

Accelerated Mutation Accumulation in Asexual Lineages of a Freshwater Snail

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Abstract

Sexual reproduction is both extremely costly and widespread relative to asexual reproduction, meaning that it must also confer profound advantages in order to persist. One theorized benefit of sex is that it facilitates the clearance of harmful mutations, which would accumulate more rapidly in the absence of recombination. The extent to which ineffective purifying selection and mutation accumulation are direct consequences of asexuality and whether the accelerated buildup of harmful mutations in asexuals can occur rapidly enough to maintain sex within natural populations, however, remain as open questions. We addressed key components of these questions by estimating the rate of mutation accumulation in the mitochondrial genomes of multiple sexual and asexual representatives of *Potamopyrgus antipodarum*, a New Zealand snail characterized by mixed sexual/asexual populations. We found that increased mutation accumulation is associated with asexuality and occurs rapidly enough to be detected in recently derived asexual lineages of *P. antipodarum*. Our results demonstrate that increased mutation accumulation in asexuals can differentially affect coexisting and ecologically similar sexual and asexual lineages. The accelerated rate of mutation accumulation observed in asexual *P. antipodarum* provides some of the most direct evidence to date for a link between asexuality and mutation accumulation and implies that mutational buildup could be rapid enough to contribute to the short-term evolutionary mechanisms that favor sexual reproduction.

Key words: sex, asexual, parthenogenetic, Muller's ratchet, mtDNA, Hill–Robertson, *Potamopyrgus antipodarum*.

Introduction

Why sexual reproduction and recombination are so common in nature has puzzled biologists for over a century (e.g., Darwin 1862; Weismann 1889; Morgan 1914; Fisher 1930; reviewed in Meirmans 2009). The modern effort to understand the maintenance of sex in natural populations was largely inspired by Williams (1966, 1975), who emphasized the need to identify individual-level advantages of sex, and Maynard Smith (1971, 1978), who pointed out that the production of sons by sexual females creates a 2-fold cost of sex that should culminate in its rapid elimination. The predominance of sexual reproduction in spite of this “cost of males” means that sex must confer major advantages that have yet to be fully understood.

Theorized benefits of sex typically fall into one of two main classes (Kondrashov 1993): ecological advantages, where the generation of novel variants is favored by selection in a rapidly changing environment (e.g., the Red Queen; Jaenike 1978; Hamilton 1980), and mutational advantages, where sex aids in the clearance of deleterious mutations (e.g., Muller's ratchet; Muller 1964). The extent that either class of mechanism can explain the maintenance of sex in natural populations, however, remains unclear (Lynch and Gabriel 1990; Otto and Nuismer 2004; Peters and Lively 2007; Neiman et al. 2009; Otto 2009).

The power of the Red Queen, for example, is limited by the fact that it favors rare genotypes rather than sexually

produced offspring, per se, meaning that its ability to maintain sex is greatly diminished when asexual competitors are genetically diverse enough for there to always be rare asexual genotypes (Lively and Howard 1994; Lythgoe 2000). Although mutation accumulation should inevitably cause asexual lineage extinction (Muller 1964; Lynch and Gabriel 1990; Charlesworth et al. 1993), it can only maintain sex if fitness loss due to mutational buildup is rapid enough to drive asexual lineages extinct before the sexuals are competitively excluded. Other models show that different selective mechanisms may interact synergistically to maintain sex (e.g., Howard and Lively 1994), but they depend on those mechanisms occurring simultaneously in natural populations.

Potamopyrgus antipodarum is a New Zealand freshwater snail which is characterized by mixed populations of obligately sexual and obligately asexual individuals and which is subject to infection by a sterilizing trematode parasite *Microphallus* sp. (Lively 1987). Several lines of evidence suggest that Red Queen dynamics in the *P. antipodarum*–*Microphallus* system are likely to play a central role in the persistence of sexual *P. antipodarum* (Jokela et al. 2009; King et al. 2009). However, the high within-population genetic diversity of asexual *P. antipodarum* (Dybdahl and Lively 1995; Jokela et al. 2003; Neiman and Lively 2004) means that parasite pressure alone is unlikely to explain why sex persists.

Here, we estimated the rate of accumulation of potentially harmful substitutions at nonsynonymous coding sites and functional noncoding sites in the mitochondrial genomes of a diverse array of sexual and asexual *P. antipodarum* as a means of evaluating whether asexual lineages experience increased mutation accumulation relative to sexual competitors. We also used these data to determine whether mutation accumulation occurs rapidly enough to be detected in asexual lineages that coexist with sexual *P. antipodarum*—that is, before sex is extirpated from populations.

Mitochondrial DNA (mtDNA) evolves rapidly enough in animals to serve as a marker of reduced efficacy of selection at an intraspecific scale (e.g., Paland and Lynch 2006; Johnson and Howard 2007). Moreover, though mitochondrial genomes are (usually) asexually transmitted, they are nevertheless affected by the transition to asexuality because they are thrust into complete linkage with the nuclear genome (Normark and Moran 2000). Mitochondrial–nuclear linkage is relevant in this context because tight linkage is expected to reduce effective population size and, hence, the ability of purifying selection to clear deleterious mutations (Hill and Robertson 1966; Ohta and Kimura 1971; Birky and Walsh 1988). This leads to the prediction that harmful substitutions should accumulate more rapidly in the mtDNA of asexuals because of selective interference from the linked nuclear genome (Normark and Moran 2000).

