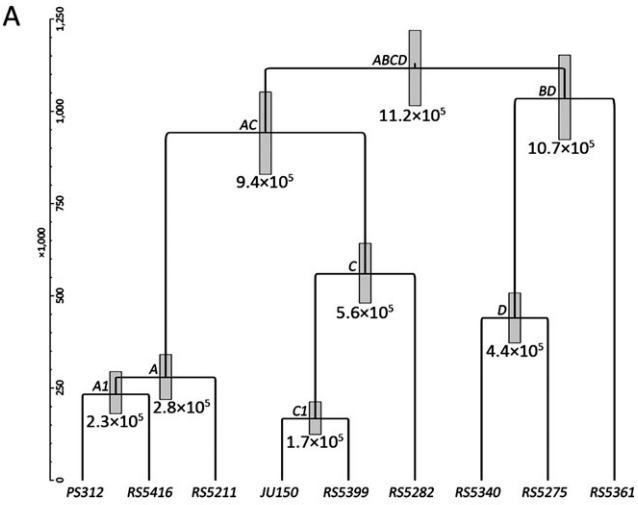
To study the genetic variation in the mtDNA of *P. pacificus* wild isolates, we have selected eight representative strains based on the available material from around the world to compare it to PS312 (table 2) (Herrmann et al. 2010). We amplified the entire mtDNA except for the D-loop for each strain with primers designed on the reference strain and then calculated the nucleotide differences relative to *P. pacificus* PS312. In the metazoan mtDNA, the D-loop is considered to be the most variable region, in addition to having a high AT content, making it very hard to amplify. We have been unable to amplify the D-loop from the other strains, even by using multiple alternative PCR primer sets. For all *P. pacificus* strains, the mtDNA coding region sums up to 10,281 bp, with very little variation in the AT content, at both the level of the entire coding region and the level of individual genes. The codon usage is similar between the tested strains with only a few exceptions: GCG (arginine) is used in all the strains except RS5361 and RS5211, the strain RS5340 is the only one that uses the codon CTC (leucine), and RS5275 is the only one that uses CGC (arginine). The genes *nad4L*, *nad2*, *cox3*, and *nad5* end with incomplete STOP codons in all the strains indicating that this feature of the *P. pacificus* mtDNA is conserved between wild isolates.

The comparison of synonymous and nonsynonymous changes between the different strains of *P. pacificus* (table 4) suggests that the strain RS5416 from the island of La Réunion is most closely related to PS312 with 165 (1.6%) synonymous and 30 (0.3%) nonsynonymous changes. The most distantly related strain is RS5399, also from La Réunion, with 482 (4.7%) synonymous and 45 (0.4%) nonsynonymous changes. These findings support the original observation of an unusually high genetic diversity of *P. pacificus* strains found on the island of La Réunion

(Herrmann et al. 2010). More general, these findings provide strong evidence for purifying selection with a magnitude similar to that reported for *C. elegans* and *C. briggsae* natural isolates (Howe and Denver 2008).

To obtain first insight into minimal divergence time estimates in *P. pacificus*, the information on the genetic variation in the mtDNA of wild isolates can be related to the mutation rate estimates from the MA line experiment. For the Bayesian analysis of the *P. pacificus* strains, we considered the first and second codon positions separately from the third codon position. We analyzed the data set in multiple ways and compare divergence time estimates obtained from three different procedures (see Materials and Methods). In a first approach, we only considered the third codon position, assuming complete neutrality (Cutter 2008). Considering the near absence of selection in the MA lines, we have used the overall mutation rate of 7.6×10^{-8} per site per generation for this analysis. In a second model, we partitioned the data set into two alignments composed of the first and second codon position together and, separately from that, the third codon position. This approach has the advantage of not eliminating the neutral mutations at the first and second codon positions. In a third model, we considered the entire coding region of the mtDNA.

