High Mutation Rates in the Mitochondrial Genomes of *Daphnia pulex*

Sen Xu,^{1,*} Sarah Schaack,^{2,†} Amanda Seyfert,² Eunjin Choi,² Michael Lynch,² and Melania E. Cristescu¹

¹Great Lakes Institute for Environmental Research, University of Windsor, Windsor, Ontario, Canada

²Department of Biology, Indiana University

[†]Present address: Department of Biology, Reed College

*Corresponding author: E-mail: xu11n@uwindsor.ca

Associate editor: Lauren McIntyre

Abstract

Despite the great utility of mitochondrial DNA (mtDNA) sequence data in population genetics and phylogenetics, key parameters describing the process of mitochondrial mutation (e.g., the rate and spectrum of mutational change) are based on few direct estimates. Furthermore, the variation in the mtDNA mutation process within species or between lineages with contrasting reproductive strategies remains poorly understood. In this study, we directly estimate the mtDNA mutation rate and spectrum using *Daphnia pulex* mutation-accumulation (MA) lines derived from sexual (cyclically parthenogenetic) and asexual (obligately parthenogenetic) lineages. The nearly complete mitochondrial genome sequences of 82 sexual and 47 asexual MA lines reveal high mtDNA mutation rate of 1.37×10^{-7} and 1.73×10^{-7} per nucleotide per generation, respectively. The *Daphnia* mtDNA mutation rate is among the highest in eukaryotes, and its spectrum is dominated by insertions and deletions (70%), largely due to the presence of mutational hotspots at homopolymeric nucleotide stretches. Maximum likelihood estimates of the *Daphnia* mitochondrial effective population size reveal that between five and ten copies of mitochondrial genomes are transmitted per female per generation. Comparison between sexual and asexual lineages reveals no statistically different mutation rates and highly similar mutation spectra.

Key words: asexuality, mitochondrial DNA, mutation-accumulation, mutation hotspots, mitochondria effective population size, mitochondrial evolution.

Introduction

Our understanding of the process of mutation in the mitochondrial genome remains limited (Denver et al. 2000; Paland 2004; Haag-Liautard et al. 2008; Howe et al. 2010), despite its wide application in evolutionary genetics. Studies of population differentiation (Avice 2000), population/species divergence time (Knowlton and Weigt 1998), species identification (Hebert et al. 2003), forensic medicine (Ivanov et al. 1996), and human disease (Taylor and Turnbull 2005) often require a rigorous understanding of the mitochondrial DNA (mtDNA) mutation rate and spectrum. The lack of data concerning mtDNA mutation process and the less well-characterized population-genetic environment of mitochondria compared with nuclear genomes leaves the evolution of mitochondrial genomes poorly understood.

In most metazoans, mtDNA shows an elevated mutation rate compared with nuclear DNA, likely due to less efficient DNA repair, a more mutagenic local environment (putatively caused by oxidative radicals), and an increased number of replications per cell division (Birky 2001; reviewed in Lynch 2007). This high mutation rate may result in accelerated mutation-accumulation (MA) via Muller's ratchet (Muller 1964; Felsenstein 1974), that is, the inability to reconstitute genomes with fewer mutations due to the absence of recombination in mtDNA (but see Piganeau et al. 2004), which may eventually affect the

long-term survival of populations and/or species (Loewe 2006). Mitochondrial genomes are thought to undergo severe within individual bottlenecks during transmission (Rand 2001), further reducing the efficacy of selection against mildly deleterious mutations as they accumulate (e.g., Hasegawa et al. 1998). Thus, to understand the evolution of mitochondrial genomes in the face of high rates of deleterious mutation requires detailed data on the mtDNA mutation process and characterization of the population-genetic environment of the mitochondrial genomes in various phylogenetic lineages.

Two approaches are generally used for estimating germ line mtDNA mutation rates. One category of studies employs phylogenetic methods that measure substitutions at silent sites between a pair of species with known or estimated divergence dates based on, for example, geological evidence (reviewed in Lynch 2007). This approach likely provides downwardly biased estimates because it assumes the neutrality of silent sites (which may be influenced by codon bias) and does not always take into account the presence of mutation hotspots that result in multiple substitutions. These two factors can result in large disparities between rates of phylogenetic divergence and actual mutation rates (reviewed in Lynch 2007). To minimize these biases, another category of studies directly estimates germ line mutation rates of mtDNA using long-term MA lines. These MA lines are generally propagated in benign

environments and bottlenecked at each generation by randomly picking one/a pair of the offspring from either clonal/hermaphroditic broods or from full-sib matings. This results in an extremely low effective population size for each MA line, thus reducing the efficiency of natural selection to a minimum and allowing the majority of mutations, except those having extreme effects, to accumulate over time in a neutral manner (Keightley and Caballero 1997).