Comparisons of patterns of evolution at synonymous versus nonsynonymous sites in protein-coding DNA are frequently used to infer mutation accumulation (e.g., Charlesworth and Wright 2001; Funk et al. 2001; Glémin 2007). A common approach is to estimate and compare the ratio (d_N/d_S) of the number of nonsynonymous substitutions per nonsynonymous site (d_N) to the number of synonymous substitutions per synonymous site (d_S) among the lineages of interest. Because nonsynonymous mutations change amino acid sequence, they are often slightly deleterious (Nachman 1998), and it is these slightly deleterious mutations that are expected to accumulate at increasing rates in asexual lineages (Gabriel et al. 1993). In contrast, synonymous substitutions (which do not affect protein sequence and are presumed to have much smaller, if any, harmful effects) accumulate at rates much closer to the rate at which mutations occur.

This means that estimating d_N/d_S can provide a measure of the extent to which harmful mutations are accumulating while controlling for the underlying mutation rate of the lineage. In particular, higher values of d_N/d_S at loci that are presumed not to be the direct targets of positive selection imply a higher rate of accumulation of deleterious mutations. Based on this logic (Li et al. 1985), d_N/d_S estimates have been used successfully to detect evidence for an increased rate of fixation of harmful nonsynonymous mutations in the mitochondrial genomes of asexual lineages (e.g., Paland and Lynch 2006; Barraclough et al. 2007). We used this approach in our study of mutation accumulation in the 13 protein-coding genes in the mitochondrial genomes of sexual versus asexual *P. antipodarum*.

One cannot so easily discriminate between relatively “neutral” versus relatively “deleterious” mutations within noncoding functional DNA sequence. Mutations in noncoding functional loci such as transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs) that lower fitness, however, should still be subject to removal by natural selection (following Moran 1996; Lynch 2007). Given the inverse relationship between the efficacy of selection and tightness of genetic linkage and because most noncoding functional mtDNA mutations are probably deleterious (Lynch 2007; also see fig. 2), asexual lineages should experience more rapid divergence in these loci than sexual lineages. These predictions are supported by evidence for increased rRNA (Moran 1996; Woolfit and Bromham 2003) and tRNA (Lynch 1997) substitution rates in genomes that experience relatively small effective population size. Thus, we used the rate of evolution of mitochondrial tRNAs and rRNAs to estimate and compare mutation accumulation in the noncoding functional mtDNA of sexual versus asexual *P. antipodarum*.

We used previously established phylogeographic data (Neiman and Lively 2004) and evidence for the bimodal distribution of asexual lineage age in *P. antipodarum* (Neiman et al. 2005) to select 18 *P. antipodarum* lineages for mitochondrial genome sequencing that captured most of the genetic diversity and asexual lineage age variation within the species (fig. 1). The wide variation in asexual lineage age allowed us to assess whether increased mutation accumulation was evident in recently derived asexual lineages and whether old asexual lineages were characterized by especially high mutation loads.

Materials and Methods

Cloning the Initial Genomic mtDNA Sequence

Total DNA was isolated with the Qiagen DNeasy Plant Kit following the standard miniprep protocol. The complete mtDNA of a single asexual female *P. antipodarum* was amplified from total DNA in four overlapping fragments. Primer pair 1a (F-ACTAGGATCCGTTGATTTAGCT; R-GCTGGTTTACATAGGATCCAT) amplified a 7.6-kb fragment; primer pair 2a (F-TACAACGTCAGCAGAAGATAAC; R-GCACTATACAGCACACGTAGA) amplified a 7.6-kb fragment; primer pair 3a (F-GCTGGTTTACATAGGATCCAT; R-CATACAAAGCTTCCATCACAGT) amplified a 2.3-kb fragment; and primer pair 4a (F-GGAGTGAACGGAAATCA; R-AGACGTAAAGATGGCAAAG) amplified a 3.3-kb fragment. Reactions contained 3.75 U Expand Long Polymerase (Roche, Indianapolis, IN), 1× Expand buffer 3, 0.5 mM each dNTP, 0.3 μM each primer, and 50 ng genomic DNA. Following denaturation of samples at 94 °C for 2 min, amplification was performed in two stages: 10 cycles of 94 °C for 10 s, 51 °C for 30 s, and 68 °C for 12 min, and 25 cycles of 94 °C for 15 s, 51 °C for 30 s, and 68 °C for 12 min with a 20-s extension in the polymerization step for each additional cycle. Reactions were then incubated at 68 °C for 7 min. PCR fragments were gel purified using QIAquick Gel Extraction Kit (Qiagen, Inc., Valencia, CA), inserted into pCR2.1 XL TOPO (Invitrogen Corp., Carlsbad, CA), and

