Full Title:

Radical Amino Acid Changes Persist Longer in the Absence of Sex

Running Title:

Radical Amino Acid Changes Persist Longer in Asexuals

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Abstract

Harmful mutations are ubiquitous and inevitable, and the rate at which these mutations are removed from populations is a critical determinant of evolutionary fate. Closely related and otherwise similar sexual and asexual taxa provide a particularly powerful setting in which to study deleterious mutation elimination because sex should facilitate mutational clearance by reducing selective interference between sites. Here, we compared the rate of removal of conservative and radical nonsynonymous mutations in sexual vs. asexual populations of *Potamopyrgus antipodarum*, a New Zealand freshwater snail species featuring coexisting and ecologically similar sexual and asexual lineages. Our analyses revealed that radical changes are removed from populations at significantly higher rates than conservative changes and that sexual lineages eliminate these radical changes more rapidly than asexual counterparts, especially over relatively short time scales. Taken together, these results indicate that reduced efficacy of purifying selection in asexual lineages allows harmful mutations to remain polymorphic longer than in sexual lineages, potentially influencing the outcome of competition between sexual and asexual lineages. The fact that our ability to detect differential patterns of mutational clearance in sexual vs. asexual individuals required polymorphism data emphasizes the critical importance of population-level sampling for characterizing evolutionary phenomena.

Introduction

One of the primary hypothesized advantages for sexual reproduction is the clearance of harmful mutations, which is expected to be much more effective when linkage disequilibria (LD) are disrupted by sex (Hill and Robertson 1966). A particularly striking and important example of how sex facilitates LD breakdown is provided by the transfer of mitochondrial (mt) genomes to new and potentially divergent nuclear genomic backgrounds from parents (usually but not always mothers; Barr et al. 2005) to sexually produced offspring. The close interactions between nuclear and mt gene products mean that the consequences of breakdown of mitonuclear LD are likely to be substantial. A good example of these consequences is provided by the common observation that changes in nuclear genomic background can substantially decrease mitochondrial function (Ellison and Burton 2006, Meiklejohn et al. 2013, Pichaud et al. 2013), likely a result of coevolution between the mt genome and the nuclear genes that encode the interacting protein subunits of the oxidative phosphorylation (OXPHOS) pathway. Indeed, proper function of these subunits appears to be an important determinant of eukaryotic health (e.g., Chen et al. 2007, Pike et al. 2007, Barreto and Burton 2013, Muir et al. 2016).

Evolution of mitochondrial genomes in the absence of sex. With respect to coevolved mitonuclear protein complexes like those found in the OXPHOS pathway, the loss of sex in the nuclear genome is expected to result in at least one of two non-mutually exclusive evolutionary consequences. First, the biparental inheritance and meiotic recombination that the nuclear genome experiences during canonical sexual reproduction will decrease linkage disequilibrium (LD) in both the nuclear and mt genomes, increasing the efficacy of natural selection by decreasing interference from linked sites (Hill and Robertson 1966, Neiman and Taylor 2009).

By contrast, the uniparental (e.g., maternal) inheritance and reduced or absent meiotic recombination that is a feature of asexual reproduction will decrease effective population size (N_e) and increase LD across both genomes, decreasing the efficacy of selection by increasing selective interference (i.e., Hill-Robertson effect, Hill and Robertson 1966). More specifically, nuclear and mt genomes "trapped" in asexual lineages are co-transmitted as a single genetic unit, such that the two genomes are effectively linked (Normark and Moran 2000). This scenario underlies the expectation that asexual lineages will accumulate deleterious mutations more rapidly than otherwise similar sexual lineages in both the nuclear (Birky and Walsh 1988, Charlesworth 1993, Lynch et al. 1993) and mt genomes (Normark and Moran 2000, Neiman and Taylor 2009). Because mitonuclear incompatibilities are expected to increase with divergence (Burton and Barreto 2012) and mitonuclear linkage decreases the efficacy of selection (Normark and Moran 2000, Neiman and Taylor 2009), accelerated accumulation of mildly deleterious mutations within asexual lineages should also allow novel mitonuclear combinations, untested by nature, to arise over time.

Second, the absence of sex may allow for elevated rates of mitonuclear coevolution if the co-transmission of mt and nuclear genomes in asexuals allows selection to act more effectively on multilocus (e.g., mitonuclear) genotypes that interact epistatically but tend to be disrupted by recombination (Neiman and Linksvayer 2006). Thus, one important potential consequence of co-transmittance of nuclear and mt genomes in the context of mutation accumulation is that the permanent linkage of these two genomes may allow for relatively strong and effective selection for compensatory mutations in response to accumulated deleterious mutations in OXPHOS genes.

The first scenario (increased mutation accumulation in asexuals) is expected to favor sexual lineages that can effectively remove deleterious mutations, while the second scenario

(tighter coevolution in asexuals) should increase the cost of sex (i.e., recombination load, see Maynard Smith 1978), thereby favoring asexual lineages. Both scenarios are expected to result in more rapid accumulation of nonsynonymous mutations in the mt genomes of asexual lineages than sexual lineages, making it essential to evaluate the relative fitness effects (i.e., deleterious, neutral, or beneficial, and to what extent) of the mutations that accumulate in asexual lineages.

Differential fitness effects of mutations and the efficacy of selection. The prediction that the mt genomes of asexuals should experience a higher rate of accumulation of nonsynonymous mutations has found support from animal (Cutter and Payseur 2003, Neiman et al. 2010, Henry et al. 2012) and plant (Horandl and Hojsgaard 2012, Voigt-Zielinsji et al. 2012, Hollister et al. 2015) taxa. While these results represent important steps towards understanding the genomic consequences of asexuality, the evolutionary mechanisms underlying these observations remain unclear, in large part because the extent to which accumulated mutations are actually deleterious in asexuals has not been evaluated. Here, we depart from previous studies assessing mutational load in asexuals that treat all nonsynonymous mutations as a monolithic "deleterious" class (but see Henry et al. 2012) despite evidence that nonsynonymous mutations are likely to vary widely in fitness effects (Keightley and Eyre-Walker 2007). Predicting and characterizing the fitness effects of these nonsynonymous mutations remains a major challenge in evolutionary biology (Keightley and Charlesworth 2005, Xue et al. 2008, Eyre-Walker and Keightley 2009, Halligan et al. 2011). Yet, partitioning nonsynonymous changes into "conservative" changes (amino acid changes in which the derived amino acid has similar biochemical properties to the ancestral amino acid) and "radical" changes (amino acid changes in which the derived amino acid has markedly different biochemical properties compared to the ancestral amino acid) represents a

116 relatively straightforward method for inferring effects of mutations on protein phenotype (Zhang
117 2000, Smith 2003, Hanada et al. 2007, Popadin et al. 2007).

118 Because mutational severity largely governs the efficacy with which selection can act
119 upon any given mutation (Fisher 1930), partitioning nonsynonymous changes into conservative
120 and radical mutational types also allows for an intuitive means of comparing the efficacy of
121 selection in sexual vs. asexual genomes. At present, commonly used methods for inferring
122 selection on DNA sequence data (e.g., π, θ, Tajima's D, d_N/d_S, *etc*.) do not typically incorporate
123 information from the distribution of fitness effects (DFE) of mutations (but see Kryazhimskiy
124 and Plotkin 2008, Schneider et al. 2011). Here, we provide a proof of principle for using
125 mutational information (i.e., radical vs. conservative changes) to infer the type, intensity, and
126 efficacy of selection using traditional tests of selection (e.g., McDonald-Kreitman test of
127 selection, π, θ, K_A/K_S), applied to the mt genomes of sexual vs. asexual snails.
128
129 ***Potamopyrgus antipodarum*: a snail model for the evolutionary maintenance of sex.** The
130 removal of deleterious mutations (and the fixation of beneficial mutations) depends upon the
131 efficacy of selection as well as the fitness effect of the mutation(s), such that elevated N_e and
132 reduced LD in sexual vs. asexual populations should result in more rapid removal of deleterious
133 mutations for the former (Birky and Walsh 1988). As such, comparing rates and patterns of
134 evolution across reproductive modes while incorporating DFE information will provide a unique
135 and powerful glimpse into the evolutionary dynamics governing the removal of deleterious
136 mutations. The New Zealand freshwater snail *Potamopyrgus antipodarum* is ideally suited to
137 evaluate this critically important evolutionary process because otherwise similar obligately
138 sexual and obligately asexual *P. antipodarum* frequently coexist within New Zealand lake

139 populations (Lively 1987, Jokela et al. 1997), enabling direct comparisons across reproductive
140 modes, and, thereby, across lineages that vary in the efficacy of selection. Asexual lineages of *P.*
141 *antipodarum* are the product of multiple distinct transitions from sexual *P. antipodarum* (Neiman
142 and Lively 2004, Neiman et al. 2011), meaning that these asexual lineages represent separate
143 natural experiments into the consequences of the absence of sex. Neiman et al. (2010) showed
144 that asexual lineages of *P. antipodarum* experience a higher rate of nonsynonymous substitution
145 in their mt genomes than sexual lineages. Here, we use an expanded mt genomic dataset to
146 evaluate whether sexual lineages distinguish between radical and conservative changes more
147 effectively than asexual lineages at interspecific and intraspecific levels. This approach allowed
148 us to evaluate for the first time whether harmful mutation accumulation is visible at the
149 polymorphic level and, if so, whether this phenomenon is driven by more effective selection in
150 sexual lineages vs. relatively rapid mitonuclear coevolution in asexual lineages. The outcome of
151 these analyses emphasizes fundamental differences in the rate of accumulation of conservative vs.
152 radical nonsynonymous mutations and suggests that radical mutations persist longer in mt
153 genomes of asexual lineages of *P. antipodarum* compared to sexual counterparts, likely a
154 consequence of reduced efficacy of purifying selection.
155
156 **Materials & Methods**
157 **Sequencing.** We analyzed 31 whole *P. antipodarum* mt genomes from 8 sexual lineages and 23
158 asexual lineages, representing the natural range of this species in New Zealand along with
159 several invasive lineages (European, North American, see Table S1, Figure 1). Eighteen of these
160 genomes (4 sexual, 14 asexual) were obtained from Genbank (Accession Nos.: GQ996416 –
161 GQ996433), along with the whole mt genome of an outgroup species, *Potamopyrgus estuarinus*

(Accession No.: GQ996415.1) (Neiman et al. 2010). Five mt genomes (2 sexual lineages, 3 asexual lineages) were assembled from the DNA sequence data generated via Illumina technology from the ongoing *P. antipodarum* nuclear genome project. The remaining eight mt genomes (1 sexual lineage, 7 asexual lineages) were newly sequenced via bi-directional Sanger sequencing on an ABI 3730. DNA for the Sanger-sequenced lineages was extracted with a mollusk-adapted phenol-chloroform extraction protocol (Fukami et al. 2004). Mt genomes were amplified in four overlapping fragments using primers and programs designed in Neiman et al (2010). PCR products were cleaned with Shrimp Exo shrimp alkaline phosphatase (Werle et al. 1994) and directly sequenced with internal sequencing primers (Table S2). The newly generated mt genome sequence data were assembled and manually edited in Sequencher 5.0. For these eight newly generated mt genome sequences, only unambiguous sites with ≥ 2 Sanger reads were used in our analyses. All new sequences will be deposited in GenBank.