The analysis at the third codon position yielded a point estimate of the time to the most recent common ancestor (TMRCA) for PS312 and its closest relative, RS5416 from La Réunion, of 2.9×10^5 generations (fig. 3). The most recent split occurred between the strain JU150 for Madagascar and RS5399 also from La Réunion, 2.3×10^5 generations ago. The divergence times of the other nodes show that the deepest analyzed divergence between the different strains of *P. pacificus* was 1.2×10^6 generations ago. Using the entire coding region or data partitioned by codon position does not significantly change the divergence time estimates (fig. 3*B*). For example, estimates based on the second model predict the divergence between PS312 and RS5416 to be 2.3×10^5 generations and the deepest split among all strains to be 1.1×10^6. Taken together, the divergence time estimates under these three models do not change significantly resulting in consistent relationships among strains and suggest a TMRCA at 10^5–10^6 generations (fig. 3*B*).



B

Dataset	A1 G×10^5	A G×10^5	AC G×10^5	C1 G×10^5	C G×10^5	D G×10^5	BD G×10^5	ABCD G×10^5
	Time to the Most Recent Common Ancestor							
I) 3rd codon position								
	2.9	3.4	10.7	2.3	6.0	4.4	10.9	12.0
95% HPD lower	2.5	3.0	9.4	1.9	5.2	3.9	9.3	11.0
95% HPD upper	3.4	4.0	12.5	2.8	6.9	5.1	12.4	13.0
II) Partitioned by codon position								
	2.3	**2.8**	**9.4**	**1.7**	**5.6**	**4.4**	**10.7**	**11.2**
95% HPD lower	1.8	2.2	8.3	1.2	4.8	3.7	9.3	10.1
95% HPD upper	2.9	3.4	1.1	2.1	6.4	5.1	12.2	12.2
III) Coding								
	1.2	1.4	4.3	0.9	2.4	1.8	4.5	4.7
95% HPD lower	1.0	1.2	3.7	0.7	2.1	1.5	3.9	4.3
95% HPD upper	1.4	1.6	5.0	1.1	2.7	2.0	5.0	5.1

Fig. 3. Phylogenetic relationship of *Pristionchus pacificus* isolates and time to the most recent common ancestor. (*A*) Phylogenetic relationship of the nine *P. pacificus* isolates based on the coding sequence of the mtDNA. The scale indicates the time in generations. The letters in the phylogram indicate the nodes for divergence calculations. (*B*) TMRCA for all eight nodes in generations. Calculations are based on 1) the third codon position, 2) the data set partitioned by codon position, and 3) the entire coding region. In bold are numbers represented in *A*.

Discussion

In this study, we provide mutation rate estimates of the mitochondrial genome of *P. pacificus* after 142 generations of inbreeding in 82 MA lines and compare these data with the genetic variation of the mtDNA of nine *P. pacificus* strains. The *P. pacificus* mitochondrial genome is 15,954 bp in size and is typical for nematodes, the difference in size resulting from the length of the noncoding regions (table 2). Almost one-sixth of the genome is noncoding, comprising two large and a number of small intergenic regions, from which, of additional importance, is the 51 bp region between *nad4* and *cox1*. This seems to be homologous to a region that in *C. elegans* has 109 bp, in *A. suum*, 117 bp (Okimoto et al. 1992), and in *Steinernema carpocapsae*, 44 bp (Montiel et al. 2006), located in the same boundaries in all species. Also, it can be folded in a hairpin structure with a run of four Ts in the loop. Such hairpin structures have been found in the noncoding regions of mtDNAs of other species, and in human and mouse, it has been shown to be the initiation sites of the synthesis of the second (L) strand of the molecule (Chang et al. 1985).

Although *P. pacificus* contains all known mitochondrial genes, the presence of a suppressor tRNA for the codon UAA is unusual. The presence of such a suppressor tRNA has most likely influenced the spectrum of observable mutations in the MA line experiments. Of the 12 obtained

Table 4. Pairwise Differences Between the Strains and Genes of *Pristionchus pacificus* Used in This Study.