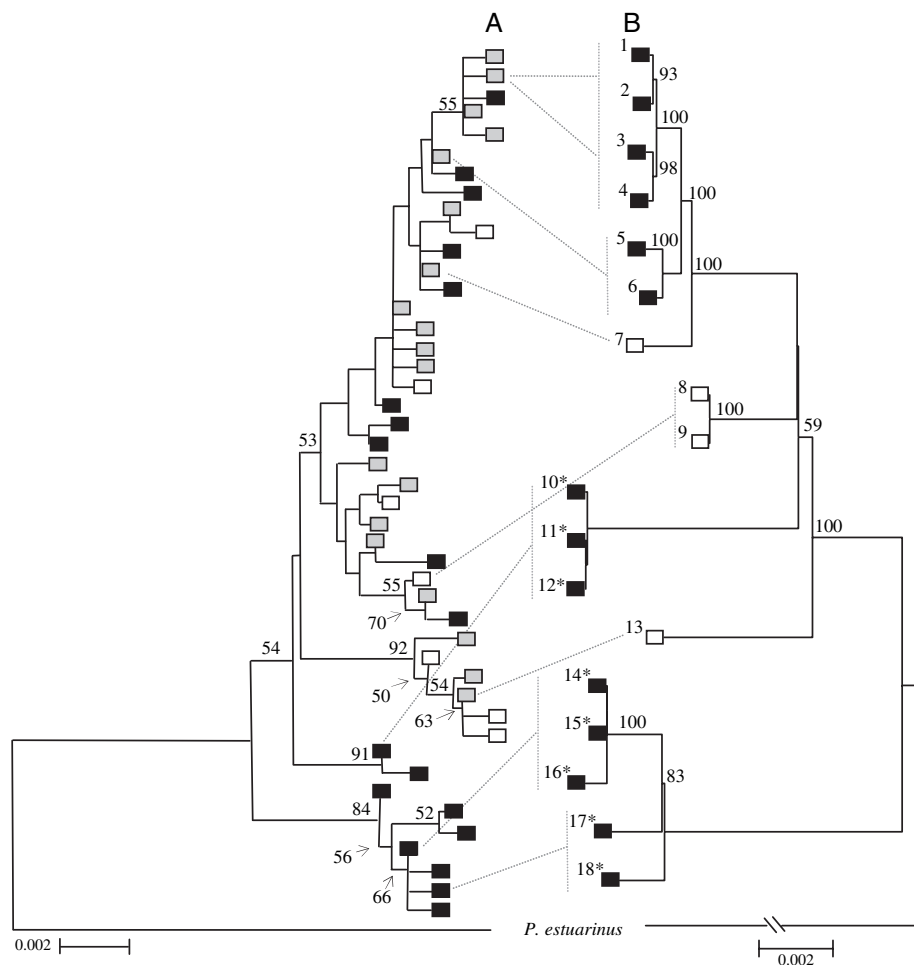


Fig. 1. (A) The maximum likelihood phylogeny determined from mitochondrial cytochrome b DNA sequence (“cytochrome b tree”; adapted from Neiman et al. 2005) used to assign lineage sexuality and age for the mitochondrial genomic data (B). Maximum likelihood bootstrap values $>50\%$ are given (500 replicates). The square at the end of each branch indicates whether the haplotype is represented by only sexual individuals (white), both sexual and asexual individuals (gray), or only asexual individuals (black). The two entirely asexual clades at the bottom of the cytochrome b tree appear to have been diverging from sexuals for at least 500 000 years (“old asexual clades” and “old asexual lineages”); all other asexual lineages are $<40\,000$ years old and likely to be considerably younger (“young asexual lineages”). The lines from tree A to tree B indicate where the snails used for the genome trees were represented on the cytochrome b tree. Asexual individuals from “mixed” sexual/asexual cytochrome b haplotypes (haplotypes 1 and 18, Neiman and Lively 2004) were used to represent young asexual lineages for the genomic data set. Because asexual lineages 1–4 and 5 and 6 do not share a most recent sexual common ancestor, they represent two independently derived sets of young asexual lineages. The cytochrome b tree was generated from an extensive species-wide sample (Neiman et al. 2005), allowing us to assign asexual lineage ages to the 14 asexual and 4 sexual *Potamopyrgus antipodarum* lineages represented in the genome tree. (B) Maximum likelihood tree constructed from the entire protein-coding (B, ~ 11 kb) mitochondrial genome DNA sequence of 14 asexual (black) and 4 sexual (white) *P. antipodarum* lineages. “*” Indicates old asexual lineages; all other asexual lineages are young. The numbers to the left of the boxes on each tree represent lineage identity across the genomic trees. Bootstrap values $>50\%$ are given. Branch lengths from a maximum likelihood baseml analysis with no clock constraint were used to provide a representation of branch length before correction for differences in evolutionary rate among different branch types due to sexuality or branch position. A tree generated with ~ 4 kb of functional noncoding mtDNA sequence is topologically identical and is not shown here.

transformed into TOP10 cells (Invitrogen Corp.). Both strands of the inserts were sequenced using vector primers and by sequence walking.

Amplification of other mtDNAs

Complete mtDNAs from four *P. antipodarum* individuals (both sexual and asexual females) were amplified from total DNA in six overlapping fragments. Primer pair 1b (F-AC-TAGGATCCGTTGATTTAGCT; R-CTCTTGAGTATGCT-GAGTACA) amplified a 3.2-kb fragment; primer pair 2b

(F-CTTTGCCAGGGAGAACT; R-CAAGCCTCAGAGGTT-GA) amplified a 4.0-kb fragment; primer pair 3b (F-CCA-TTCGACTTTGCAGAG; R-GCAGCCAGAACTATTGAC) amplified a 3.2-kb fragment; primer pair 4b (F-CAAGC-TAGGTGGTGTATTG; R-CCTCTCAGAAAGTAGCAGA) amplified a 3.6-kb fragment; primer pair 5b (F-GCATT-CTTCTACTCTTGTC; R-GCCTCAAAGTGTTAAAGC) amplified a 3.6-kb fragment; and primer pair 6b (F-GGAGT-GAACGGAAATCA; R-AGACGTAAAGATGGCAAAG) amplified a 3.3-kb fragment. Reactions contained 1 U Phusion