We used flow cytometry (following the protocol outlined in Neiman et al. 2011, Neiman et al. 2012, Paczesniak et al. 2013, Krist et al. 2014) to assign ploidy and thus reproductive mode (diploid – sexual; polyploid – asexual) to the newly sequenced lineages for which ploidy had not already been determined.

Phylogenetic analysis. Concatenated mt genome sequences have been shown to produce more accurate tree topologies than single gene trees (Rokas et al. 2003, Gadagkar et al. 2005). Accordingly, we concatenated nucleotide sequences from ~11 kbp protein-coding nucleotides from each of the 31 *P. antipodarum* lineages and from *P. estuarinus*, for a total of 32 concatenated sequences. The concatenated sequences were aligned in the correct reading frame using the ClustalW package implemented in MEGA 5.2.2 (Kumar et al. 2008) and manually

185 edited (alignment available upon request). To minimize the effects of selection on tree topology,
186 we used only 3rd-position sites to infer the mt genome phylogeny using the maximum likelihood
187 (ML) methods implemented in MEGA 5.2.2 software (Kumar et al. 2008), using the ML model
188 selection tool in MEGA 5.2.2 to select the Tamura-Nei model of molecular evolution with
189 gamma-distributed sites (Tamura and Nei 1993). Tree topology was assessed using 1,000
190 bootstrap replicates and visualized using FigTree v1.4 (Raumbaut 2007): only nodes with
191 bootstrap support > 60 were relied upon for tests of molecular evolution. The tree topology that
192 we obtained (Figure 1) is qualitatively identical to previously published mitochondrial trees for *P.*
193 *antipodarum* (Neiman et al. 2004, Neiman et al. 2010, and Paczesniak et al. 2013).
194
195 **Quantifying the rate of radical** vs**. conservative mutation and substitution.** We used seven
196 different amino acid classification schemes drawn from Zhang (2000), Hanada et al. (2007), and
197 a modified Grantham scheme based on amino acid composition, polarity, and volume (Grantham
198 1974) to evaluate patterns of radical and conservative nonsynonymous polymorphism and
199 substitution in the mt genome of *P. antipodarum* (Table 1). We defined radical mutations as
200 mutations in which the derived amino acid was from a different category than the ancestral
201 amino acid, while conservative mutations were defined as mutations in which the derived amino
202 acid and the ancestral amino acid were in the same category. While there is some overlap
203 between different classification schemes, each scheme highlights different amino acid properties
204 that are likely to shape protein evolution. To wit, amino acid charge is a major determinant of
205 protein folding (Perutz et al. 1965, Anfinsen 1973, Nakashima et al. 1986, Bashford et al. 1987,
206 Wright et al. 2005) and three-dimensional structure (Lesk and Chothia 1980, Geisler and Weber
207 1982, Doms et al. 1988, Rumbley et al. 2001), and uncharged to charged amino acid changes

208 (and vice versa) are rarely maintained (see Table S3, K_R/K_S). Amino acid polarity is particularly
209 important for proper membrane integration, as phospholipid membranes are highly hydrophobic,
210 and changes between polar and non-polar amino acids may expose or bury key interaction
211 residues (Von Heijne 1992). Volume and aromaticity can both affect protein folding (e.g.,
212 proline is a structure breaker) and can play a role in protein-protein interactions (Burley and
213 Petsko 1985). Classification schemes 4 and 7 are unique in that they are based on evolutionary
214 information (although classification scheme 7 largely fits with charge and polarity
215 classifications), meaning that these schemes incorporate aspects of other amino acid
216 characteristics into their classifications. All mutational types were defined relative to the
217 invertebrate mt genetic code (http://www.ncbi.nlm.nih.gov/Taxonomy/Utils/wprintgc.cgi#SG5).
218 The number of synonymous, conservative nonsynonymous, and radical nonsynonymous sites per
219 codon are detailed in Table S4.
220 Because the number of substitutions per site can be used as an estimate of the rate of
221 substitution (Li et al. 1985), we counted the number of nucleotide substitutions and determined
222 the type of substitution (i.e., synonymous, conservative nonsynonymous, and radical
223 nonsynonymous) in the protein-coding regions of the mt genomes (excluding codons with >1
224 change) of each lineage relative to *P. estuarinus*. We then calculated the number of mutational
225 target sites per lineage for a given type of mutation (see Table S4). To confirm that the number
226 of each of type of site was properly calculated, we checked that the number of nonsynonymous
227 sites and the number of synonymous sites per codon summed to three and that the number of
228 conservative nonsynonymous sites and the number of radical nonsynonymous sites per codon
229 summed to the number of nonsynonymous sites per codon (Hanada et al. 2007). Of particular
230 note is that the “GTG”, “TTG”, “ATT”, “ATC”, and “ATA” codons can all be used as

alternative start codons in invertebrate mt genomes (only the GTG alternative start codon was observed in the present dataset), which we accounted for in our site calculations. We then used custom-built Python scripts (available upon request) to calculate substitution rates for each mutational type and used the Jukes-Cantor correction to account for multiple hits (Jukes and Cantor 1969).

Substitutions between species are generally older than polymorphisms within species (McDonald 1996), such that different analyses implicitly assume particular and distinct time scales (i.e., substitutions: relatively old time scale, hereafter "long time scale"; polymorphisms: relatively recent time scale, hereafter "short time scale"; ratio of polymorphism to divergence – composite of recent and old time scales, hereafter "composite time scale"). We compared the mean rate of conservative nonsynonymous substitution (Jukes-Cantor corrected; number of conservative nonsynonymous substitutions/conservative nonsynonymous site; K_C) to the mean rate of radical nonsynonymous substitution (Jukes-Cantor corrected; number of radical nonsynonymous substitutions/radical nonsynonymous site; K_R) from each of the seven amino acid classification schemes to the Jukes-Cantor-corrected mean synonymous substitution rate (number of synonymous substitutions/synonymous site; K_S) and to one another using pairwise Mann-Whitney U (MWU) tests and the Holm procedure for modified Bonferroni correction for multiple comparisons (Holm 1979). All statistical tests were performed in R (R Core Team 2012). In subsequent analyses, we corrected for systematic across-lineage differences in underlying mutation rate by dividing the estimate of K_R and K_C for each lineage by K_S.

Because polymorphisms within species give rise to divergences between species, and because higher ratios of polymorphism to divergence are indicative of more intense purifying selection (McDonald and Kreitman 1991), we used McDonald Kreitman (MK) tests of selection

to evaluate whether conservative changes were more likely than radical changes to reach fixation using a Fisher's Exact Test (FET) with the Holm modification to the Bonferroni correction for multiple comparisons for each of the seven amino acid categories. As an additional test of the intensity of selection acting on conservative vs. radical changes, we compared the ratios of polymorphism to divergence for conservative vs. radical changes by performing 10,000 bootstrap replicates. *P*-values were inferred by comparing the resulting bootstrap distributions (Efron and Tibshirani 1993) and significance was evaluated using the Holm modification to the Bonferroni correction for multiple comparisons for each of the seven amino acid categories.

Finally, we evaluated nucleotide diversity, π (Tajima 1989), nucleotide heterozygosity, θ (Watterson 1975), and Tajima's *D* (Tajima 1989) in conservative vs. radical sites to test whether conservative and radical sites differ at the intraspecific (i.e., relatively recent) level. Polymorphism at sites under more stringent purifying selection will tend to exist at lower relative frequencies than at sites under less stringent purifying selection. To compare these intraspecific measures of molecular evolution in all seven amino acid classification schemes we performed 10,000 bootstrap replicates, inferred *p*-values from the resulting distributions, and evaluated statistical significance using the Holm modification to the Bonferroni correction for multiple comparisons.

Comparisons between sexual and asexual lineages. Although our sampling scheme provided a comprehensive picture of the mitochondrial diversity present in *P. antipodarum* (see Figure 1, Neiman and Lively 2004, Neiman et al. 2011, Paczesniak et al. 2013), we lacked the statistical power to use standard Phylogenetic Independent Contrast methods (Felsenstein 1985) to compare sexuals and asexuals while accounting for phylogenetic non-independence. Instead, we

277 employed custom Python scripts to randomly sample (with replacement) sexual and asexual
278 lineages to compare rates and patterns of conservative and radical nonsynonymous evolution at
279 short, long, and composite time scales. We used 10,000 bootstrap replicates to estimate means
280 and 95% CIs for K_C/K_S, K_R/K_S, P_C/D_C, P_R/D_R, π_C/π_S, π_R/π_S, θ_C/θ_S, and θ_R/θ_S in sexual vs. asexual
281 lineages in all seven amino acid classification schemes. We estimated two-tailed *p*-values by
282 determining the probability of overlap of the bootstrap distributions and determined statistical
283 significance using the Holm procedure to perform a modified Bonferroni correction for multiple
284 comparisons.

285 To account for possible effects of differential sampling of sexual (n = 8) vs. asexual (n =
286 23) lineages, we used the methods described above to compare rates and patterns of conservative
287 vs. radical molecular evolution across reproductive modes by generating 10,000 bootstrap
288 replicates using equal (n = 8) and randomly-chosen-with-replacement, sample sizes for sexual
289 and asexual lineages. The results of this equivalent sampling analysis are depicted in Figure 3
290 and Table S3.