To date, the direct estimates of mtDNA mutation rates have been limited to a few model species: *Caenorhabditis elegans* (Denver et al. 2000), *C. briggsae* (Howe et al. 2010), *Drosophila melanogaster* (Haag-Liautard et al. 2008), and *Saccharomyces cerevisiae* (Lynch et al. 2008). From these studies, the mtDNA mutation rate (including both base substitutions and indels) appears to be on the order of 10^{-8} to 10^{-7} per site per generation, which is hundreds of times higher than previous phylogenetic-based estimates (Denver et al. 2000). Upon closer examination, however, the spectrum and rate of mtDNA mutation vary widely across species. For example, the mtDNA mutation rate in *C. elegans* (1.6×10^{-7} per site per generation) is approximately two times higher than in *D. melanogaster* (7.8×10^{-8} per site per generation). In *D. melanogaster*, concordant with the nearly universal A/T bias in the mitochondrial genomes, 82% of the base substitutions appear to be G $\rightarrow$ A (Haag-Liautard et al. 2008). In contrast, only 15% of the base substitutions in *C. elegans* increase the A + T content of the mitochondrial genome (Denver et al. 2000), and all the base-substitution mutations in *S. cerevisiae* appear to be A/T $\rightarrow$ G/C (Lynch et al. 2008), despite the strong A/T bias in their mitochondrial genomes. Because germ line cells usually harbor multiple copies of mtDNA (e.g., $\sim 2,000$ in mice primordial germ cells; Cao et al. 2007), a neutral mutation originating in a single mtDNA copy must go through a drift process to reach fixation. Consistent with this scenario, extensive heteroplasmic base-substitution mutations are found in *D. melanogaster*. However, little heteroplasmy is observed in *C. elegans* (Denver et al. 2000), suggesting a lower mitochondrial effective population size in *C. elegans* compared with *D. melanogaster* (Haag-Liautard et al. 2008). Furthermore, the *C. briggsae* mitochondrial genome is characterized by a high rate (~ 0.001 to 0.0013 per site per generation) of heteroplasmic large deletions that encompass hundreds of base pairs, indicating the unique attributes of the DNA replication and repair machinery in this species (Howe et al. 2010).

Although the differences in mtDNA mutation patterns between distantly related taxa are starting to emerge, how closely related species and intraspecific populations/strains vary in their mitochondrial mutation patterns remains poorly understood (Howe et al. 2010). The evolution of mutation rate is thought to be determined by the costs of exact replication, the cost of deleterious mutations, and advantages of beneficial mutations (but see Lynch 2010). Given that in asexual species, recombination is usually negligible, the cost of replication fidelity and the effects of deleterious mutation are expected to yield a low mutation

rate (Dawson 1998). However, if beneficial mutations are common, mutation rates in asexual species may be higher relative to sexual species, even rising to an intolerable level that can lead to extinction (Johnson 1999; Andre and Godelle 2006; Gerrish et al. 2007). To date, few empirical studies investigate how sexual and asexual species differ in both nuclear and mitochondrial mutation rates (but see Baer et al. 2010). *Daphnia pulex*, a freshwater microcrustacean that typically reproduces by cyclical parthenogenesis (i.e., clonal reproduction with annual bouts of sex), but in which numerous obligate asexual lineages have arisen (Hebert et al. 1989), provides a great system to examine mtDNA mutation rates in sexual and asexual lineages. For mitochondrial genomes that experience “asexual” inheritance in both asexual and sexual species, the mitochondrial–nuclear linkage in asexual species can result in reduced efficiency of selection (Normark and Moran 2000). Asexual lineages of *Daphnia* (Paland and Lynch 2006), snails (Neiman et al. 2010), and rotifers (Barracough et al. 2007) have been shown to accumulate deleterious mutations at a much faster rate compared with sexual lineages. Furthermore, the mitochondrial–nuclear linkage is important in determining the mtDNA mutation rate because all the proteins and enzymes involved in mtDNA replication and repair are encoded by nuclear genes.

In this study, we describe the mtDNA mutation process in *D. pulex* MA lines and compare rates among those derived from one cyclically parthenogenetic (hereafter, sexual) and two obligately parthenogenetic (hereafter, asexual) individuals. By direct sequencing of more than 1.6 million mtDNA nucleotides in 84 sexual and 47 asexual *D. pulex* MA lines, our results show that the per generation mtDNA mutation rates in this species (1.37×10^{-7} per nucleotide per generation for the sexual clone and 1.40×10^{-7} and 2.28×10^{-7} for the two asexual clones, respectively) are among the highest rates reported. Although the mean mtDNA mutation rates are different, no statistically significant difference was detected between the mutation rate in sexual and asexual clones and their mutation spectrum appears to be very similar. Furthermore, the effective population size of mitochondrial genomes in *D. pulex* is estimated to range between 5 and 10 copies per generation.