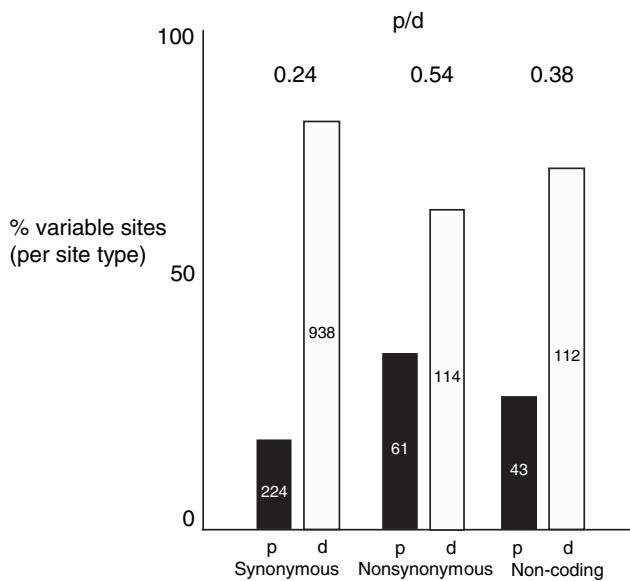


Fig. 2. Percent polymorphic versus divergent sites across all variable sites in the mitochondrial genome of the four sexual *P. antipodarum* lineages; fixed differences (divergent sites) are relative to *Potamopyrgus estuarinus*. For each site type, polymorphism (p) and divergence (d) are indicated by black and white bars, respectively; numbers inside each bar indicate the total number of each type of site. There was a significantly higher ratio of polymorphism in nonsynonymous/synonymous sites than divergence in nonsynonymous/synonymous sites (Fisher's exact test, $P < 0.0001$). There was a significantly higher ratio of polymorphism/divergence in nonsynonymous versus synonymous sites (Fisher's exact test, $P < 0.0001$) and in functional noncoding versus synonymous sites (Fisher's exact test, $P < 0.0001$) but no significant difference in the ratio of polymorphisms to divergence in nonsynonymous sites versus noncoding sites (Fisher's exact test, $P = 0.192$).

Polymerase (New England BioLabs, Ipswich, MA), $1\times$ HF buffer, 0.2 mM each dNTP, 0.5 μ M each primer, and 50 ng genomic DNA. Following denaturation of samples at 98 °C for 30 s, amplification was performed for 35 cycles of 98 °C for 20 s, 60 °C for 30 s, and 72 °C for 2 min 30 s. Reactions were then incubated at 72 °C for 10 min.

Complete mtDNAs from 14 individual *P. antipodarum* (both sexual and asexual females) were amplified from total DNA in four overlapping fragments. Primer pair 1c (F-GAGGTAGGAGACTGTAGT; R-GAGTCCTAAGCCCAATGCA) amplified a 4.3-kb fragment; primer pair 2c (F-GCTAGTATGAATGGTTTGACG; R-CACTAGAGCTGAACTGGT) amplified a 5.8-kb fragment; primer pair 3c (F-TCAGCTTGTGGATCTGA; R-GCCTAATCAGTATGAGGAAG) amplified a 3.5-kb fragment; and primer pair 4c (F-GGAGTGAACGGAAATCA; R-CTCTTGAGTATGCTGAGTACA) amplified a 5.1-kb fragment. Reaction reagents and conditions were as described above, except primer pair 1c and 2c used 64 °C for annealing and 2 min 30 s for extension.

The complete mtDNA extracted from a single *Potamopyrgus estuarinus* individual was amplified from total DNA in three overlapping fragments. Primer pair 1d (F-GAGGTAGGAGACTGTAGT; R-GAGTCCTAAGCCCAATGCA)

amplified a 9.2-kb fragment. Primer pairs 3c and 4c were as described above. Reaction reagents and conditions were as described above, except primer pair 1d used 58 °C for annealing and 4 min 30 s for extension.

DNA Sequencing

PCR fragments were purified by QIAquick PCR Purification (Qiagen, Inc.). Both strands were directly sequenced using primers (as described above) and, where necessary, by sequence walking. Sequencing was performed on an ABI Prism 3730 (AB Applied Biosystems, Foster City, CA), and the profiles were analyzed and edited with ChromasPro (Technelysium Pty Ltd, Tewantin, Australia).

Statistical and Phylogenetic Analyses

DNA sequences from the 19 *Potamopyrgus* genomes were aligned using Clustal within Mega 4.0 and corrected manually. We then used a contingency table-based variation of the McDonald–Kreitman test (MK test) and Fisher's exact test to test the baseline assumption that most nonsynonymous mutations are deleterious (e.g., Paland and Lynch 2006). Specifically, we evaluated whether the ratio of nonsynonymous to synonymous polymorphisms apparent within sexual *P. antipodarum* was higher than the ratio of nonsynonymous to synonymous substitutions separating *P. antipodarum* from *P. estuarinus*. We also used a modified MK approach to compare the ratio of functional noncoding polymorphism/divergence in sexual *P. antipodarum* to the ratio of synonymous polymorphism/divergence (e.g., Andolfatto 2005). We used only sexual *P. antipodarum* for these comparisons in order to assess whether nonsynonymous mutations and mutations in noncoding functional regions are generally deleterious in lineages where purifying selection is expected to be effective (at least relative to asexuals).

To generate the phylogenies used in this study, we used the "FindModel" application of Modeltest 3.7 (Posada and Crandall 1998) to select the model of nucleotide substitution that best fit each of the two (coding and functional noncoding) *P. antipodarum* data sets. For both data sets, the best-fitting model was general time reversible + gamma. We used this model to generate a maximum likelihood tree with the DNAmI function of PHYLIP 3.68 (Felsenstein 1989) for each data set. We then used Seqboot (500 replicates), DNAmI, and Consense (as implemented within PHYLIP 3.68) to perform a maximum likelihood bootstrapping analysis on each tree.

Next, we used PAML (Yang 2007) to determine whether nonsynonymous and functional noncoding substitutions accumulated at different rates in sexual versus asexual lineages (following Paland and Lynch 2006; Johnson and Howard 2007). This software works by calculating the goodness of fit of a particular, user-specified model of evolution to a phylogenetic data set. Here, we used PAML to compare the fit of simple models where the entire *P. antipodarum* phylogeny experienced the same rate of evolution to more complex models where, for example,

sexual and asexual lineages were allowed to evolve at different rates. Following Paland and Lynch (2006), the sexual outgroup *P. estuarinus* was excluded from the trees used in these analyses because it cannot be classified as either a sexual or asexual lineage of *P. antipodarum*.