291 Because population demography can influence patterns of molecular evolution (Tajima
292 1989), and because transitions to asexuality and/or the rapid growth expected for a newly-
293 generated asexual lineage might influence important demographic parameters (e.g., N_e; Kaiser
294 and Charlesworth 2009), it is important to account for potential effects of demography when
295 evaluating molecular evolution in sexuals vs. asexuals (Wright and Charlesworth 2001). We
296 dealt with this issue by modifying the Watterson estimator of nucleotide heterozygosity θ
297 (Watterson 1975) by calculating the mean number of unique mutations per mt genome in sexuals
298 vs. asexuals, θ_U:

299 $$(1) \qquad \theta_U = \frac{S_U}{L\, a_n}$$

where S_U is the number of unique polymorphisms (i.e., changes that appear only within a single lineage), L is the number of mutational target sites, and a_n is:

$$a_n = \sum_{i=1}^{n-1} \frac{1}{i} \quad (2)$$

in which n is equal to the number of lineages per reproductive mode. Fu and Li (1993) have shown that θ_U is an estimate of nucleotide heterozygosity, θ, that only uses the external branch lengths of a population (see equation 18, Fu and Li 1993). We compared the mean number of unique polymorphisms per site for each mutational type across reproductive modes using 95% CIs generated by 10,000 bootstrap replicates (Figure 4, Table S3).

One group of asexual *P. antipodarum* that we included in these analyses is quite genetically distinct (mean pairwise distance between clade A and (clade B, clade C) = 0.035) from other lineages (mean pairwise distance within (clade B, clade C) = 0.013) (Figure 1). Therefore, including clade A may cause us to overestimate the extent of mutation accumulation in asexual *P. antipodarum* as a whole. In order to exclude the possibility that this divergent group of asexual lineages may be driving the observed pattern of amino acid evolution, we repeated the comparisons of molecular evolution with clade A excluded, using the clade B sexuals as an outgroup to clade C (see Figure 1, Table S3).

Results & Discussion

Relative harm conferred by conservative vs**. radical mutations.** We used ~11 kbp of mt protein-coding sequence representing ~8520 nonsynonymous sites generated from eight sexual and 23 asexual *P. antipodarum* lineages (Table S1, Figure 1) and seven distinct amino acid classification schemes (Table 1) to evaluate the relative harmfulness of radical vs. conservative mutations and address whether reproductive mode influences the rate of accumulation of these

two types of mutations in mt genomes. We quantified the number of each mutation type present across these 31 *P. antipodarum* lineages relative to an outgroup, *P. estuarinus* (Table S1, Figure 1). We detected a total of 35 nonsynonymous fixed differences between these two species (mean conservative nonsynonymous fixed differences = 27.14, SD = 4.49; mean radical nonsynonymous fixed differences = 6.71, SD = 3.59).

Before comparing mutation accumulation across reproductive modes, we established the type and intensity of selection acting on conservative vs. radical changes in the *P. antipodarum* mitochondrial genome. To infer the type and intensity of selection acting on conservative vs. radical changes at a relatively long time scale, we compared the Jukes-Cantor corrected rates of conservative nonsynonymous substitution (K_C) and radical nonsynonymous substitution (K_R) to the Jukes-Cantor corrected synonymous substitution rate (K_S). We found that conservative and radical nonsynonymous substitutions accumulate at significantly lower rates than synonymous substitutions in all amino acid classification schemes (Figure 2A, Table S3). Additionally, conservative amino acid changes contribute to divergence between *P. antipodarum* and *P. estuarinus* significantly more than radical changes (Figure 2A) in each of the seven amino acid classification schemes (MWU: $p < 0.0001$ for all seven schemes, Table S3). These results indicate that while both conservative and radical mutations are fixed at discernably lower rates than synonymous changes, radical amino acid changes are fixed significantly less often than conservative amino acid changes.

We next compared the ratio of polymorphism to divergence for synonymous, nonsynonymous, conservative, and radical differences by performing MK tests of selection (Figure 2B, Table S3). We calculated the mean number of polymorphisms and divergences across amino acid classification schemes and found that conservative polymorphisms (mean

346 number of conservative polymorphisms = 122.14, SD = 27.25) and radical polymorphisms
347 (mean number of radical polymorphisms = 63.86, SD = 27.25) were significantly less likely to
348 reach fixation than synonymous polymorphisms (number of synonymous polymorphisms = 619)
349 (FET: $p < 2.2 \times 10^{-16}$ for both mutational types). We also found that conservative polymorphisms
350 were significantly more likely to contribute to divergence than radical polymorphisms (FET: $p =$
351 0.049, see Table S3 for MK tests of individual amino acid classification schemes). We also
352 compared the mean ratio of polymorphism to divergence for conservative (P_C/D_C) vs. radical
353 (P_R/D_R) changes using 10,000 bootstrap replicates. We found that mean P_R/D_R was significantly
354 higher than mean P_C/D_C by comparing bootstrap distributions ($p < 0.0002$, see Table S3 for
355 P_C/D_C vs. P_R/D_R of individual amino acid classification schemes). These findings reveal that
356 while both conservative and radical changes appear to be evolving under strong purifying
357 selection in *P. antipodarum* mt genomes, radical nonsynonymous changes are eliminated even
358 more rapidly than conservative nonsynonymous changes.
359 We next compared nucleotide diversity, π, at synonymous, nonsynonymous, conservative,
360 and radical sites, which provides a picture of the strength and efficacy of selection at a relatively
361 short time scale. Sites under purifying selection are expected to exhibit lower levels of nucleotide
362 diversity than relatively neutral sites (e.g., nonsynonymous vs. synonymous sites, respectively)
363 (Nei and Gojobori 1986). Consistent with this prediction, we used 10,000 bootstrap replicates to
364 compare nucleotide diversity across site types and found that mean nonsynonymous nucleotide
365 diversity ($\pi_A = 0.0050$) was significantly lower than mean synonymous nucleotide diversity ($\pi_S =$
366 0.066) in *P. antipodarum* ($p < 0.0002$, Table S3). Similarly, mean conservative nonsynonymous
367 nucleotide diversity ($\pi_C = 0.0060$) and mean radical nonsynonymous nucleotide diversity ($\pi_R =$
368 0.0027) were significantly lower than mean π_S ($p < 0.0002$ for π_C and π_R, see Table S3 for

individual schemes). When correcting for π_S (e.g., π_A/π_S), we found that mean synonymous-corrected radical nucleotide diversity ($\pi_R/\pi_S = 0.042$) was over two-fold lower than mean synonymous-corrected conservative nucleotide diversity ($\pi_C/\pi_S = 0.090$) ($p < 0.0002$, see Table S3 for individual schemes), indicating that radical polymorphisms are maintained at lower frequencies than conservative polymorphisms (Figure 2B, Table S3). Thus, even at a relatively short time scale, radical amino acid changes appear to experience more stringent purifying selection than conservative amino acid changes in *P. antipodarum* mt genomes.

Together, these results are consistent with the expectation that radical mutations are usually more harmful than conservative mutations (Rand et al. 2000, Freudenberg-Hua et al. 2003, Smith 2003, Yampolsky et al. 2005). Our results are important in demonstrating that radical and conservative mutations appear to experience very different histories of selection in natural populations. In particular, we provide some of the first evidence from a non-model system featuring reproductive mode polymorphism (a primary determinant of N_e) that there exists more stringent purifying selection on radical vs. conservative mutational types, a pattern detectable even at the intraspecific level. While there is substantial overlap amongst classification schemes, the consistent signal of more stringent purifying selection acting on radical changes is evidence that the grouping strategies employed by each scheme (*e.g.*, charge, polarity, volume, *etc*.) are founded on important biological properties of amino acids. Our results also emphasize that the relative degree of amino acid change should be an important consideration in evaluating patterns of selection and suggest that the standard grouping of radical and conservative mutational types into a monolithic "nonsynonymous" class is often overly simplistic and may be positively misleading (for an example, see Summary & Implications).

391 Comparisons across long, short, and composite time scales (e.g., substitution rates vs.
392 nucleotide diversity vs. ratio of polymorphism to divergence) reveal qualitatively similar results
393 across all three time scales (Figure 2). In particular, radical changes clearly experience a higher
394 intensity of purifying selection than do conservative changes, indicating that radical changes
395 usually impart substantially more severe fitness effects than conservative changes and should
396 thus return to mutation-selection-drift equilibrium more rapidly than their conservative
397 counterparts (Figure 2, Fisher 1930). As such, we can use comparisons of these two mutational
398 types across different time scales to compare the efficacy with which sexual vs. asexual lineages
399 remove relatively mild (i.e., conservative) and relatively severe (i.e., radical) deleterious
400 mutations.
401
402 **Estimating the efficacy of purifying selection in sexuals** vs. **asexuals.** To estimate the relative
403 differences in rates of evolution in sexual vs. asexual lineages of *P. antipodarum* over a
404 relatively long time scale, we compared synonymous substitution rates (K_S), conservative
405 nonsynonymous substitution rates (K_C), and radical nonsynonymous substitution rates (K_R) for
406 sexuals vs. asexuals using 10,000 bootstrap replicates. We found that mean K_S of asexual *P.*
407 *antipodarum* is significantly higher than K_S of sexual *P. antipodarum* ($p < 0.0002$), indicating
408 that asexual *P. antipodarum* experience a higher rate of synonymous substitution than sexuals
409 (Table S2; see also Neiman et al. 2010). To account for this difference, we corrected estimates of
410 K_C and K_R by K_S and then compared K_C/K_S (mean sexual = 0.021, mean asexual = 0.022) and
411 K_R/K_S (mean sexual = 0.0059, mean asexual = 0.0063) across reproductive modes using 10,000
412 bootstrap replicates. While we did not detect significant differences between sexual and asexual
413 K_C/K_S or K_R/K_S ($p = 0.061$ and $p = 0.18$, respectively, Table S3), we did recapitulate Neiman et

al. (2010) in detecting significantly higher K_A/K_S in asexuals vs. sexuals ($p = 0.0034$). Thus, by subdividing the number of substitutions into conservative and radical categories, we likely lost substantial power in our ability to detect differences across reproductive modes. This explanation is especially likely considering there are only a total of 35 nonsynonymous fixed differences between the *P. antipodarum* and *P. estuarinus* mt genome sequences. Absence of evidence for significant differences in the conservative and radical substitution rates might also be linked to the relatively recent and multiple derivations of asexuality in *P. antipodarum* (Neiman and Lively 2004, Paczesniak et al. 2013). Indeed, our results suggest that the resolution with which two groups of organisms (e.g., sexual vs. asexual) can be differentiated with substitutions might decrease with increasing divergence from the outgroup. In other words, a large class of deleterious mutations that are still evident on the intraspecific level have long since disappeared at the interspecific level, rendering them invisible to methods of quantifying mutation accumulation that only focus on divergence.