Materials and Methods

MA Lines of *Daphnia*

The mtDNA genome was sequenced for three sets of MA lines of *D. pulex*. The first set of MA lines was derived from a sexual individual from the Slimy Log population (abbreviated S, $n = 82$ lines), collected in Oregon. The other two sets of MA lines originated from two obligately asexual individuals collected from temporary ponds in Linwood, Ontario, Canada (abbreviated A1, $n = 26$ lines) and Barry County, Michigan (abbreviated A2, $n = 21$ lines), respectively. The protocols for maintaining *Daphnia* MA lines are described in Lynch (1985). Briefly, all lines were maintained in a benign laboratory environment and asexually

propagated. The generation time for all of these MA lines is 10–13 days on average. Every generation, each line was bottlenecked by randomly picking a single individual to continue the line. Simultaneously, two additional females of each brood were maintained as backups in case the focal individual died or produced no offspring. The occasional use of backups causes difference in the number of generations for the lines derived from the same individual. The average number of generations for S and A lines was 61 and 100 (116 for A1 and 81 for A2), respectively.

PCR and Sequencing Protocols

A total of 21–24 partially overlapping fragments of mtDNA were amplified (average length ~750 bp). DNA from approximately ten individuals from each MA line was extracted using a cetyltrimethylammonium bromide method (Doyle JJ and Doyle JL 1987). Polymerase chain reaction (PCR) reactions used 18 µl Eppendorf PCR master mix, 0.5 µM primers, and 10–100 ng DNA template. The following thermocycling regime was used for all PCR reactions: 4 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 51 °C for 30 s, and extension at 72 °C for 1–2 min. Sequencing was performed with ABI BigDye Terminator v3.1 on an ABI 3730 DNA sequencer (Applied Biosystems, Foster City, CA). All putative substitutions and indel mutations were confirmed by sequencing with both forward and reverse primers. Assuming a high sequencing error rate of 10^{-4} (Tindall and Kunkel 1988) and the same error in both sequencing reactions, an error rate of $\sim 0.33 \times 10^{-8}$ is expected for mutations confirmed on both strands at a given site. With this conservative estimate, only ~ 0.005 mutations would be expected to be due to sequencing error in the total pool of ~ 1.65 million base pairs that we examined. Our assumption of a negligible sequencing error rate was supported by the fact that our sequences showed high Phred scores ranging between 38 and 60 (corresponding to a sequencing error rate between $\sim 10^{-4}$ and 10^{-6}).

Detection and Quantification of Heteroplasmy

Sequences for each amplified fragment were aligned against the complete *D. pulex* mitochondrial genome sequence (Genbank accession no. AF117817; Crease 1999) using CodonCode Aligner 2.0.6 (CodonCode Aligner Corporation, MA). Homoplasmic mutations were scored by eye, whereas the mutation detection function in CodonCode Aligner was used for identifying heteroplasmic mutations. This software implements the algorithms of Polyphred (Nickerson et al. 1997) that uses drops in ancestral peak intensity and rise of secondary peak as evidence for heteroplasmic mutations. The option of low sensitivity was selected to minimize the false positive rate. Each candidate mutant was confirmed by manually checking the trace files sequenced from both directions.

Because many mutations were heteroplasmic, we used the method developed by Haag-Liautard et al. (2008) to estimate the mutation frequencies based on a peak height comparison of DNA trace files. This method takes into

account the effect of the preceding base on peak heights of the wild type and mutant alleles. Assuming P is the base preceding the heteroplasmic site, X is the wild type nucleotide, and Y is the mutated nucleotide, the nearest nucleotide combinations of PX and PY (reference sites) were searched within 100 bp flanking the heteroplasmic site. The frequency of mutants at the site in question (D_{mut}) was defined as $(H_{\text{mut}}/H'_{\text{mut}})/(H_{\text{mut}}/H'_{\text{mut}} + H_{\text{wild}}/H'_{\text{wild}})$, where H_{mut} and H_{wild} represent the peak height of the mutated and wild type nucleotide, respectively, and H'_{mut} and H'_{wild} denote the peak height of X and Y at the reference sites, respectively. This method has been shown to yield estimates of heteroplasmy that are very consistent with a pyrosequencing approach (Haag-Liautard et al. 2008).

Estimation of Mutation Rate and Mitochondrial Effective Population Size

Assuming a neutral evolution model for mtDNA, the fates of newly arisen mutants are solely determined by genetic drift and the fixation rate at drift-mutation equilibrium is proportional to the mutation rate (Haag-Liautard et al. 2008). The probability of ultimate fixation of a neutral mutation (i) is its current frequency within an MA line. Thus, the mutation rate (per nucleotide site per generation) was calculated using the equation $\mu = \sum_i D_i / (LnT)$, where D_i is the frequency of a mutant allele, L is the total number of MA lines, n is the number of nucleotides surveyed, and T is the average number of generations. Nonparametric bootstrap technique (10,000 replicates) was used to calculate a 95% confidence interval (CI) for the average mutation rate as implemented in the R language package boot (www.r-project.org). To test whether the mtDNA mutation rate differs among the three sets of MA lines, we employed nonparametric bootstrap (10,000 replicates) to examine if the difference between the mean mutation rates of any two sets of MA lines is significantly different from zero, the difference being considered significant if the 95% CI for the difference between means did not contain zero.