For the protein-coding sequence, we used the “codeml” implementation of PAML to apply a one-ratio maximum likelihood model to the data that estimated a single d_N/d_S value for the (unrooted) *P. antipodarum* phylogeny. We then ran a two-ratio model where sexual and asexual lineages were allowed to have different d_N/d_S (sex vs. asex model) and used a likelihood ratio test to assess whether the two-ratio versus one-ratio model provided a better fit to the data. This test evaluated whether asexual lineages had a different rate of mutation accumulation relative to sexual lineages. A higher estimated d_N/d_S value for the asexual lineages and a significantly better fit of the two-ratio model to the data would indicate that asexual *P. antipodarum* experience accelerated accumulation of harmful mutations.

Because the time lag between the appearance of a new mutation and its clearance via purifying selection means that mildly deleterious mutations are expected to be at higher frequency in terminal versus internal branches (Paland and Lynch 2006), apparent differences between sexual and asexual d_N/d_S could be confounded by differences in branch length between sexual and asexual lineages. We dealt with this issue by using PAML to evaluate the fit of two additional models to the data: where terminal versus internal branches were allowed to have different d_N/d_S (terminal vs. internal model) and where terminal sexual, terminal asexual, internal sexual, and internal asexual branches were allowed to have different d_N/d_S (four-ratio model). We then used likelihood ratio tests to compare the fit of both of the two-ratio models (sex vs. asex and terminal vs. internal) to the one-ratio model and to compare the four-ratio model to both two-ratio models and to the one-ratio model.

We used an identical approach for the functional noncoding sequence but with a noncodon-based approach, baseml. Here, we used likelihood ratio tests to compare the fit of a one-rate model to a two-rate model where sexuals and asexuals were allowed to evolve at different rates (sex vs. asex model), a two-rate model where terminal and internal branches could evolve at different rates (terminal vs. internal model), and a four-ratio model where terminal versus internal sexual branches and terminal versus internal asexual branches were all allowed to evolve at different rates (four-rate model). Because these analyses require the implementation of a molecular clock at various scales (global for one-rate models, local for >1 rate models), they also require the use of rooted trees.

Finally, we used one-ratio versus two-ratio (sex vs. asex) tests and one-rate versus two-rate (sex vs. asex) likelihood ratio tests to assess whether the overall finding of significantly higher rates of mutation accumulation in asexual lineages held up when only young asexual lineages were included in the phylogeny. We conducted these compar-

isons in order to evaluate whether mutation accumulation occurs rapidly enough to be detected in very recently derived asexual lineages.

Results and Discussion

We sequenced the mitochondrial genomes of four sexual individuals, an individual from each of six “young” asexual lineages that are known to be of recent derivation from sexual ancestors (and of at least two independent transitions to asexuality; fig. 1), an individual from each of eight “old” asexual lineages from two independently derived asexual clades, and one *P. estuarinus* individual. The data set comprised 11 196 bp of coding mtDNA from 13 protein-coding genes and 3,896 bp of noncoding functional DNA (22 tRNAs and 2 rRNA loci). All 19 mitochondrial genome sequences were deposited in GenBank (accession nos GQ996415–GQ996433).

A contingency table-based variation of the MK test showed that the ratio of nonsynonymous/synonymous polymorphisms within sexual *P. antipodarum* (0.27) was significantly higher than the ratio of nonsynonymous/synonymous substitutions separating sexual *P. antipodarum* from *P. estuarinus* (0.12; see fig. 2). This result indicates that when nonsynonymous mtDNA mutations occur in *P. antipodarum*, they are less likely to proceed to fixation and, hence, contribute less to interspecific divergence (Hasegawa et al. 1998; Meiklejohn et al. 2007; Marshall et al. 2008; Rand 2008). The implication is that these mutations are usually deleterious and are, thus, removed by natural selection. A similar conclusion follows from the significantly higher d_N/d_S in terminal versus internal branches (table 1), suggesting that nonsynonymous mutations in terminal branches are often cleared by purifying selection before they contribute to divergence in internal branches (Paland and Lynch 2006).

We used another variation of the MK approach to evaluate whether mutations in functional noncoding mtDNA are deleterious as compared with relatively “neutral” synonymous mutations. We found a significantly higher ratio of functional noncoding polymorphism/divergence (0.38) than synonymous polymorphism/divergence (0.24; see fig. 2) in sexual *P. antipodarum* versus *P. estuarinus*. The fact that these noncoding mutations are less likely to contribute to divergence between species than synonymous mutations suggests that they are generally deleterious. The somewhat higher rate of evolution of functional noncoding mtDNA in terminal versus internal branches (table 1) is also consistent with this conclusion. Other studies of substitution and polymorphism in both coding and noncoding functional mtDNA have reached similar conclusions (reviewed in Lynch 2007; Rand 2008), pointing toward the likelihood that mtDNA evolution in *P. antipodarum* is generally governed by the balance between purifying selection and genetic drift.

The PAML analyses indicated that asexual lineages experienced a substantially higher rate of accumulation of deleterious nonsynonymous substitutions than sexual

Table 1. Summary of PAML Analyses of Comparisons of d_N/d_S Ratios in Coding mtDNA and the Rate of Evolution of Functional Noncoding mtDNA in Sexual versus Asexual *Potamopyrgus antipodarum*.