To compare the efficacy of selection in different sexuals vs. asexuals at a composite time scale, we compared the mean ratios of polymorphism to divergence for conservative (P_C:D_C) and radical (P_R:D_R) changes across reproductive modes in all seven amino acid classification schemes. Because our sample included an unequal number of sexual lineages (n = 8) compared to the number of asexual lineages (n = 23), and because the number of polymorphisms increases with sample size, we performed pairwise comparisons of P_C:D_C and P_R:D_R across reproductive modes by performing 10,000 bootstrap replicates of eight randomly sampled (with replacement) sexual lineages and eight randomly sampled (with replacement) asexual lineages. While sexual and asexual lineages exhibited significantly lower mean P_C:D_C than P_R:D_R ($p = 0.0080$ and $p = 0.0082$, respectively), the fact that mean sexual P_CD_C is significantly lower than mean asexual

437 P_RD_R ($p = 0.0010$), but that mean asexual P_CD_C is statistically indistinguishable from mean
438 sexual P_RD_R ($p = 0.11$, Figure 3, see Table S3 for individual classification schemes) indicates
439 that the probability of radical polymorphisms proceeding to fixation is higher in asexual lineages
440 than in sexual lineages. These results from a composite time scale are consistent with sexual
441 lineages eliminating radical, but not conservative, polymorphisms more rapidly than asexual
442 lineages.

443 We next compared nucleotide diversity (π) and nucleotide heterozygosity (θ) across
444 reproductive modes using 10,000 bootstrap replicates. To account for differential effects of
445 neutral processes (e.g., demographic changes, mutation rate, *etc*.) on nucleotide diversity in
446 sexuals vs. asexuals (Charlesworth and Wright 2001, Kaiser and Charlesworth 2009), and
447 because sexual π_S was significantly lower than asexual π_S ($p = 0.011$, Table S3), we corrected π_C
448 and π_R by dividing each value by π_S. Similarly, because asexuals exhibited significantly higher θ_S
449 than sexual lineages ($p = 0.013$, Table S3), we corrected estimates of θ_C and θ_R by dividing by θ_S.
450 We did not find any significant differences in mean π_C/π_S, π_R/π_S, θ_C/θ_S, or θ_R/θ_S between sexuals
451 and asexuals (Table S3), indicating that sexual and asexual *P. antipodarum* harbor similar
452 numbers of conservative and radical polymorphisms and at similar relative frequencies. At face
453 value, these results are consistent with a scenario in which sexual and asexual lineages
454 experience similar efficacies of selection. What must be taken into account, however, is that at
455 the time of asexual origin, only a single founding mt genome is transmitted to a newly asexual
456 lineage. The consequence of this bottleneck at the transition to a new asexual lineage is that
457 ancestrally polymorphic sites become immediately monomorphic *within* the new asexual lineage.
458 This phenomenon should cause rapid changes in allele frequencies and thus, changes in
459 frequency-dependent measures of polymorphism (e.g., π) of the sampled population. The

implications are that such comparisons of nucleotide diversity and heterozygosity between sexuals and asexuals do not necessarily provide a complete or accurate picture of evolutionary processes.

To account for changes in allele frequency caused by the transition to asexuality itself, we compared the mean number of unique polymorphisms per genome (see Methods) across reproductive modes. By sampling only unique polymorphisms (i.e., relatively new mutations), we were able to estimate mutation accumulation *since* the transition to asexuality. Sexuals and asexuals did not differ in terms of the mean number of unique synonymous polymorphisms per site ($\theta_{U\text{-}S}$), indicating that new mutations arise at similar rates across reproductive modes (Figure 4). Sexuals exhibited significantly lower mean $\theta_{U\text{-}R}/\theta_{U\text{-}S}$ than $\theta_{U\text{-}C}/\theta_{U\text{-}S}$ ($p < 0.0002$), while $\theta_{U\text{-}C}/\theta_{U\text{-}S}$ and $\theta_{U\text{-}R}/\theta_{U\text{-}S}$ were statistically indistinguishable in asexual lineages ($p = 0.75$), indicating that selection recognizes and removes deleterious changes more rapidly in sexual lineages than in asexual lineages (Figure 4, see Table S3 for individual classification schemes). Further, these results indicate that differences in the efficacy of selection across reproductive modes become apparent relatively quickly after the transition to asexuality, especially with respect to mutational changes with relatively large selection coefficients (i.e., radical mutations).

One group of asexuals was particularly genetically distinct relative to the rest of the *P. antipodarum* dataset (clade A, Figure 1), raising the question of whether this group might be contributing disproportionately to our observations of slowed radical mutation elimination in asexual *P. antipodarum*. We addressed this possibility by performing the same analyses described above on a subsample of *P. antipodarum* lineages (clade C) but treated the clade B sexuals as an outgroup (see Methods, Table S3) and excluded clade A entirely. The results of this more limited analysis are largely consistent with the outcomes of analyses from the whole

483 dataset (Table S3), indicating that the inclusion of the relatively divergent asexual clade A did
484 not substantively affect the original analysis. Notably, sexual lineages in clade C maintain radical
485 polymorphisms as significantly lower frequencies than conservative polymorphisms (mean π_C/π_S
486 = 0.11, mean π_R/π_S = 0.058, $p < 0.0002$, see Table S2 for individual classification schemes),
487 while conservative and radical polymorphisms were maintained at statistically indistinguishable
488 levels in clade C asexual lineages (mean π_C/π_S = 0.13, mean π_R/π_S = 0.082, $p = 0.27$, see Table S2
489 for individual classification schemes), consistent with ineffective selection in asexual lineages
490 contributing to the retention of radical polymorphisms.
491
492 **Summary & Implications.** Asexual *P. antipodarum* have already been found to exhibit elevated
493 accumulation of nonsynonymous substitutions in their mt genomes relative to sexual *P.*
494 *antipodarum* (Neiman et al. 2010). Here, we provide evidence that asexual *P. antipodarum*
495 exhibit elevated ratios of polymorphism to divergence for radical changes and harbor more
496 unique radical polymorphisms than sexuals, a particularly harmful type of nonsynonymous
497 mutation. Our analyses of the relative effects of conservative vs. radical changes provide a novel
498 line of evidence that the mutations these asexual lineages are accumulating are deleterious.
499 Together, these findings indicate that asexual lineages of *P. antipodarum* likely experience an
500 increased rate of accumulation of harmful mutations than sexual conspecifics, a pattern that is
501 observable at both relatively long (substitution) (Neiman et al. 2010, present study) and
502 relatively short (polymorphism) time scales (present study).
503 Radical mutations appear more likely to be deleterious than conservative mutations and
504 asexual *P. antipodarum* appear to be accumulating these mutations more rapidly than sexual *P.*
505 *antipodarum*, raising the intriguing possibility that asexual *P. antipodarum* might exhibit

506 decreased mitochondrial function compared to sexual counterparts. The presumed severity of
507 some mutations in these genomes (e.g., a nonsense mutation in *nd2* of one asexual lineage that
508 would truncate ND2 by three amino acids) suggests that either mitochondrial function is
509 decreased in at least some asexual lineages or that asexuals possess one or more mechanisms to
510 compensate for deleterious mutation load (e.g., RNA editing). RNA editing of mt-encoded
511 transcripts has been observed in a variety of plant taxa (Covello and Gray 1989, Gualberto et al.
512 1989) and in land snail mt-encoded tRNAs (Yokobori and Paabo 1995), but it is unclear whether
513 *P. antipodarum* employs similar strategies. Future work evaluating mitochondrial function at the
514 organelle and organismal levels in *P. antipodarum* will be essential to understanding how the
515 efficacy of selection influences the maintenance and distribution of sex in this system.
516 Our study also provides a clear demonstration of a situation where inclusion of all
517 nonsynonymous changes in a monolithic "deleterious" category can obscure important
518 evolutionary dynamics, especially at the polymorphic level. A particularly illuminating example
519 of the potential for this type of grouping to result in misleading conclusions is that when
520 nonsynonymous changes are treated as a single group, the number of unique nonsynonymous
521 mutations per genome ($\theta_{U\text{-}A}/\theta_{U\text{-}S}$) in sexual vs. asexual lineages is statistically indistinguishable,
522 in stark contrast to the clear distinctions between sexual and asexual *P. antipodarum* that are
523 revealed by taking mutational effect into account. The implications are, that by partitioning
524 mutations into conservative and radical bins, we gain substantial resolution at the intraspecific
525 level. Similarly, we find that sexual and asexual P/D ratios are statistically indistinguishable if all
526 nonsynonymous mutations are grouped together. At a longer time scale (i.e., divergence), the
527 relative dearth of radical nonsynonymous changes appears to present temporal sampling issues,

such that differences between sexual and asexual *P. antipodarum* can no longer be detected. This result highlights the importance of representative intraspecific sampling and analysis.

While we interpret these results as resulting from less effective selection in asexual lineages, another possible (and non-mutually exclusive) explanation is that the co-transmission (and thus, effective linkage) between the nuclear and mt genomes in asexuals has facilitated the persistence and spread of beneficial nonsynonymous mutations via selection imposed by cooperation with nuclear-encoded genes (Blier et al. 2001, Meiklejohn et al. 2007). Because asexuals co-transmit their nuclear and mt genomes, mutations in either genome may cause decreases in mitochondrial function. Therefore, long-term co-transmission of the nuclear and mt genomes may provide a scenario in which asexuals experience relatively strong selection favoring compensatory mutation(s). We have not detected any evidence of positive selection acting in the mt genome of *P. antipodarum* (e.g., codon-by-codon $d_N/d_S < 1$, Neutrality Index > 1 for all 13 protein-coding genes, sliding window $\pi_A/\pi_S < 1$ at all sites, data not shown), though indirect evidence that a particular mt haplotype is spreading amongst asexual lineages hints that selection favoring particular mt haplotypes or mitonuclear combinations might be involved (Paczesniak et al. 2013). Evaluation of rates and patterns of evolution in the nuclear-encoded mt genes that make up ≥95% of the genes that influence mitochondrial function (Sardiello et al. 2003), coupled with the functional analyses mentioned above, will ultimately be needed to determine whether mitonuclear linkage in asexuals is at least in part responsible for elevated retention of apparently harmful mutations in mt genomes.

Our results taken from different time points in the *P. antipodarum* evolutionary history lead us to conclude that patterns of protein evolution in this species are being driven both by mutational severity and reproductive mode. In particular, at the shortest time scale (i.e., number

of unique polymorphisms), sexuals and asexuals differ in the number of radical but not conservative changes. At an older though still population-level scale (i.e., nucleotide diversity for clade C only), radical polymorphisms reside at higher frequencies in asexuals rather than sexuals. Differences across reproductive modes disappear at even older time scales (i.e., species-wide nucleotide diversity, rates of substitution), although the composite time scale revealed that asexuals have higher ratios of polymorphism to divergence than sexuals, particularly with respect to radical changes. While these observations might appear inconsistent, the implicit time scale assumption of each respective measurement instead hints that the selective process plays out at different rates in sexuals vs. asexuals. Namely, our data indicate that mutations in asexuals experience smaller $N_e|s|$ than in sexuals, meaning that the elimination of apparently deleterious mutations occurs more slowly in asexual than in sexual lineages. Theoretical (Ohta 1987, Charlesworth et al. 1993, Charlesworth and Wright 2001) and empirical (Wright et al. 2008, Katju et al. 2015) work support this conclusion, in that populations with low N_e are expected to harbor a larger proportion of "effectively neutral" mutations than populations with large N_e. Our data are consistent with this phenomenon, revealing that amino acid-changing mutations (especially radical changes) in the mt genome remain polymorphic longer in asexual than in sexual lineages, an observation only possible with a multiple time scales approach.