Genes in *P. pacificus*	Length (bp)	Nonsynonymous/Synonymous Differences Relative to PS312							
	PS312	RS5416	RS5211	JU150	RS5399	RS5282	RS5340	RS5275	RS5361
nad6	435	3/8	3/9	4/19	5/19	9/19	8/20	5/21	6/19
nad4L[a]	232	1/3	1/3	1/10	2/9	0/8	0/7	0/9	3/12
nad1	876	1/12	1/11	0/21	1/24	3/41	2/38	2/42	4/42
atp6	600	1/5	0/2	2/7	1/6	2/22	0/21	0/16	0/17
nad2[a]	845	3/13	5/14	6/32	7/32	4/51	4/47	5/49	4/44
cyt b	1,104	2/13	5/14	5/34	5/44	6/34	5/38	3/45	6/40
cox3[a]	767	0/16	0/16	1/28	1/29	1/31	0/36	1/39	4/38
nad4	1,230	3/17	3/21	7/51	6/52	5/54	6/59	5/55	4/52
cox1	1,578	0/31	0/34	1/57	1/60	0/60	0/73	1/75	1/76
cox2	696	0/12	0/17	1/24	1/26	1/21	0/29	0/35	2/27
nad3	336	0/17	0/6	1/14	1/15	2/13	0/12	1/14	4/9
nad5[a]	1,582	7/28	5/29	5/95	7/95	5/97	8/96	6/93	7/106
Total	10,281	30/165	23/176	34/392	38/411	38/451	33/476	29/493	45/482
%AT[b]	75.93	75.86	75.84	75.62	75.62	75.85	75.92	75.97	75.39

[a] Incomplete STOP codon.
[b] Calculated only for the protein-coding regions.

mutations, six result in nonsense mutations, all of which can be suppressed by the suppressor tRNA. In *C. elegans* in comparison, similar MA line experiments resulted in the identification of 26 mutations after 214 generations in 74 MA lines, but only four of these mutations resulted in a premature STOP codon. However, given the small number of total mutations, it remains unclear if the values observed in *P. pacificus* and *C. elegans* are statistically different from one another. Considering the number of mutations, *P. pacificus* has nearly three times as many nonsense mutations than expected. From the *C. elegans* data set (Denver et al. 2000) of four nonsense mutations in a total of 26, only two nonsense mutations would have been expected for *P. pacificus*. In contrast, the number of expected and observed mutations in *P. pacificus* is the same when considering the number of nucleotides scanned; based on the four nonsense mutations in *C. elegans* from 10,428 bp in 74 lines, the expected number in *P. pacificus* from 13,500 bp in 82 lines would be six. Although the statistical significance of the different number of nonsense mutations remains open, we speculate that the *P. pacificus* nonsense mutations did not result in a lethal phenotype and were not subject to purifying selection. Thus, the presence of a suppressor tRNA apparently influenced the type and number of mutations that have been accumulated in *P. pacificus* MA lines. It should be noted however, that no premature STOP codons have been found in the mtDNA genes of the *P. pacificus* natural isolates, which argues for presumptive fitness affects under natural conditions.

The *P. pacificus* MA line–derived mutation rate is 7.6×10^{-8} ($\pm 2.2 \times 10^{-8}$) per site per generation, different than the values found in *C. elegans* (Denver et al. 2000) or in *C. briggsae* (Howe et al. 2010). However, the differences among these nematodes are not statistically significant. Recent studies indicated that mutation rates might vary according to the genetic background (Howe et al. 2010). Although this observation influences the estimation of divergence times, the real consequences cannot currently be evaluated. Future studies can reveal if features of the mitochondrial genomes of *C. elegans* and *P. pacificus* also hold true for nuclear genomes. One advantage of MA line experiments is that the resulting lines can be analyzed for multiple traits nearly indefinitely, making them a powerful tool for evolutionary investigations.