Model	No. Free Parameters	lnL	χ^2 (df)	d_N/d_S or Rate ^a
Coding				
One ratio	1	-19680.08	NA	0.101
Two ratio (sex vs. asex)	2	-19674.046	vs. one ratio: 12.068 (1)***	Sex: 0.084 Asex: 0.161
Two ratio (T, I) ^b	2	-19675.866	vs. one ratio: 8.428 (1)**	T: 0.143 I: 0.0851
Four ratio (Sex _T , Asex _T , Sex _I , Asex _I)	4	-19671.78	vs. one ratio: 16.6 (3)*** vs. two ratio (sex/asex): 4.532 (2) vs. two ratio (T vs. I): 8.172 (2)**	Sex _T : 0.103 Asex _T : 0.204 Sex _I : 0.079 Asex _I : 0.123
One ratio (pruned) ^c	1	-16807.664	NA	0.098
Two ratio (pruned, sex vs. asex)	2	-16805.709	vs. one ratio: 3.91 (1)*	Sex: 0.086 Young asex: 0.162
Functional noncoding				
One rate	1	-6114.496	NA	NA
Two rate (sex vs. asex)	2	-6112.204	vs. one rate: 4.584 (1)*	Sex: 1.00 Asex: 1.598
Two rate (T vs. I)	2	-6113.924	vs. one rate: 2.144 (1)	T: 1.283 I: 1.00
Four rate (Sex _T , Asex _T , Sex _I , Asex _I)	4	-6109.782	vs. one rate: 9.428 (3)** vs. two rate (sex/asex): 4.844 (2) vs. two rate (T vs. I): 8.284 (2)**	Sex _T : 1.526 Asex _T : 5.973 Sex _I : 1.00 Asex _I : 1.962
One rate (pruned)	1	-5554.967	NA	NA
Two rate (pruned, sex vs. asex)	2	-5554.812	vs. one rate: 0.155 (1)	Sex: 1.00 Asex: 1.367

NOTE.—df, degrees of freedom; NA, not applicable.

^a First branch type entered in the control file automatically set to 1 by baseml; all other rates are estimated relative to this value.

^b “T” indicates terminal branches and “I” indicates internal branches.

^c “Pruned” represents analyses from pruned trees containing only sexuals and young asexual lineages.

*Indicates significance at $P < 0.05$, **Indicates significance at $P < 0.025$, and ***Indicates significance at $P < 0.001$.

lineages, as evidenced by the significantly better fit of the two-ratio sex versus asex model to the protein-coding data and the $\sim 2\times$ higher point estimate of d_N/d_S in asexual (0.161) versus sexual lineages (0.084; table 1). These d_N/d_S values for sexuals versus asexuals are similar to those reported in Paland and Lynch (2006) and Johnson and Howard (2007), both in terms of the value of d_N/d_S and the magnitude of discrepancy between sexual and asexual lineages. The four-ratio model (contrasting sexual, asexual, internal, and external branches) provided a significantly better fit than the two-ratio model contrasting terminal versus internal branches but not the two-ratio model contrasting sexual versus asexual branches. This, and the fact that asexual branches had higher d_N/d_S regardless of whether they were internal or external, demonstrates that the sex/asex differences in d_N/d_S are not due to discrepancies in branch length.

We obtained a result similar to the outcome of the analyses described above using a pruned tree containing only young asexual and sexual lineages. Here, we found that the two-ratio model where sexual and asexual branches could have different d_N/d_S was a significantly better fit to the data than the one-ratio model and that the young asexual d_N/d_S was $\sim 2\times$ higher than that of the sexuals (table 1). This result indicates that young asexual lineages have experienced a discernable increase

in the rate of accumulation of deleterious nonsynonymous substitutions when compared with close sexual relatives.

We addressed the possibility that high d_N/d_S in asexuals could be due to low d_S rather than elevated d_N by comparing a tree with branch lengths generated only by variation at synonymous sites to a tree with branch lengths determined only by variation at nonsynonymous sites. This comparison shows that although d_S varies among lineages, it is not systematically decreased in asexuals relative to sexuals. By contrast, the tree based upon only nonsynonymous sites showed that all the asexual lineages had longer branches than their closest sexual relative. This difference indicates that the increased d_N/d_S in asexual *P. antipodarum* is due to increased d_N in asexual relative to sexual lineages (fig. 3), rather than variation in the underlying mutation rate (Sloan et al. 2009) or decreased mutation rate in asexual lineages (Schön et al. 1998).

In noncoding functional sequence (e.g., tRNAs and rRNAs), any mutations that lower fitness can be removed from a lineage by purifying selection. Therefore, we used comparisons of the substitution rate in these loci as an additional assay of the efficacy of selection, where accelerated rates are assumed to reflect less effective purifying selection (e.g., Lynch 2007). As above,