Ultimately, less efficient removal of deleterious mutations in asexual lineages is only important to the maintenance of sex and/or the persistence of asexual lineages if those mutations in fact negatively affect fitness. Recent empirical evidence suggests that harmful mutations indeed play a role in asexual lineage deterioration: Tucker et al. (2013) found that obligately asexual *Daphnia* suffer from gene conversion-type processes that decrease heterozygosity and subsequently expose deleterious recessive alleles, leading to lineage deterioration (Tucker et al.

574 2013). By contrast, our data indicate that asexual *P. antipodarum* harbor elevated numbers of
575 deleterious mutations due to less efficient removal of existing mutations rather than accelerated
576 acquisition of new mutations. Because mt genotype is critically important to organismal function
577 and fitness (Ellison and Burton 2006, Meiklejohn et al. 2013, Pichaud et al. 2013, Muir et al.
578 2016), this increased load of likely harmful mutations could potentially contribute to negative
579 phenotypic consequences in asexuals, though slowed deleterious mutation removal in asexuals
580 would likely also need to be prevalent in the nuclear genome in order to provide the short-term
581 advantages necessary to maintain sexual reproduction within a population (Lynch et al. 1993).
582 Given that the nuclear genome is the site of the vast majority of gene content and recombination
583 in sexual lineages, our observation of elevated retention of deleterious mutations in mt genomes
584 of asexual *P. antipodarum* leads us to predict that the nuclear genome is also likely to exhibit
585 substantial differences in deleterious mutational load and efficacy of selection across
586 reproductive modes.
587

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595

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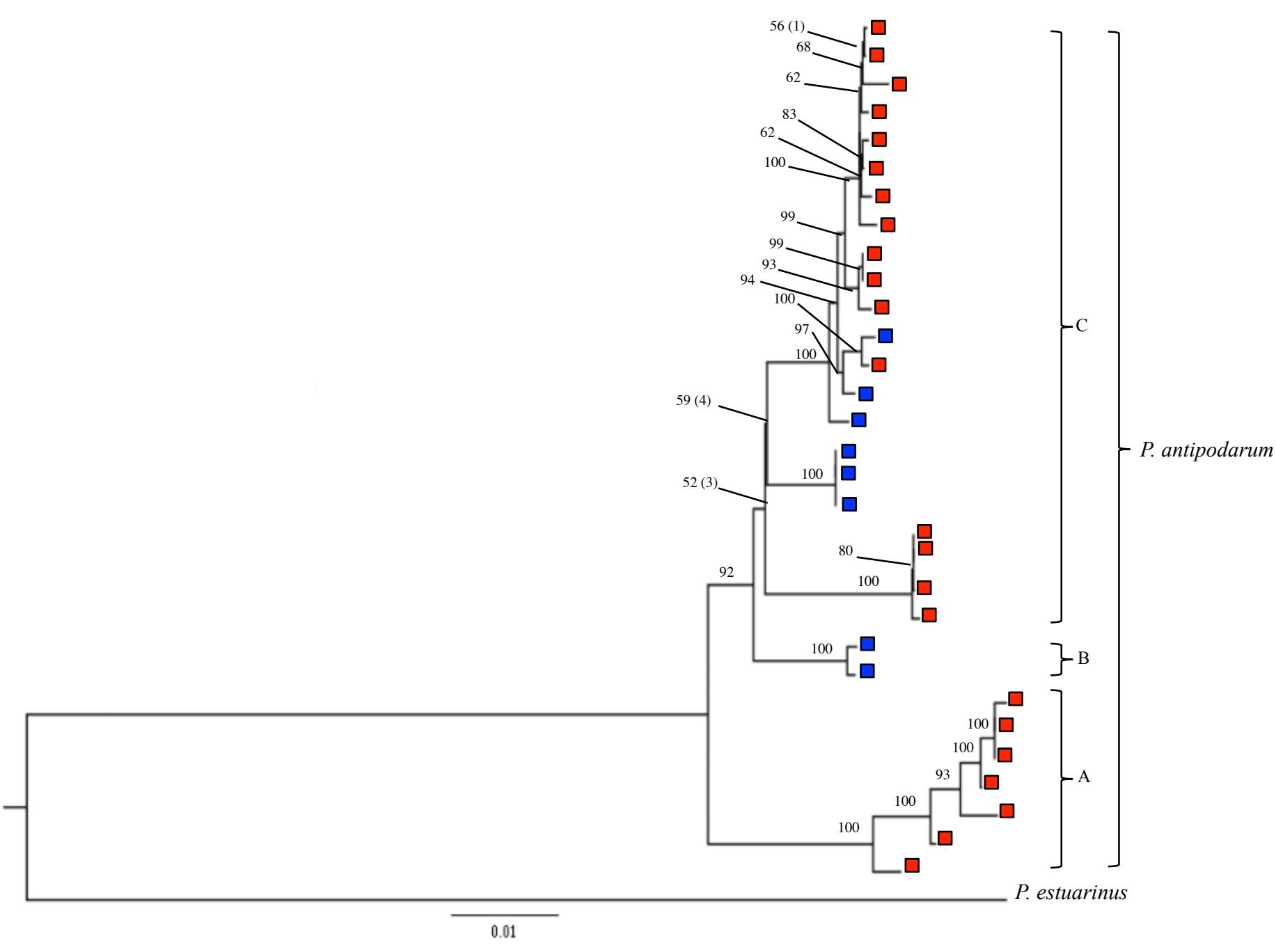
Figure 1. Whole-mt genome maximum likelihood phylogeny for *P. antipodarum* and an outgroup, *P. estuarinus*. Maximum likelihood-based phylogeny of 23 asexual (red) and eight sexual (blue) *Potamopyrgus antipodarum* lineages using only 3rd position nucleotides of the ~11kbp protein-coding region of the mitochondrial genome. Branch support was estimated using 1,000 bootstrap replicates; only bootstrap values >50 are shown. Because clade A represents a particularly distinct group of asexuals (mean pairwise distance between clade A and (clade B, clade C) = 0.035, mean pairwise distance within (clade B, clade C) = 0.013), we removed clade A from the re-analysis of rates and patterns of amino acid sequence evolution; clade C was used as the ingroup and clade B as an outgroup in this analysis.

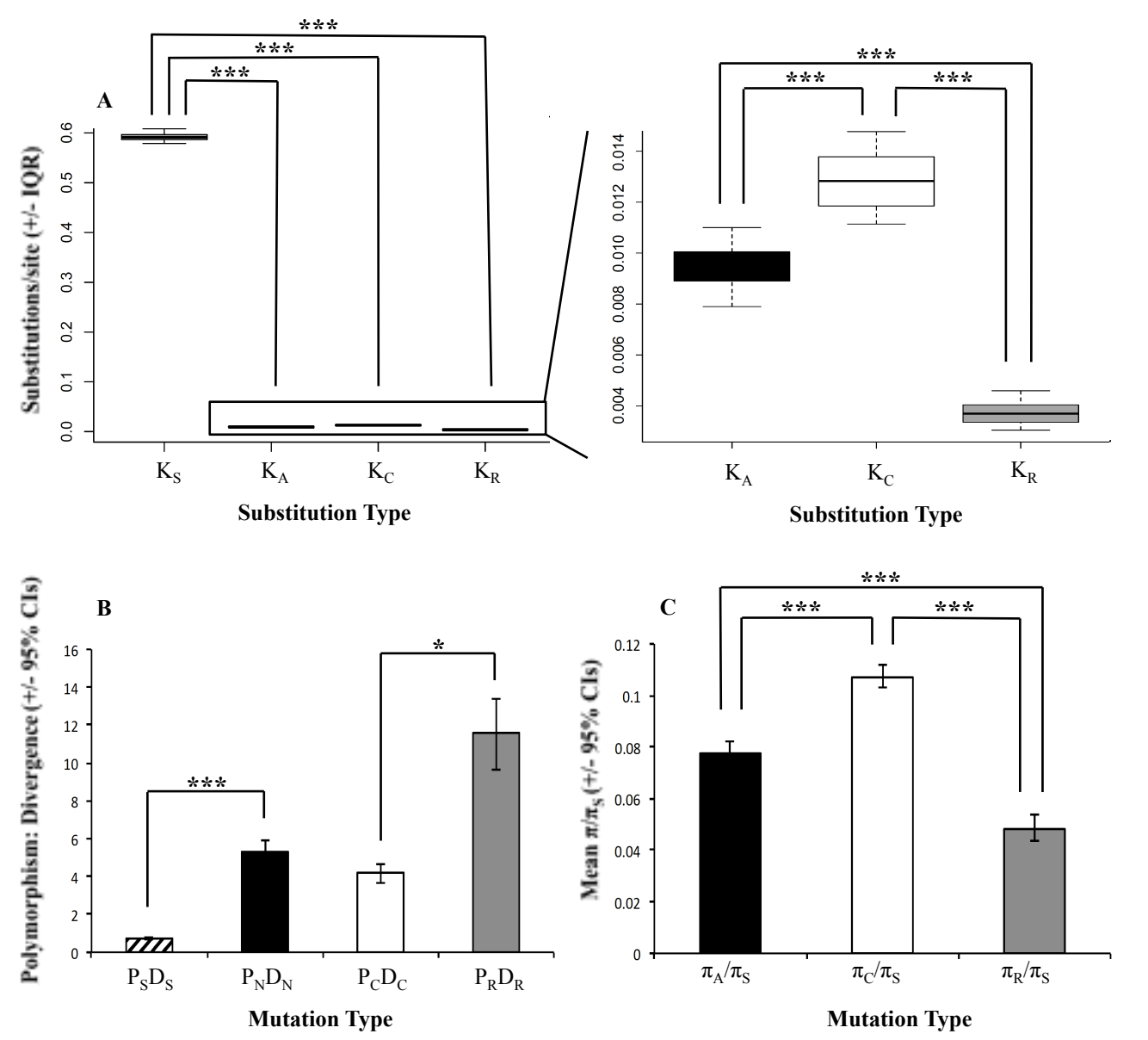
Figure 2. Molecular evolution of conservative and radical changes in mt genomes of *P. antipodarum*. A) Comparison of the Jukes-Cantor-corrected substitution rates across different mutational types. Left: Substitutions per site; K_S – synonymous, K_A – nonsynonymous, K_C – conservative nonsynonymous, and K_R – radical nonsynonymous. Right: Inset of boxed-in region depicting only K_A, K_C, and K_R. Error bars represent inner-quartile ranges (IQR). Statistical significance was assessed using a Mann-Whitney U test. B) Ratio of polymorphism to divergence in sites with conservative (white) vs. radical (gray) changes. Ratios for synonymous