One major aim of this study was to relate genetic variation in wild isolates to the mutation rate estimates from MA lines in order to obtain first insight into minimal divergence times in *P. pacificus* (fig. 3). The divergence time estimates derived from the three tested models show consistent relationships among strains and suggest a minimal divergence of 10^5–10^6 generations. The number of generations that *P. pacificus* produces in nature each year depends on several biological and ecological parameters. From a theoretical perspective, this number might vary between 1 and 100 generations per year, with 100 generations representing the maximum number of generations that can be produced under optimal growth conditions when worms go through a direct life cycle without entering the arrested dauer stage (Sommer et al. 1996). In contrast, one generation per year represents the absolute minimum, which we have observed by studying the survival rate of *P. pacificus* dauer larvae (Mayer and Sommer 2011). Several considerations favor the hypothesis that the actual number of generations of *P. pacificus* per year is closer to our lower theoretical estimate than to the maximum number of generations that is observed in the laboratory. First, our field studies indicate that *Pristionchus* nematodes occur on scarab beetles exclusively in the arrested dauer stage (Weller et al. 2010). Second, we have no evidence that *Pristionchus* would reproduce on the living beetle; rather, they wait for the beetle's death in the dauer stage. Therefore, we assume that there is only one *Pristionchus* generation on a particular beetle (Herrmann et al. 2006a, 2007). Third, most scarab beetle species have very slow generation times, for example, the *P. pacificus* host *E. orientalis* in the USA and Japan has an annual life cycle (Herrmann et al. 2007). Fourth, our limited data on the soil reservoir of *Pristionchus* nematodes, which are based on studies in German

forests, also indicate that the majority of *Pristionchus* nematodes found in nature are in the dauer stage (Weller AM, Sommer RJ, unpublished observation). Finally, although our experimental work so far only provided evidence for a necromenic association with scarab beetles, we can formally not rule out that under certain conditions, *P. pacificus* might have a phoretic relationship with these beetles.

Assuming a number of one to ten generations per year, the data provided in this study would suggest that the divergence between the nine analyzed strains of *P. pacificus* is somewhere between 10^4–10^5 years. Considering a fairly complete sampling of major groups of haplotypes within the species, these estimates might represent the most basal divergence within *P. pacificus*. This number is consistent with the available molecular phylogeny (Mayer et al. 2007, 2009) as well as with the spectrum of beetle hosts, and the biogeography of islands that *P. pacificus* is found on (Herrmann et al. 2006a, 2006b, 2007, 2010). Further insight and confirmation of the natural history of the *P. pacificus* require the identification and characterization of a closely related sister species, an endeavor that is still ongoing.

Supplementary Material

Supplementary table S1 and figure S1 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

Acknowledgments

We thank Werner E. Mayer, Katy Morgan, Richard Neher, and Erik Ragsdale for critically reading the manuscript and for fruitful discussions, Dan Bumbarger, Angela McGaughran, Martin Mechelke, Michael Matschiner, Robbie Rae, Amit Sinha, and Adrian Streit for fruitful discussions. We would like to thank T. Eirich and S. Maeck for preparing the plates during the MA lines experiment and H. Haussmann for freezing the MAC lines. Accession codes: The nucleotide sequence reported here has been deposited at GenBank with the accession number JF414117. This work was funded by the Max-Planck Society.

References

Armstrong MR, Blok VC, Phillips MS. 2000. A multipartite mitochondrial genome in the potato cyst nematode *Globodera pallida*. *Genetics* 154:181–192.

Avise JC. 1998. The history and purview of phylogeography: a personal reflection. *Mol Ecol*. 7:371–379.

Baer CF, Shaw F, Steding C, et al. (11 authors). 2005. Comparative evolutionary genetics of spontaneous mutations affecting fitness in rhabditid nematodes. *Proc Natl Acad Sci U S A*. 102:5785–5790.

Barrick JE, Yu DS, Yoon SH, Jeong H, Oh TK, Schneider D, Lenski RE, Kim JF. 2009. Genome evolution and adaptation in a long-term experiment with *Escherichia coli*. *Nature* 461:1243–1247.