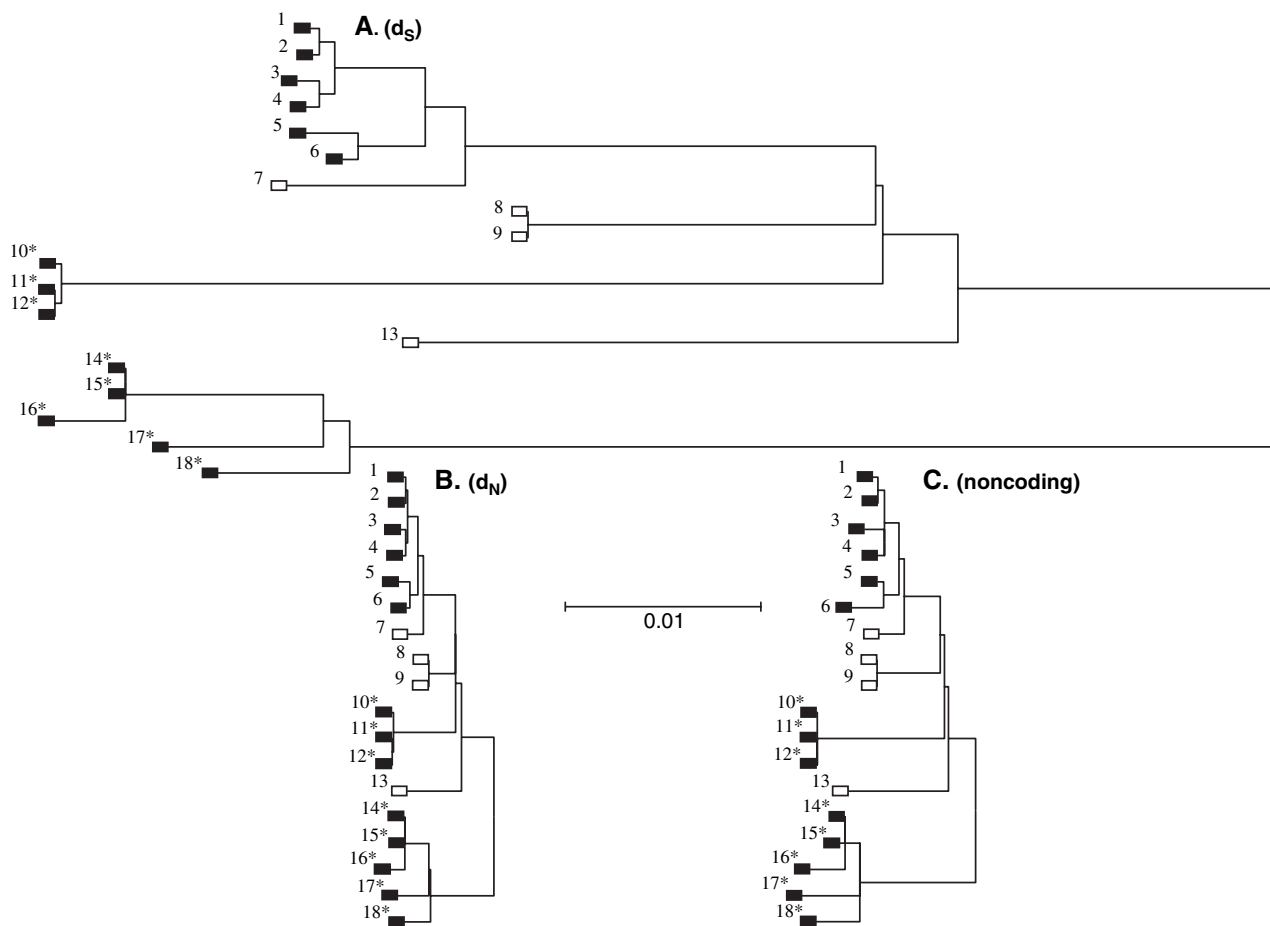


Fig. 3. A–C) A comparison of trees with (A) branch lengths determined by the number of synonymous substitutions/synonymous site (d_S), (B) the number of nonsynonymous substitutions/nonsynonymous site (d_N), and (C), the number of functional noncoding substitutions/functional noncoding site (noncoding). We used the d_S and d_N branch lengths generated by the best-fitting (four ratio) codeml model for the coding mtDNA. Because the best-fitting baseml model, the four-rate model, requires the implementation of a molecular clock, all branches are constrained to end at the same point. Thus, in order to provide a representation of branch lengths for the functional noncoding mtDNA that could be compared with the d_S and d_N trees, we used branch lengths from a baseml analysis applied to the noncoding data set in the absence of a clock. Although these trees are shown unrooted, rooted trees are required for analyses involving a molecular clock and, accordingly, were used for all baseml analyses of evolutionary rate. Box colors and numbering follow figure 1B. Branch lengths are shown at the same scale of 0.01 substitutions/site, as indicated in the scale bar in the middle of the figure.

we compared the fit of a one-rate model to two types of two-rate models (sex vs. asex, terminal vs. internal) and a four-rate model to the one-rate and two-rate models. These analyses suggested that asexual *P. antipodarum* experienced a two to four times higher substitution rate in their functional noncoding mtDNA than sexual counterparts, even when branch length was taken into account (table 1). The rate acceleration experienced in the functional noncoding mtDNA of asexual *P. antipodarum* is evident in a tree generated using only functional noncoding sites, where asexual lineages generally have longer branches than their closest sexual relatives (fig. 3).

As with the coding sequence, we also ran PAML analyses with pruned trees containing only young asexual and sexual lineages. Although the direction of differences between sexuals and asexuals was similar to the analyses using the full data set in that asexual branches had higher rates of

substitution than sexual branches, the two-rate sex versus asex model did not provide a significantly better fit to the data than the single-rate model. This difference in outcome between the PAML analyses with coding versus noncoding mtDNA is, perhaps, not surprising given that there was a somewhat higher rate of fixation of functional noncoding mutations than nonsynonymous mutations (fig. 2), suggesting that, on average, mutations in noncoding functional mtDNA are subject to weaker purifying selection than nonsynonymous mutations.

Although variation in the rate of accumulation of apparently harmful mutations between asexual and sexual lineages is likely due to differences in the efficacy of selection, we cannot exclude a role for other factors associated with asexual *P. antipodarum*. For example, the triploidy of asexuals may influence the rate of mutation accumulation within asexual lineages, either directly by affecting the rate and spectrum of mutation or, indirectly, by altering gene

expression or phenotype (reviewed in Otto 2007). We also cannot rule out the possibility that asexual *P. antipodarum* experience less effective and/or relaxed purifying selection due to factors such as smaller census population size or lowered selective constraint. However, sexual and young asexual lineages of *P. antipodarum* coexist within many lakes (Lively 1987, 1992) and in habitats within lakes (Jokela and Lively 1995; Fox et al. 1996) and often make up a sizeable fraction of these sympatric populations (Lively and Jokela 2002; Neiman et al. 2005). It is therefore unlikely that asexual *P. antipodarum* experience a systematically lower census population size.