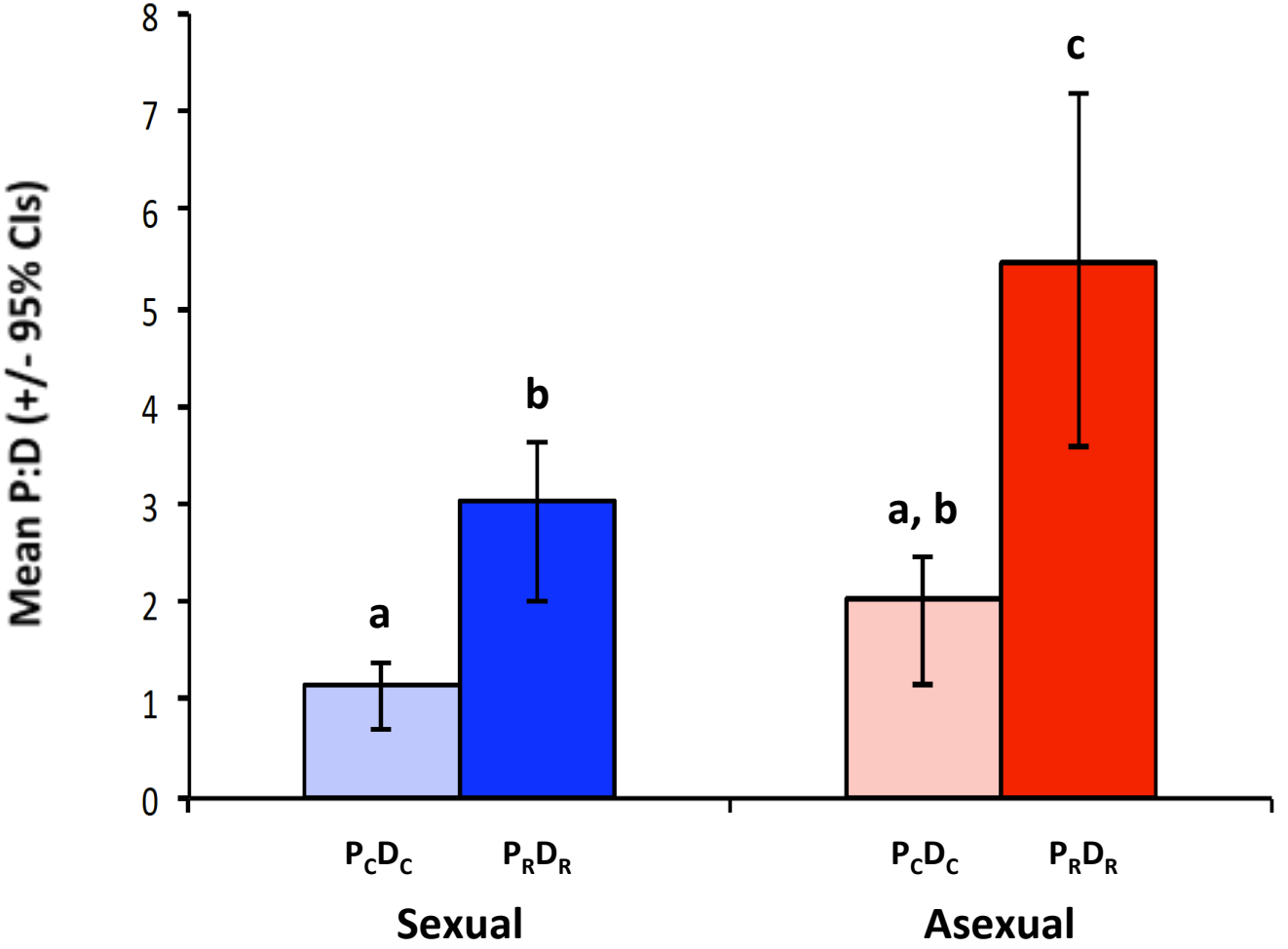
(diagonal stripes) and nonsynonymous (black) mutational types are shown for comparison. Error bars represent 95% confidence intervals generated using 10,000 bootstrap replicates. Statistical significance in P_C:D_C vs. P_R:D_R was assessed using Fisher's Exact Test. C) Mean synonymous-corrected nucleotide diversity for conservative (white) vs. radical (gray) sites in *P. antipodarum*. Nucleotide diversity at nonsynonymous sites is shown for comparison. Error bars represent 95% confidence intervals generated using 10,000 bootstrap replicates. Asterisks indicate significant differences (* = $p < 0.05$, *** = $p < 0.0002$).

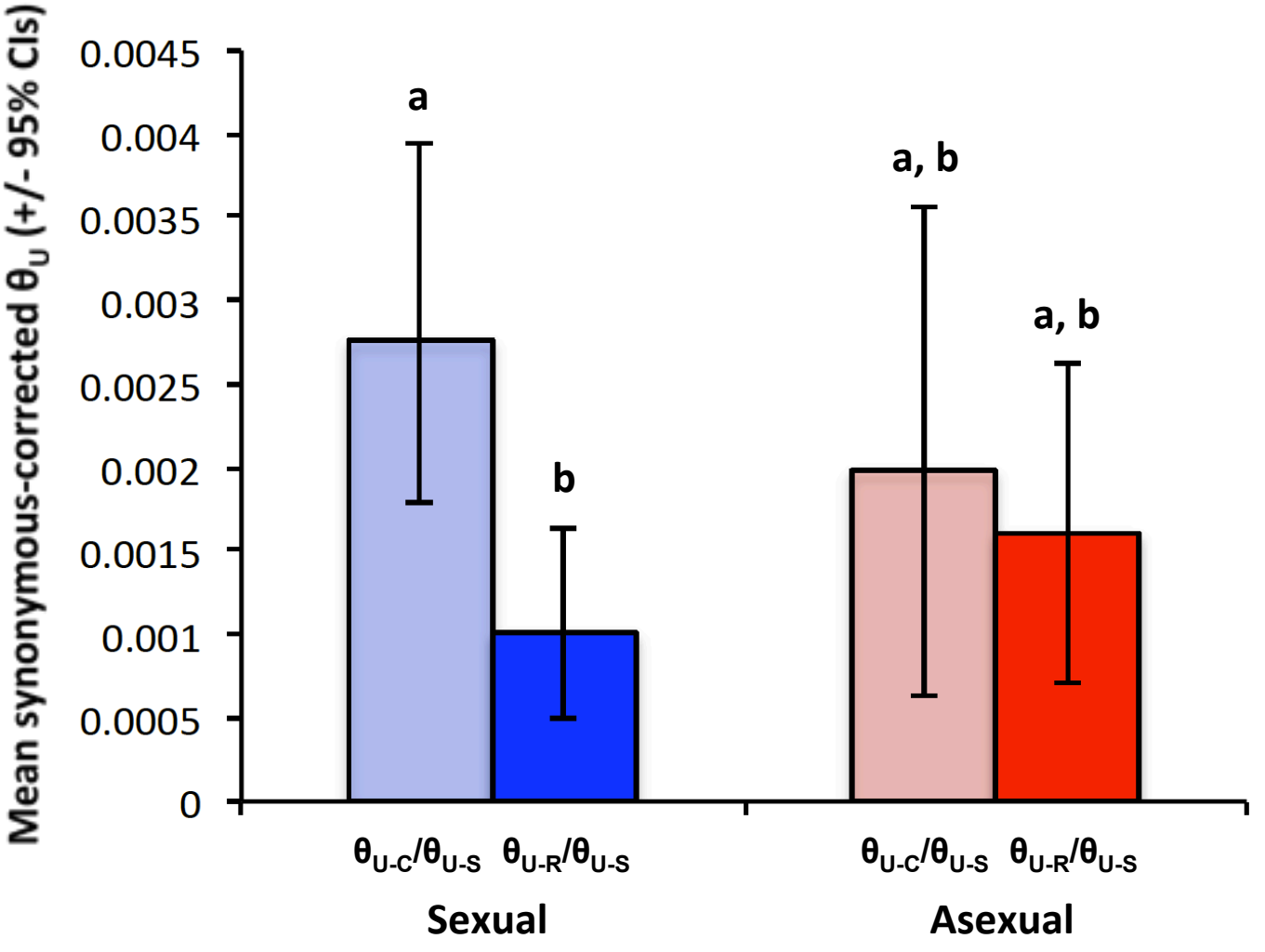
Figure 3. Ratios of polymorphism to divergence for conservative and radical changes in sexual vs. asexual lineages of *P. antipodarum*. Comparison of ratios of polymorphism to divergence for sexual (blue) vs. asexual (red) lineages for conservative (semi-transparent) vs. radical (solid) changes. Error bars indicate 95% CIs generated using 10,000 bootstrap replicates. Lower-case letters indicate statistical groupings determined using the Holm-modified Bonferroni correction for multiple comparisons.

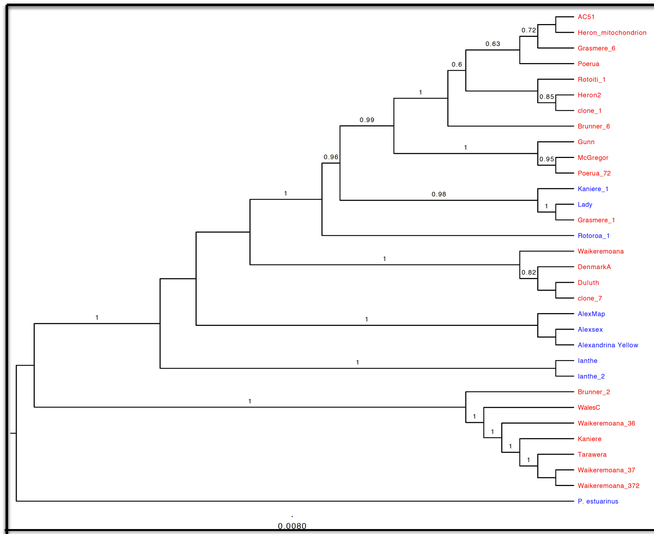
Figure 4. Mean number of unique polymorphisms per genome (θ_U) for sexual vs. **asexual lineages of *P. antipodarum*.** Mean number of conservative (semi-transparent) and radical (solid) unique polymorphisms per site for sexual (blue) vs. asexual (red) lineages of *P. antipodarum*. Error bars indicate 95% CIs generated using 10,000 bootstrap replicates. Lower-case letters indicate statistical groupings determined using the Holm-modified Bonferroni correction for multiple comparisons.