Furthermore, we used a maximum likelihood (ML) modeling method (Haag-Liautard et al. 2008) to infer the mtDNA mutation rate as well as the effective population size of mitochondrial genomes transmitted to each generation. Briefly, under the assumption of neutrality, this method estimates the mutation rate from the proportion of unmutated sites, and the effective number of mitochondrial genomes (N_e) is modeled using a haploid Wright–Fisher transition matrix method. For both sexual and asexual (A1 and A2 combined) MA lines, analyses were performed with base substitutions, indels, and the combined data set of substitutions and indels, respectively.

Results

We directly sequenced a total of 1,653,703 bp of mtDNA from 129 MA lines, covering $\sim 87\%$ (13,410 bp) of the 15,333-bp *Daphnia* mitochondrial genome for sexual and

Table 1. List of the mtDNA Mutations Detected in *Daphnia pulex* MA Lines.

Line	Generation	Position	Mutation	Effect	Frequency	Context
S-109	62	4401	G → A	Met > Ile	0.50	GATACCTTTATGTTCTTATCGAA
S-60	59	8298	A → C	Met > Val	0.80	GGGGGGGAGCTATATTACTAATAG
S-58	62	8841	A(8) → A(7)	Frameshift	0.44	GCCCTA8GTAGTCAAGACT
S-35	63	9165	G → T	Tyr > Asn	0.42	TCAAATAGATAAGTATCTTAGAAAG
S-13	59	12300	C(7) → C(8)	Frameshift	0.22	AGAATC7AAGCCTACTTTAT
S-78	63	14411	C(3) → C(2)	Small rRNA	1	AAAAATTTAC3TATCAACAAAAT
S-21	61	14716	AC deletion	D-loop	0.76	AAGTATTTAGCGACCGCTTTGAAATT
S-11	54	14766	C(9) → C(8)	D-loop	1	TTTTGAGGGGATC9TAGAACCCCCC
S-51	61	14766	C(9) → C(8)	D-loop	1	TTTTGAGGGGATC9TAGAACCCCCC
S-67	57	14766	C(9) → C(8)	D-loop	1	TTTTGAGGGGATC9TAGAACCCCCC
S-68	51	14766	C(9) → C(8)	D-loop	1	TTTTGAGGGGATC9TAGAACCCCCC
S-32	90	14840	T(9) → T(10)	D-loop	1	ATCTCTTTTATT9AGATTATAATT
A1-1	110	7216	G → A	Synonymous	0.78	TAGAAATAAATAAATAACAGTGCTGA
A1-12	146	11696	C(6) → C(7)	Frameshift	0.23	ACAATCAC6AAAAAAATGACAC
A2-13	113	12661	A → G	Large rRNA	0.58	AACAGACTTTCCAACAAAACCTCTG
A1-18	111	12949	T → A	Large rRNA	1	CCCAAACGCATTAAAGCTTTTTCAC
A2-4	85	14766	C(8) → C(9)	D-loop	1	TTTCGAGGGGATC8TAGAACCCCCC
A2-17	102	14766	C(8) → C(10)	D-loop	1	TTTCGAGGGGATC8TAGAACCCCCC
A1-9	130	14840	T(10) → T(9)	D-loop	1	AATCTCTCTTTAT10AGATTATAATT
A1-16	125	14840	T(10) → T(9)	D-loop	1	AATCTCTCTTTAT10AGATTATAATT
A1-18	111	14840	T(10) → T(9)	D-loop	1	AATCTCTCTTTAT10AGATTATAATT
A2-12	104	14840	T(10) → T(11)	D-loop	1	AATCTCTCTTTAT10AGATTATAATT
A2-18	80	14840	T(10) → T(9)	D-loop	1	AATCTCTCTTTAT10AGATTATAATT

NOTE.—Underlined letters indicate the sites where mutations occur.

~77% (11,789 bp) for asexual MA lines (full alignment files available in [supplementary file S1, Supplementary Material](#) online). We detected 12 mutations in the sexual lines and 11 mutations in the pooled data set of asexual (A1 and A2) lines ([table 1](#) and [supplementary fig. S1, Supplementary Material](#) online), yielding an overall mutation rate of 1.37×10^{-7} (95% CI $0.64\text{--}2.18 \times 10^{-7}$) and 1.73×10^{-7} ($0.83\text{--}2.71 \times 10^{-7}$) per nucleotide per generation for sexual and asexual *D. pulex*, respectively ([table 2](#)). mtDNA mutation rate for the A1 lines was 1.40×10^{-7} ($0.41\text{--}2.53 \times 10^{-7}$), whereas that for the A2 lines was 2.28×10^{-7} ($0.58\text{--}4.07 \times 10^{-7}$). The mutation rate becomes a bit lower (1.30×10^{-7}) for the sexual lines if we consider the same regions that were sequenced in asexual lines. There was no significant difference in mutation rate between the sexual lines and the pooled asexual (A1 and A2) lines. Pairwise comparisons among the three sets of MA lines also revealed no statistical difference in their mtDNA mutation rates. The ML estimate of the mtDNA mutation is largely consistent with our direct estimation, suggesting a mutation rate of 1.51×10^{-7} for sexual and 1.75×10^{-7} for asexual lines ([table 2](#)). Nine of the 23 de novo mutations detected (39%) appeared to be heteroplasmic ([table 1](#)), with the frequency of mutants ranging between 0.22 and 0.78. Moreover, ML estimates of the effective mitochondrial population size using substitutions, indels, and the combined data set for both species consistently suggested that five to ten copies of mitochondria genomes were transmitted per female *Daphnia* per generation ([table 2](#)).