Blaxter ML, De Ley P, Garey JR, et al. (12 co-authors) 1998. A molecular evolutionary framework for the phylum Nematoda. *Nature* 392:71–75.

Borchert N, Dieterich C, Krug K, Schütz W, Jung S, Nordheim A, Sommer RJ, Macek B. 2010. Proteogenomics of *Pristionchus pacificus* reveals distinct proteome structure of nematode models. *Genome Res*. 20:837–846.

Chang DD, Hauswirth WW, Clayton DA. 1985. Replication priming and transcription initiate from precisely the same site in mouse mitochondrial DNA. *EMBO J*. 4:1559–1567.

Charlesworth B. 2009. Fundamental concepts in genetics: effective population size and patterns of molecular evolution and variation. *Nat Rev Genet*. 10:195–205.

Cutter AD. 2008. Divergence times in Caenorhabditis and Drosophila inferred from direct estimates of the neutral mutation rate. *Mol Biol Evol*. 25:778–786.

Denver DR, Morris K, Lynch M, Thomas WK. 2004. High mutation rate and predominance of insertions in the *Caenorhabditis elegans* nuclear genome. *Nature* 430:679–682.

Denver DR, Morris K, Lynch M, Vassilieva LL, Thomas WK. 2000. High direct estimate of the mutation rate in the mitochondrial genome of *Caenorhabditis elegans*. *Science* 289:2342–2344.

Dieterich C, Clifton SW, Schuster L, et al. (17 co-authors). 2008. The *Pristionchus pacificus* genome provides a unique perspective on nematode lifestyle and parasitism. *Nat Genet*. 40:1193–1198.

Drummond AJ, Rambaut A. 2007. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol Biol*. 7:214.

Edgar RC. 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics*. 5:113.

Gernhard T. 2008. The conditioned reconstructed process. *J Theor Biol*. 253:769–778.

Guindon S, Gascuel O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol*. 52:696–704.

Haag-Liautard C, Coffey N, Houle D, Lynch M, Charlesworth B, Keightley PD. 2008. Direct estimation of the mitochondrial DNA mutation rate in *Drosophila melanogaster*. *PLoS Biol*. 6:1706–1714.

Haag-Liautard C, Dorris M, Maside X, Macaskill S, Halligan DL, Houle D, Charlesworth B, Keightley PD. 2007. Direct estimation of per nucleotide and genomic deleterious mutation rates in *Drosophila*. *Nature* 445:82–85.

He Y, Jones J, Armstrong M, Lamberti F, Moens M. 2005. The mitochondrial genome of *Xiphinema americanum sensu stricto* (Nematoda: Enoplea): considerable economization in the length and structural features of encoded genes. *J Mol Evol*. 61:819–833.

Herrmann M, Kienle S, Rochat J, Mayer WE, Sommer RJ. 2010. Haplotype diversity of the nematode *Pristionchus pacificus* on Réunion in the Indian Ocean suggests multiple independent invasions. *Biol J Linn Soc*. 100:170–179.

Herrmann M, Mayer WE, Hong R, Kienle S, Minasaki R, Sommer RJ. 2007. The nematode *Pristionchus pacificus* (Nematoda: Diplogastridae) is associated with the oriental beetle *Exomala orientalis* (Coleoptera: Scarabaeidae) in Japan. *Zool Sci*. 24:883–889.

Herrmann M, Mayer WE, Sommer RJ. 2006a. Nematodes of the genus *Pristionchus* are closely associated with scarab beetles and the Colorado potato beetle in Western Europe. *Zoology*. 109:96–108.

Herrmann M, Mayer WE, Sommer RJ. 2006b. Sex, bugs and Haldane's rule: the nematode genus *Pristionchus* in the United States. *Front Zool*. 3:14.

Hong RL, Sommer RJ. 2006. *Pristionchus pacificus*: a well-rounded nematode. *BioEssays* 28:651–659.