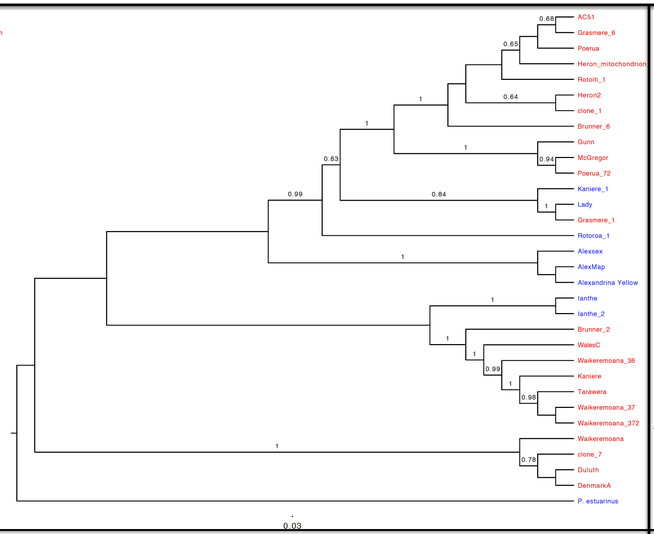

56 (1)
68
62
83
62
100
99
99
93
94
100
97
100
59 (4)
100
52 (3)
80
100
92
100
100
100
93
100
100
C
B
A
P. antipodarum
P. estuarinus
0.01

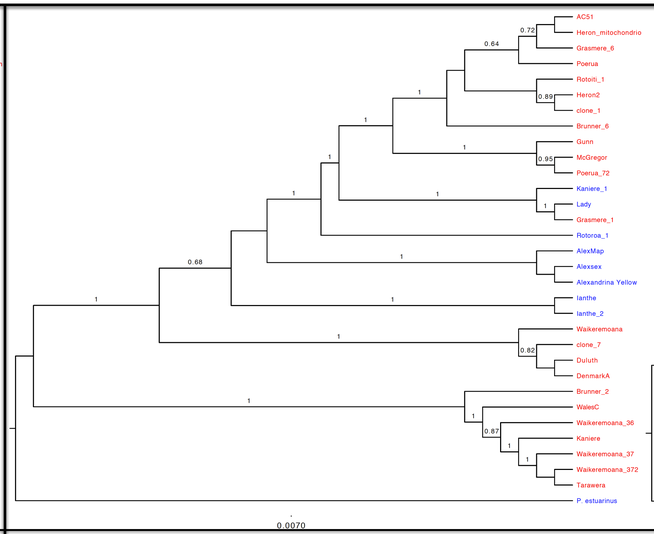

A
***
***
***
Substitutions/site (+/- IQR)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
K_S
K_A
K_C
K_R
Substitution Type
***
***
***
0.004
0.006
0.008
0.010
0.012
0.014
K_A
K_C
K_R
Substitution Type
B
Polymorphism: Divergence (+/- 95% CIs)
***
*
0
2
4
6
8
10
12
14
16
P_SD_S
P_ND_N
P_CD_C
P_RD_R
Mutation Type
C
***
***
***
Mean π/π_S (+/- 95% CIs)
0
0.02
0.04
0.06
0.08
0.1
0.12
π_A/π_S
π_C/π_S
π_R/π_S
Mutation Type

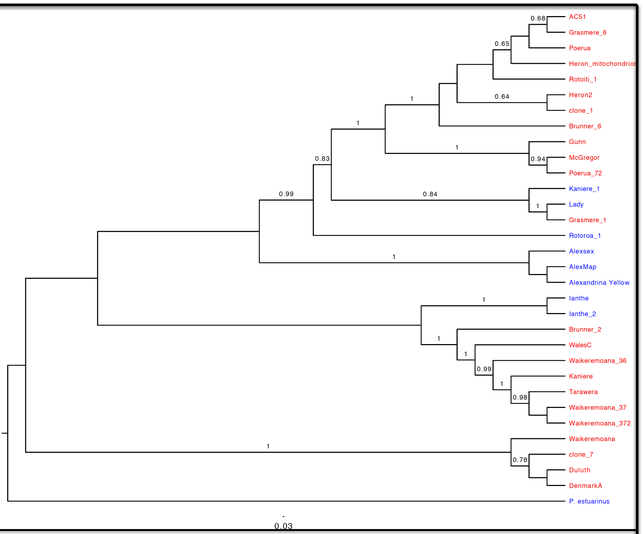

Mean P:D (+/- 95% CIs)
8
7
6
5
4
3
2
1
0
a
b
a, b
c
P_CD_C
P_RD_R
P_CD_C
P_RD_R
Sexual
Asexual

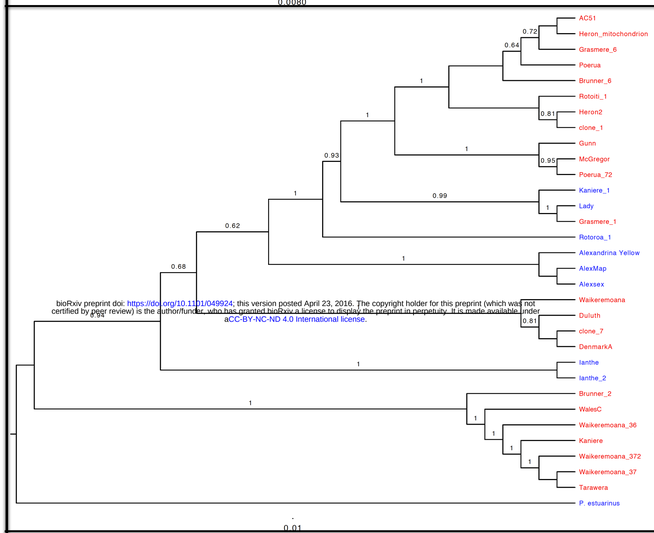

Mean synonymous-corrected θU (+/- 95% CIs)
0.0045
0.004
0.0035
0.003
0.0025
0.002
0.0015
0.001
0.0005
0
a
b
a, b
a, b
θU-C/θU-S
θU-R/θU-S
θU-C/θU-S
θU-R/θU-S
Sexual
Asexual