Base Substitutions

The sexual and asexual lines, respectively, showed three base substitutions ([table 1](#)) that appeared to be heteroplasmic with mutant frequency ranging between 0.42 and 0.78.

For sexual lines, the single base substitutions consisted of one transition (A → C) and two transversion (G → T and G → A) events and resulted in amino acid changes ([table 1](#)). For asexual lines, two transitions (G → A and A → G) and one transversion (T → A) were observed; however, none of these base substitutions produced amino acid changes ([table 1](#)). The base-substitution rate for sexual lines was 2.0×10^{-8} , whereas that for asexual lines was 4.3×10^{-8} .

Homopolymer Mutations

Both the sexual and the asexual lines showed a large proportion of indels (75% and 73%, respectively) among the detected mutations. Except for one deletion event, all indels occurred in homopolymeric regions that resided in both protein-coding and noncoding regions, which is consistent with the expectation that new mutations occurring in these MA lines were not eliminated by selection. A similar proportion of indels in asexual (2 of 8 indels) and sexual lines (1 of 9 indels) affected protein-coding regions. All the indels in the coding region appeared to be segregating at relatively low frequency, ranging between 0.22 and 0.44, whereas all the indels in noncoding regions appeared to be fixed. Mutation hotspots were identified in both lineages in the D-loop region. At site 14766, four of the sexual MA lines (S-11, 51, 68, and 67) showed a deletion in a homopolymeric run of nine Cs, whereas two asexual MA lines (A2-4 and A2-17) had a 1-bp insertion and a 2-bp insertion of C, respectively. Ancestral sequences were checked to ensure that these mutations were not due to sorting of ancestral heteroplasmy. At site 14840, in the homopolymeric run of nine/ten Ts, the MA lines S-32 and A2-12 appeared to have a 1-bp insertion, whereas lines, A1-9, A1-16, A1-18, and A2-18 showed a 1-bp deletion.

Table 2. Approximate and ML Estimates of Mutation Rates (per site per generation) and ML Estimates of the Effective Population Size of Mitochondrial Genomes (N_e) per Generation.

Mutation Type	No. of Mutations	μ (approximately) $\times 10^{-8}$	μ (ML) $\times 10^{-8}$	N_e (ML)
S lines (61 generation)				
Base substitution	3	2.0	2.7	10
Indel	9	11.7	12.2	5
All events	12	13.7	15.1	10
A lines (100 generation)				
Base substitution	3	4.3	4.8	5
Indel	8	13.0	12.7	5
All events	11	17.3	17.5	5
A1 lines (116 generation)				
All events	6	14.0	N/A	N/A
A2 lines (81 generation)				
All events	5	22.8	N/A	N/A

NOTE.—N/A, not applicable.

Discussion

The individual effects of mutation, natural selection, tight linkage, and uniparental inheritance on the evolution of mitochondrial genomes are usually difficult to tease apart. Nonetheless, MA experiments alleviate the effects of natural selection, allowing us to examine the *Daphnia* mtDNA mutation process largely unbiased by natural selection. Furthermore, the comparison between MA lines initiated from sexual and asexual ancestors offers insight into the differences in mutation propensity between clones with different reproductive strategies.

Mutation Rate and Spectrum in *Daphnia* mtDNA

Our results show that the overall mtDNA mutation rate (including both base substitutions and indels) in sexual (1.37×10^{-7} per nucleotide per generation) and asexual MA lines (1.73×10^{-7}) are similar to that for *C. elegans* (1.6×10^{-7}) but significantly higher than *D. melanogaster* (7.8×10^{-8}). To better understand the mutagenic cellular environment in different species, it is useful to compare the mtDNA mutation rate on a per germ cell division basis. Previous studies (Lynch et al. 2008) have revealed that the per cell division rates of *S. cerevisiae* (1.29×10^{-8}) and *C. elegans* (1.16×10^{-8}) are significantly higher than that of *D. melanogaster* (0.17×10^{-8}) and humans (0.21×10^{-8}). The high per generation mutation rate in *Daphnia* could be either the result of a high number of germ cell divisions or simply due to a high mutational susceptibility. However, this hypothesis cannot be currently tested given the limited information on the gametogenesis of *Daphnia*.