Hong RL, Witte H, Sommer RJ. 2008. Natural variation in *P. pacificus* insect pheromone attraction involves the protein kinase EGL-4. *Proc Natl Acad Sci U S A*. 105:7779–7784.

Howe DK, Baer CF, Denver DR. 2010. High rate of large deletions in *Caenorhabditis briggsae* mitochondrial genome mutation processes. *Genome Biol Evol*. 2010:29–38.

Howe DK, Denver DR. 2008. Muller's Ratchet and compensatory mutation in *Caenorhabditis briggsae* mitochondrial genome evolution. *BMC Evol Biol*. 8:62.

Hu M, Chilton NB, Gasser RB. 2002. The mitochondrial genomes of the human hookworms, *Ancylostoma duodenale* and *Necator americanus* (Nematoda: Secernentea). *Int J Parasitol*. 32: 145–158.

Hu M, Chilton NB, Gasser RB. 2003. The mitochondrial genome of *Strongyloides stercoralis* (Nematoda) - idiosyncratic gene order and evolutionary implications. *Int J Parasitol*. 33: 1393–1408.

Hyman BC, Azevedo JLB. 1996. Similar evolutionary patterning nematode mitochondrial genes among repeated and single copy. *Mol Biol Evol*. 13:221–232.

Jacob JE, Vanholme B, Van Leeuwen T, Gheysen G. 2009. A unique genetic code change in the mitochondrial genome of the parasitic nematode *Radopholus similis*. *BMC Res Notes*. 2:192.

Kass R, Raftery A. 1995. Bayes factors. *J Am Stat Assoc*. 90:773–795.

Keddie EM, Higazi T, Unnasch TR. 1998. The mitochondrial genome of *Onchocerca volvulus*: sequence, structure and phylogenetic analysis. *Mol Biochem Parasitol*. 95:111–127.

Lavrov DV, Brown WM. 2001. *Trichinella spiralis* mtDNA: a nematode mitochondrial genome that encodes a putative ATP8 and normally structured tRNAs and has a gene arrangement relatable to those of coelomate metazoans. *Genetics* 157:621–637.

Librado P, Rozas J. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 25: 1451–1452.

Lowe TM, Eddy SR. 1997. tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. *Nucleic Acids Res*. 25:955–964.

Lynch M. 2007. The origin of genome architecture. Sunderland (MA): Sinauer.

Lynch M. 2010. Evolution of the mutation rate. *Trends Genet*. 26:345–352.

Lynch M, Sung W, Morris K, et al. (11 co-authors). 2008. A genome-wide view of the spectrum of spontaneous mutations in yeast. *Proc Natl Acad Sci U S A*. 105:9272–9277.

Mayer MG, Sommer RJ. 2011. Natural variation in *Pristionchus pacificus* dauer formation reveals cross-preference rather than self-preference of nematode dauer pheromones. *Proc R Soc B Biol Sci*. Advance access published February 9, 2011,doi:10.1098/rspb.2010.2760.

Mayer WE, Herrmann M, Sommer RJ. 2007. Phylogeny of the nematode genus *Pristionchus* and implications for biodiversity, biogeography and the evolution of hermaphroditism. *BMC Evol Biol*. 7:104.

Mayer WE, Herrmann M, Sommer RJ. 2009. Molecular phylogeny of beetle associated diplogastrid nematodes suggests host switching rather than nematode-beetle coevolution. *BMC Evol Biol*. 9:212.

Megen H van, Elsen S van den, Holterman M, Karssen G, Mooyman P, Bongers T, Holovachov O, Bakker J, Helder J. 2009. A phylogenetic tree of nematodes based on about 1200 full-length small subunit ribosomal DNA sequences. *Nematology* 11:927–950.

Montiel R, Lucena MA, Medeiros J, Simões N. 2006. The complete mitochondrial genome of the entomopathogenic nematode *Steinernema carpocapsae*: insights into nematode mitochondrial DNA evolution and phylogeny. *J Mol Evol*. 62:211–25.