To estimate how these accelerated substitution rates translate into increased nonsynonymous substitutions within particular classes of *P. antipodarum* lineages, we used DnaSP v5.10 (Librado and Rozas 2009) to estimate the number of nonsynonymous substitutions experienced by each of the 18 *P. antipodarum* lineages relative to *P. estuarinus*. Across the 13 protein-coding genes (~8330 nonsynonymous sites) in the *P. antipodarum* mitochondrial genome, an average of 65.5 (± 5.74 standard deviation [SD]) nonsynonymous substitutions separated sexual lineages of *P. antipodarum* from *P. estuarinus*. Asexual lineages accumulated about five additional nonsynonymous substitutions during this divergence (mean = 71.25 ± 4.67 SD). When young versus old asexual lineages were considered separately, old asexual lineages (mean = 72.81 ± 5.54 SD) had accumulated about four more nonsynonymous substitutions than young asexual lineages (mean = 69.0 ± 2.13 SD). The elevated mutation accumulation in asexual taxa may represent a substantial mutation burden, especially if this phenomenon also occurs in the nuclear genome. If we assume that the nuclear genome has approximately 2000 times more genes than the mitochondrial genome, even accounting for the nuclear genome having roughly one-tenth the mutation rate (e.g., Haag-Liutard et al. 2008), the rate acceleration we observed would burden asexual nuclear genomes with hundreds of nonsynonymous substitutions. Indeed, mutation accumulation is often cited as one of the most likely reasons for why most asexual lineages seem to be short lived and for why the success of ancient asexual lineages such as the bdelloid rotifers is at least superficially an evolutionary paradox (e.g., Maynard Smith 1986). Our data indicate that this apparent paradox can be extended to much shorter lived asexual taxa, including asexual lineages within species.

Implications for the Maintenance of Sex

All the “young” asexual *P. antipodarum* lineages that we sampled (lineages no. 1–6, fig. 1B) share cytochrome b haplotypes with sexual individuals (haplotypes 1 and 18; Neiman and Lively 2004), meaning that they are likely to have been derived from sexual ancestors within several thousand years (Dybdahl and Lively 1995; Neiman et al. 2005). These lineages also are often found coexisting with sexual *P. antipodarum* (Neiman and Lively 2004; Neiman et al. 2005). The accelerated rate of accumulation of nonsynonymous substitutions in these recently derived asexual

lineages relative to sexuals demonstrates that this process can occur rapidly enough to be detectable before the sexuals have been competitively excluded. This may be especially likely in a system like *P. antipodarum* where Red Queen dynamics are likely to be operating because they should prolong the persistence of sexuals in mixed populations (Howard and Lively 1994; Neiman et al. 2009).

Elevated rates of mutation accumulation in recently derived asexual lineages have also been reported in *Daphnia* (Paland and Lynch 2006) and *Campeloma* (Johnson and Howard 2007). However, because the geographic distributions of sexual versus asexual *Daphnia* and *Campeloma* are almost entirely nonoverlapping (Paland et al. 2005; Johnson 2006), it is not clear that accelerated mutation accumulation can be relevant to the maintenance of sex or even that sex requires an explanation in those systems. Our study involves a system where sexuals and asexuals are frequently sympatric and ecologically similar, meaning that the accelerated mutation accumulation that we documented in asexual lineages is more likely to be a direct consequence of the absence of sex. The fact that there is increased mutation accumulation in recently derived asexual lineages in a system where sexuals and asexuals frequently coexist demonstrates that these mutational processes could be rapid enough to contribute to the maintenance of sex in natural populations.

Several lines of evidence suggest that the mutation accumulation observed in asexual *P. antipodarum* could have phenotypic consequences that might influence the persistence of sex in this system. First, mutation accumulation experiments in genetic model systems show that accelerated mutational buildup resulting from a decreased efficacy of purifying selection can cause marked declines in fitness in just a few dozen to a few hundred generations (e.g., Vassilieva et al. 2000; Denver et al. 2004). Second, the results of our MK tests strongly suggest that nonsynonymous and functional noncoding mutations are, on average, deleterious. Third, empirical studies have demonstrated negative phenotypic effects of mtDNA mutations (reviewed in Ballard and Rand 2005). Finally, the reduced efficacy of selection experienced by mtDNA in asexual *P. antipodarum* should—by extension—also affect the nuclear genome, perhaps, even more severely because the nuclear genome experiences the direct loss of physical recombination in a newly asexual lineage (as predicted by Normark and Moran 2000). Thus, the rapid increase in mutation accumulation in mtDNA may therefore reflect only a small fraction of the increased mutational load experienced by asexual lineages in *P. antipodarum*.

A role for mutation accumulation in the maintenance of sex requires that the mutations that build up at a higher rate in asexuals have harmful phenotypic consequences. Although earlier studies comparing morphology and life history (Jokela et al. 1997) and response to starvation (Lively et al. 1998) in sexual versus asexual *P. antipodarum* found no persistent effect of mating system, these earlier studies were limited to snails from Lake Alexandrina, where the asexual lineages are known to be recently derived (Dybdahl

and Lively 1995; Neiman et al. 2005). Thus, the evidence for wide variation in asexual lineage age in *P. antipodarum* (Neiman et al. 2005) sets the stage for comparisons of traits associated with fitness in sexual versus asexual and young versus old asexual lineages. If mutation accumulation is relevant to the maintenance of sex, we would expect that representatives of old asexual lineages might suffer in comparison to younger asexual lineages (and sexuals) with regard to important traits such as mitochondrial function and the rate of offspring production and population growth. Because harmful effects of mutation accumulation might be heightened under stressful conditions (e.g., Vassilieva et al. 2000; Young et al. 2009), experiments utilizing some form of stressor (e.g., disease, starvation, heat, cold) might be particularly useful. Studies evaluating these hypotheses would provide an important step forward in evaluating the extent to which mutation accumulation might contribute to the predominance and persistence of sex in natural populations.

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