The base-substitution mutation rate for *Daphnia* (table 2) is significantly lower than that of *C. elegans* (9.7×10^{-8}), *C. briggsae* (7.2×10^{-8}), and *D. melanogaster* (6.2×10^{-8}) but higher than that of *S. cerevisiae* (1.2×10^{-8}). Results from recent MA studies demonstrate that the mtDNA mutation rate is several fold higher than the nuclear rate for species such as *D. melanogaster* (10-fold difference, Haag-Liautard et al. 2007, 2008), *C. elegans* (7-fold difference, Denver et al. 2000; Denver, Morris, Lynch, et al. 2004), and *S. cerevisiae* (37-fold difference, Lynch et al. 2008). Consistent with this overall pattern, the ratio between mtDNA and

nuclear mutation rate in *Daphnia* MA lines ($\sim 10^{-9}$, Lucas JJ, personal communication) is approximately 10.

The ratio of indel to base-substitution mutations in *D. pulex* mtDNA is ~ 2.3 , which is significantly higher than that for *C. elegans* (0.62; Denver et al. 2000), *S. cerevisiae* (0.58; Lynch et al. 2008), *D. melanogaster* (0.28; Haag-Liautard et al. 2008), and *C. briggsae* (1.20; Howe et al. 2010). The high frequency of indels observed in both sexual and asexual MA lines is driven by a few mutation hotspots at homopolymer sites. As a consequence of slippage during replication, homopolymers are known to be especially vulnerable to insertion–deletion mutations (Denver, Morris, Kewalramani, et al. 2004). For example, four independent sexual MA lines (S-11, S-51, S-67, and S-68) showed a deletion at position 14766 at a homopolymeric run of nine Cs (table 1). Alternatively, the large proportion of indels may be due to the fact that our direct sequencing approach is likely more sensitive for detecting indels than low-frequency base-substitution variants. This is because base substitutions originating on a single mtDNA molecule must persist through a transitional period of heteroplasmy prior to reaching fixation during which they may often segregate at low frequencies. With the lowering cost of next-generation sequencing and the development of statistical methods for analyzing high-coverage sequence data (Lynch 2008), mutation rate estimates that include low-frequency genetic variants will improve.

Intraspecific Comparison of Mutation Rate and Spectrum

Although the average mtDNA mutation rate for asexual lines (1.73×10^{-7}) is $\sim 27\%$ higher than for sexual lines (1.37×10^{-7}), no statistically significant difference was detected among the estimates of the three sets of MA lines. The patterns of indels between sexual and asexual MA lines are also not statistically different ($P = 0.3348$, Fisher's Exact test). Variation of the mtDNA mutation rate has been observed in different strains of *D. melanogaster* (Haag-Liautard et al. 2008), although the difference was not strongly supported by ML modeling. For *C. elegans* and *C. briggsae*, mtDNA base-substitution rates are highly similar, although *C. briggsae* experiences a much higher rate

of large-scale deletions in mitochondrial genomes (Howe et al. 2010). Theoretical models on the evolution of nuclear mutation rate with different reproductive modes focus on the indirect selection pressure on mutator alleles generated through linkage disequilibrium with deleterious/beneficial mutations (e.g., Johnson 1999). It is unclear how the linkage between nuclear and mitochondrial genomes influences the mtDNA mutation rate. A fundamental assumption of theoretical investigation is the absence of recombination in asexual taxa. However, recent studies have shown significant amounts of ameiotic recombination (crossing over and gene conversion) in asexual *Daphnia*, which is orders of magnitude higher than mutation rate (Omilian et al. 2006; Xu et al. 2011). The similarity of mutation rates in asexual and sexual *D. pulex* lineages shown in this study seems to suggest either that these obligately parthenogenetic lineages are not old enough to evolve a lower mutation rate than the nuclear genomes or that ameiotic recombination might play a role in the regulation of mutation rates in asexual lineages. Further theoretical work is necessary to demonstrate how ameiotic recombination impacts the evolution of mutation rate in asexuals.

The *Daphnia* mitochondrial genome is characterized by an A + T content of 62.3% (Crease 1999). However, because of the small number of base-substitution mutations observed in this study (i.e., 3 base substitutions for S and A lines, respectively), it remains challenging to determine whether the A + T content bias is due to mutation bias or due to selection. Previous investigations have shown contrasting patterns of base substitutions in MA lines of different species, indicating that different evolutionary forces are responsible for the universal compositional bias of A/T in mitochondrial genomes. In *D. melanogaster* (A/T composition 82%) and *C. briggsae* (A/T composition 75%), 86% and 87% of base substitutions increase A/T content, suggesting that substitution bias is likely driving the nucleotide composition bias. In contrast, *C. elegans* (A/T composition 76%) and *S. cerevisiae* (A/T composition 84%) show 75% and 67% of base substitutions increase G + C content, indicating that natural selection has strongly shaped the mitochondrial nucleotide composition in these species (Denver et al. 2000; Lynch et al. 2008).