Mukai T. 1964. The genetic structure of natural populations of *Drosophila melanogaster*. I. Spontaneous mutation rate of polygenes controlling viability. *Genetics* 50:1–19.

Ojala D, Montoya J, Attardi G. 1981. tRNA punctuation model of RNA processing in human mitochondria. *Nature* 290:470–474.

Okimoto R, Chamberlin HM, Macfarlane JL, Wolstenholme DR. 1991. Repeated sequence sets in mitochondrial DNA molecules of root knot nematodes (*Meloidogyne*): nucleotide sequences, genome location and potential for host-race identification. *Nucleic Acids Res*. 19:1619–1626.

Okimoto R, Macfarlane JL, Clary D, Wolstenholme DR. 1992. The mitochondrial genomes of two nematodes, *Caenorhabditis elegans* and *Ascaris suum*. *Genetics* 130:471–498.

Ossowski S, Schneeberger K, Lucas-Lledó JI, Warthmann N, Clark RM, Shaw RG, Weigel D, Lynch M. 2010. The rate and molecular spectrum of spontaneous mutations in. *Arabidopsis thaliana*. *Science* 327:92–94.

Posada D. 2008. jModelTest: phylogenetic model averaging. *Mol Biol Evol*. 25:1253–1256.

Schlager B, Wang X, Braach G, Sommer RJ. 2009. Molecular cloning of a dominant roller mutant and establishment of DNA-mediated transformation in the nematode model *Pristionchus pacificus*. *Genesis* 47:300–304.

Seyfert AL, Cristescu ME, Frisse L, Schaack S, Thomas WK, Lynch M. 2008. The rate and spectrum of microsatellite mutation in Caenorhabditis elegans and Daphnia pulex. *Genetics* 178: 2113–2121.

Shapiro B, Rambaut A, Drummond AJ. 2006. Choosing appropriate substitution models for the phylogenetic analysis of protein-coding sequences. *Mol Biol Evol*. 23:7–9.

Sommer RJ. 2009. The future of evo-devo: model systems and evolutionary theory. *Nat Rev Genet*. 10:416–422.

Sommer RJ, Carta LK, Kim S-Y, Sternberg PW. 1996. Morphological, genetic and molecular description of *Pristionchus pacificus* sp. n. (Nematoda, Diplogastridae). *Fundam Appl Nematol*. 19:511–521.

Suchard MA, Weiss RE, Sinsheimer JS. 2001. Bayesian selection of continuous-time Markov chain evolutionary models. *Mol Biol Evol*. 18:1001–1013.

Tian H, Schlager B, Xiao H, Sommer RJ. 2008. Wnt signaling by differentially expressed Wnt ligands induces vulva development in *Pristionchus pacificus*. *Curr Biol*. 18:142–146.

Vassilieva LL, Lynch M. 1999. The rate of spontaneous mutation for life-history traits in *Caenorhabditis elegans*. *Genetics* 151: 119–129.

Weller AM, Mayer WE, Rae R, Sommer RJ. 2010. Quantitative assessment of the nematode fauna present on Geotrupes dung beetles reveals species-rich communities with a heterogeneous distribution. *J Parasitol*. 96:525–531.

Wolstenholme DR. 1992. Animal mitochondrial DNA: structure and evolution. *Int Rev Cytol*. 141:173–216.

Zauner H, Sommer RJ. 2007. Evolution of robustness in the signaling network of Pristionchus vulva development. *Proc Natl Acad Sci U S A*. 104:10086–10091.

Zheng M, Messerschmidt D, Jungblut B, Sommer RJ. 2005. Conservation and diversification of Wnt signaling function during the evolution of nematode vulva development. *Nat Genet*. 37:300–304.

Zouros E, Freeman KR, Ball AO, Pogson GH. 1992. Direct evidence for extensive paternal mitochondrial DNA inheritance in the marine mussel Mytilus. *Nature* 359:412–414.