Mitochondrial Effective Population Size

An important parameter for the mitochondrial population-genetic environment is the effective population size. The mitochondrial effective population size is much smaller than the census size within a cell due to an mtDNA bottleneck during early oogenesis and/or to the fact that some mtDNA alleles in heteroplasmic cells replicate more often than others by chance or due to selective advantages (Birky 2001). To date, it has been shown that the effective number of mitochondrial genomes is approximately ten in mice and humans (Jenuth et al. 1996; Marchington et al. 1997) and two in cows (Ashley et al. 1989), implying that heteroplasmy likely lasts only a few generations. In *Drosophila*, the effective number of mitochondria ranges between 545 and 700 per generation (Solignac et al. 1984), whereas crickets have a

mitochondrial effective population size of 87–395 (Rand and Harrison 1986), indicating that heteroplasmy may be more common in these species. Our ML estimate of the effective number of mitochondrial genomes in the germ line cells of *Daphnia* (5–10 copies) implies a short number of generations (10–20) for a heteroplasmic mutation to reach fixation. However, this estimate is probably downwardly biased because missing low-frequency mutants can lead to underestimates by this method (see Haag-Liautard et al. 2008). Our data contribute to the growing literature of the rate and spectrum of mitochondrial mutation, a key parameter in understanding the genetic change within and between species. However, future studies are needed to further investigate the variation of mtDNA mutation rates with breeding systems to test theories about the evolution of mutation rates.

Supplementary Material

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

Acknowledgments

We thank Dr Peter Keightley at University of Edinburgh, United Kingdom for providing the ML computer program for estimating mitochondrial effective population sizes. This work was supported by University of Windsor doctoral scholarships to S.X., by National Institutes of Health fellowship support and National Science Foundation (NSF) grant DEB-0608254 and 0805546 to S.S., by a Natural Sciences and Engineering Research Council (Canada) grant and an Early Researcher Award from Ontario Ministry of Research and Innovation to M.E.C., and NSF award EF-0328516 to M.L.

References

- Andre JB, Godelle B. 2006. The evolution of mutation rate in finite asexual populations. *Genetics* 172:611–626.
- Ashley MV, Laipis PJ, Hauswirth WW. 1989. Rapid segregation of heteroplasmic bovine mitochondria. *Nucleic Acids Res.* 17:7325–7331.
- Avice JC. 2000. *Phylogeography: the history and formation of species*. Cambridge: Harvard University Press.
- Baer CF, Joyner-Matos J, Ostrow D, Grigaltchik V, Salomon MP, Upadhyay A. 2010. Rapid decline in fitness of mutation accumulation lines of gonochoristic (outcrossing) *Caenorhabditis* nematodes. *Evolution* 64:3242–3253.
- Barracough TG, Fontaneto D, Ricci C, Herniou EA. 2007. Evidence for inefficient selection against deleterious mutations in cytochrome oxidase I of asexual bdelloid rotifers. *Mol Biol Evol.* 24:1952–1962.
- Birky CW. 2001. The inheritance of genes in mitochondria and chloroplasts: laws, mechanisms, and models. *Annu Rev Genet.* 35:125–148.
- Cao LQ, Shitara H, Horii T, Nagao Y, Imai H, Abe K, Hara T, Hayashi JI, Yonekawa H. 2007. The mitochondrial bottleneck occurs without reduction of mtDNA content in female mouse germ cells. *Nat Genet.* 39:386–390.
- Crease TJ. 1999. The complete sequence of the mitochondrial genome of *Daphnia pulex* (Cladocera: Crustacea). *Gene* 233:89–99.
- Dawson KJ. 1998. Evolutionarily stable mutation rates. *J Theor Biol.* 194:143–157.

- Denver DR, Morris K, Kewalramani A, Harris KE, Chow A, Estes S, Lynch M, Thomas WK. 2004. Abundance, distribution, and mutation rates of homopolymeric nucleotide runs in the genome of *Caenorhabditis elegans*. *J Mol Evol*. 58:584–595.
- 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.
- Doyle JJ, Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem Bull*. 19:11–15.
- Felsenstein J. 1974. The evolutionary advantage of recombination. *Genetics* 78:737–756.
- Gerrish PJ, Colato A, Perelson AS, Sniegowski PD. 2007. Complete genetic linkage can subvert natural selection. *Proc Natl Acad Sci U S A*. 104:6266–6271.
- 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, Charlesworth B, Keightley PD. 2007. Direct estimation of per nucleotide and genomic deleterious mutation rates in *Drosophila*. *Nature* 445:82–85.
- Hasegawa M, Cao Y, Yang ZH. 1998. Preponderance of slightly deleterious polymorphism in mitochondrial DNA: nonsynonymous/synonymous rate ratio is much higher within species than between species. *Mol Biol Evol*. 15:1499–1505.
- Hebert PDN, Beaton MJ, Schwartz SS, Stanton DJ. 1989. Polyphyletic origins of asexuality in *Daphnia pulex*. 1. Breeding system variation and levels of clonal diversity. *Evolution* 43:1004–1015.
- Hebert PDN, Ratnasingham S, deWaard JR. 2003. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proc R Soc Biol Sci Ser B*. 270:596–599.
- Howe DK, Baer CF, Denver DR. 2010. High rate of large deletions in *Caenorhabditis briggsae* mitochondrial genome mutation processes. *Genome Biol Evol*. 2:29–38.
- Ivanov PL, Wadhams MJ, Roby RK, Holland MM, Weedn VW, Parsons TJ. 1996. Mitochondrial DNA sequence heteroplasmy in the Grand Duke of Russia Georgij Romanov establishes the authenticity of the remains of Tsar Nicholas II. *Nat Genet*. 12:417–420.
- Jenuth JP, Peterson AC, Fu K, Shoubridge EA. 1996. Random genetic drift in the female germline explains the rapid segregation of mammalian mitochondrial DNA. *Nat Genet*. 14:146–151.
- Johnson T. 1999. Beneficial mutations, hitchhiking and the evolution of mutation rates in sexual populations. *Genetics* 151:1621–1631.
- Keightley PD, Caballero A. 1997. Genomic mutation rates for lifetime reproductive output and lifespan in *Caenorhabditis elegans*. *Proc Natl Acad Sci U S A*. 94:3823–3827.
- Knowlton N, Weigt LA. 1998. New dates and new rates for divergence across the Isthmus of Panama. *Proc R Soc Biol Sci Ser B*. 265:2257–2263.
- Loewe L. 2006. Quantifying the genomic decay paradox due to Muller's ratchet in human mitochondrial DNA. *Genet Res*. 87:133–159.
- Lynch M. 1985. Spontaneous mutations for life-history characters in an obligate parthenogen. *Evolution* 39:804–818.
- Lynch M. 2007. The origins of genome architecture. Sunderland (MA): Sinauer Associates.
- Lynch M. 2008. Estimation of nucleotide diversity, disequilibrium coefficients, and mutation rates from high-coverage genome sequencing projects. *Mol Biol Evol*. 25:2409–2419.
- 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.
- Marchington DR, Hartshorne GM, Barlow D, Poulton J. 1997. Homopolymeric tract heteroplasmy in mtDNA from tissues and single oocytes: support for a genetic bottleneck. *Am J Hum Genet*. 60:408–416.
- Muller H. 1964. The relation of recombination to mutational advance. *Mutat Res*. 106:2–9.
- Neiman M, Hehman G, Miller JT, Logsdon JM, Taylor DR. 2010. Accelerated mutation accumulation in asexual lineages of a freshwater snail. *Mol Biol Evol*. 27:954–963.
- Nickerson DA, Tobe VO, Taylor SL. 1997. PolyPhred: automating the detection and genotyping of single nucleotide substitutions using fluorescence-based resequencing. *Nucleic Acids Res*. 25:2745–2751.
- Normark BB, Moran NA. 2000. Testing for the accumulation of deleterious mutations in asexual eukaryote genomes using molecular sequences. *J Nat Hist*. 34:1719–1729.
- Omilian AR, Cristescu MEA, Dudycha JL, Lynch M. 2006. Ameiotic recombination in asexual lineages of *Daphnia*. *Proc Natl Acad Sci U S A*. 103:18638–18643.
- Paland S. 2004. Investigations into mitochondrial genome evolution using *Daphnia* [dissertation]. [Bloomington (IN)]: Department of Biology, Indiana University.
- Paland S, Lynch M. 2006. Transitions to asexuality result in excess amino acid substitutions. *Science* 311:990–992.
- Piganeau G, Gardner M, Eyre-Walker A. 2004. A broad survey of recombination in animal mitochondria. *Mol Biol Evol*. 21:2319–2325.
- Rand DM. 2001. The units of selection on mitochondrial DNA. *Annu Rev Ecol Syst*. 32:415–448.
- Rand DM, Harrison RG. 1986. Mitochondrial DNA transmission genetics in crickets. *Genetics* 114:955–970.
- Solignac M, Genemont J, Monnerot M, Mounolou JC. 1984. Genetics of mitochondria in *Drosophila*: mtDNA inheritance in heteroplasmic strains of *Drosophila mauritiana*. *Mol Gen Genet*. 197:183–188.
- Taylor RW, Turnbull DM. 2005. Mitochondrial DNA mutations in human disease. *Nat Rev Genet*. 6:389–402.
- Tindall KR, Kunkel TA. 1988. Fidelity of DNA synthesis by the *Thermus aquaticus* DNA polymerase. *Biochemistry* 27:6008–6013.
- Xu S, Omilian AR, Cristescu ME. 2011. High rate of large-scale hemizygous deletions in asexually propagating *Daphnia*: implications for the evolution of sex. *Mol Biol Evol*. 28:335–342.