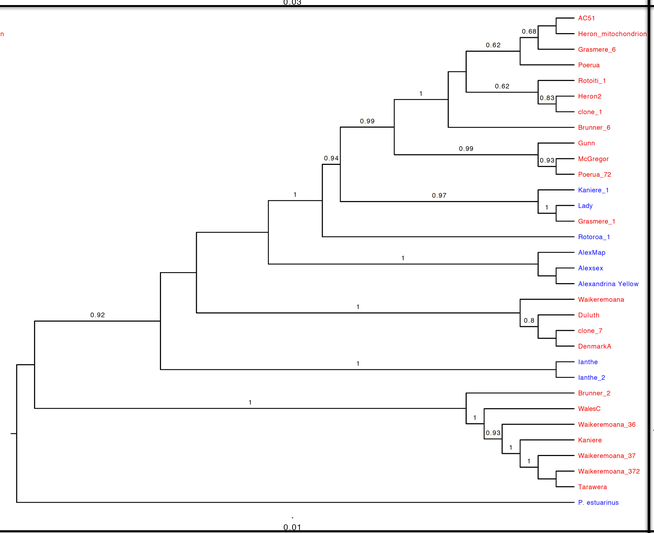


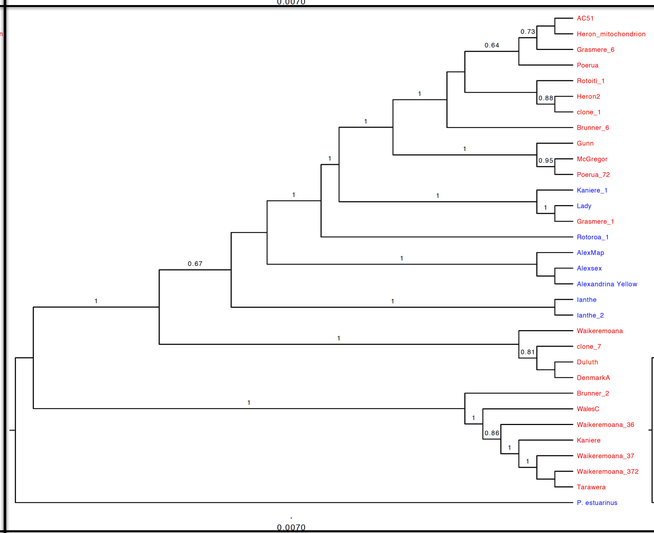


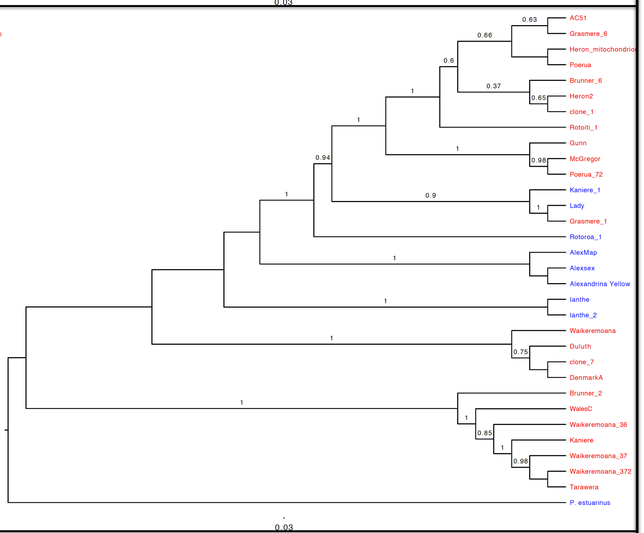


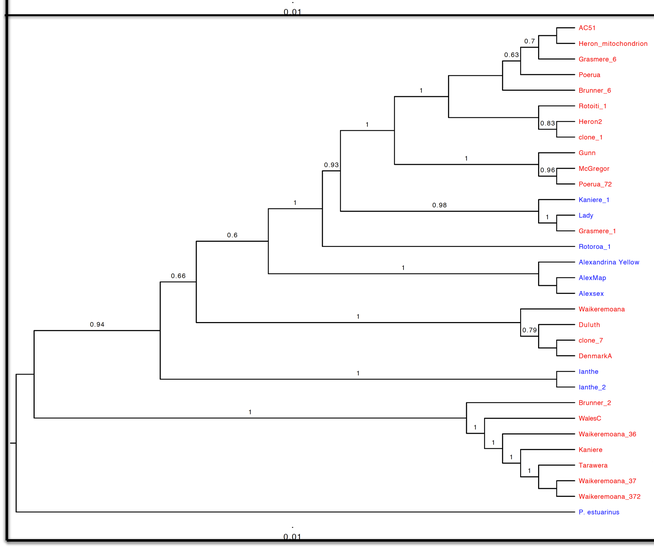


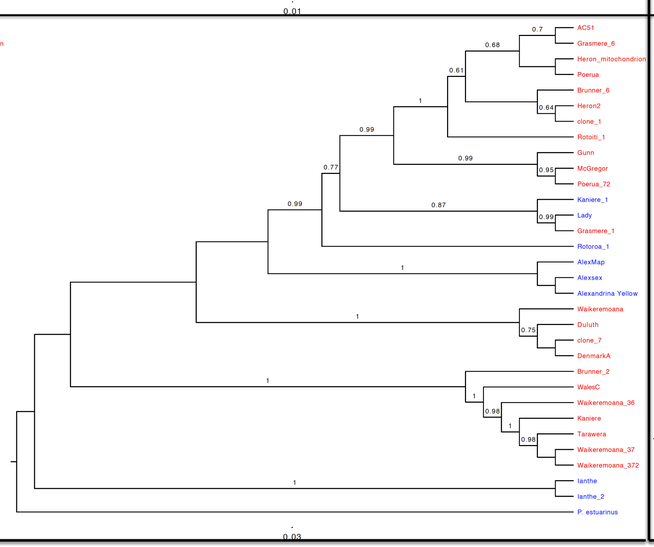


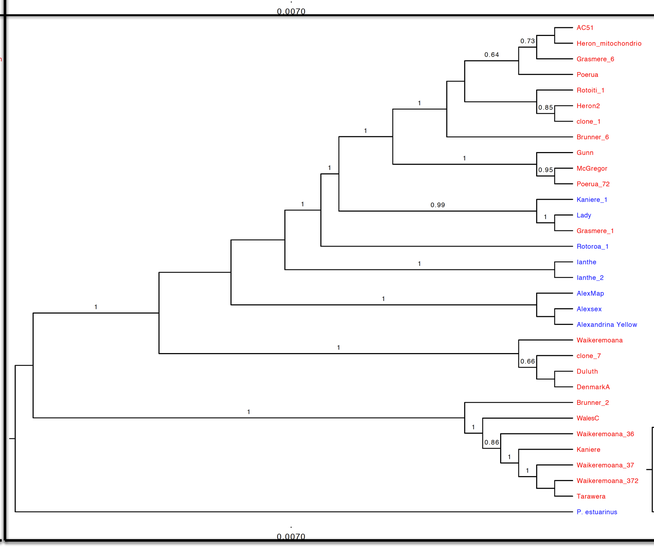












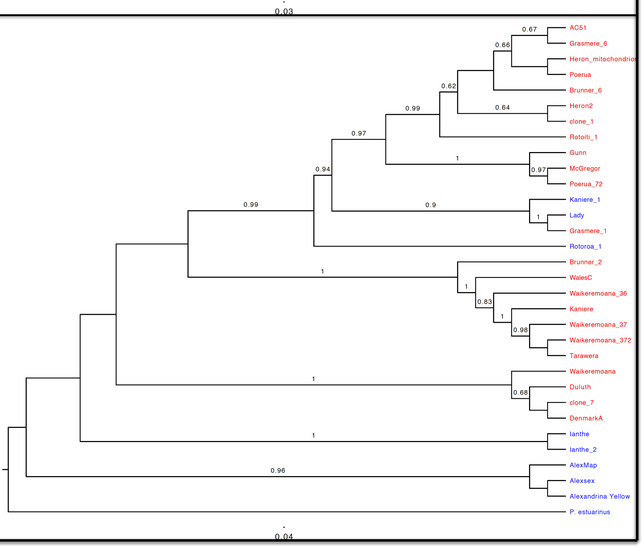


Table S1. Summary of source populations of *Potamopyrgus antipodarum.*

Lake of Origin	Latitude/Longitude	Reproductive Mode	Ploidy	Sequencing Platform	Reference
Alexandrina	-35.440603, 139.083438	Sexual	2x	ABI 3730	Neiman et al. 2010
Alexandrina	-35.440603, 139.083438	Sexual	2x	ABI 3730	Neiman et al. 2010
Alexandrina	-35.440603, 139.083438	Sexual	2x	Illumina	This study
Alexandrina	-35.440603, 139.083438	Asexual	3x	ABI 3730	Neiman et al. 2010
Alexandrina	-35.440603, 139.083438	Asexual	3x	ABI 3730	Neiman et al. 2010
Alexandrina	-35.440603, 139.083438	Asexual	3x	ABI 3730	Neiman et al. 2010
Brunner	-42.607385, 171.439636	Asexual	3x	ABI 3730	This study
Brunner	-42.607385, 171.439636	Asexual	4x	ABI 3730	This study
Denmark	56.001158, 9.207968	Asexual	3x	ABI 3730	Neiman et al. 2010
Grasmere	-43.061036, 171.774569	Asexual	3x	ABI 3730	This study
Grasmere	-43.061036, 171.774569	Asexual	4x	ABI 3730	This study
Gunn	-44.874524, 168.090031	Asexual	3x	ABI 3730	Neiman et al. 2010
Heron	-43.481012, 171.169173	Asexual	3x	ABI 3730	Neiman et al. 2010
Heron	-43.481012, 171.169173	Asexual	3x	ABI 3730	Neiman et al. 2010
Ianthe	-43.481012, 171.169173	Sexual	2x	ABI 3730	Neiman et al. 2010
Ianthe	-43.481012, 171.169173	Sexual	2x	Illumina	This study
Kaniere	-43.481012, 171.169173	Sexual	2x	ABI 3730	This study
Kaniere	-43.481012, 171.169173	Asexual	3x	Illumina	This study
Lady	-42.599558, 171.573173	Sexual	2x	ABI 3730	Neiman et al. 2010
Lake Superior	47.917620, -86.953400	Asexual	3x	ABI 3730	Neiman et al. 2010
McGregor	-43.936019, 170.470227	Asexual	3x	ABI 3730	Neiman et al. 2010
Poerua	-42.702716, 171.495638	Asexual	3x	Illumina	This study
Poerua	-42.702716, 171.495638	Asexual	4x	ABI 3730, Illumina*	This study
Rotoiti	-38.037084, 176.345628	Asexual	4x	ABI 3730	This study
Rotoroa	-41.851912, 172.642727	Sexual	2x	ABI 3730	This study
Tarawera	-38.186357, 176.429540	Asexual	3x	ABI 3730	Neiman et al. 2010
Waikaremoana	-38.771011, 177.109841	Asexual	3x	ABI 3730	Neiman et al. 2010
Waikaremoana	-38.771011, 177.109841	Asexual	3x	ABI 3730	Neiman et al. 2010
Waikaremoana	-38.771011, 177.109841	Asexual	3x	ABI 3730	Neiman et al. 2010
Waikaremoana	-38.771011, 177.109841	Asexual	3x	Illumina	This study
Wales	52.321471, -3.703571	Asexual	3x	ABI 3730	Neiman et al. 2010

* – Same DNA extraction was sequenced on both platforms

Table S2. Primers used to amplify and sequence whole mtDNA in *P. antipodarum*.

Fragment	Forward	Reverse
PCR		
1	5′-GAGGTAGGAGACTGTAGT-3′	5′-GAGTCCTAAGCCCAATGCA-3′
2	5′-GCTAGTATGAATGGTTTGACG-3′	5′-GTTATGGCAGCAATAGTAATTG-3′
3	5′-TCAGCTTGTGGATCTGA-3′	5′-GCCTAATCAGTATGAGGAAG-3′
4	5′-GGAGTGAACGGAAATCA-3′	5′-CTCTTGAGTATGCTGAGTACA-3′
Sequencing		
1.1	5′TATGAATATTCAGATTTTTTAAATA-3′	5′-CTGCCACCTTTATTATAAAG-3′
1.2	5′-GGCTCATAGTTTACTTAACTT-3′	5′-CGTCAAACCATTCATACTAGC-3′
1.3	5′-GCGGTTAGACCACGAAG-3′	5′-AACTAATAGATGTTTCTATG-3′
1.4	5′-CATAGAAACATCTATTAGTT-3′	5′-CTAATCCCAGTTTCCCTC-3′
2.1	5′-GTCTCTCTCTAATTTTATAG-3′	5′-GCTGATAGATGAAAGTCTC-3′
2.2	5′-CTCTCCTTATTTTTTAGCC-3′	5′-ATATTTGCAGGAATTCAGTG-3′
2.3	5′-GCATTGGAAGCTAAAGAC-3′	5′-GTTAACAGCTTCTGTTCG-3′
2.4	5′-CAGCACACTTGAAACATTG-3′	5′-CTCATATCTTGCTGCAAC-3′
3.1	5′-CTTCATCAATTAGCGCTTTATTT-3′	5′-GTGAAAGAAATCTTAGCCTA-3′
3.2	5′-CTTTCTACCTTAAGCCAGCTAG-3′	5′-GTACTAAGCCCCTAAAGGCAA-3′
3.3	5′-CAGCACAGCCTTTAACTAAG-3′	5′CTAAATGAAAGGGGTTACG-3′
3.4	5′-CCGCTAAATCCATTTGAAG-3′	5′-GTCATCCCTGTAGCTAG-3′
4.1	5′-CTATTGTAGTTATATTGTTGG-3′	5′-GAGATATTACAAGCGGTG-3′
4.2	5′-CAATTTCTTCCTCATACTGATT-3′	5′-CCTTAACTCCTAATCTTGGTAC-3′
4.3	5′-TTAGGGTGGATGCTATTTGC-3'	5′-GATACAAGAGCCTCTCATAC-3′
4.4	5′-GATTTAGCTATTTTTTCATTAC-3′	5′-CTGTTGTAATAAAGTTTACTG-3′

Table S4. Number of sites for different mutational types across seven amino acid classification schemes in the invertebrate mitochondrial genetic code.

				1		2		3		4		5		6		7	
Codon	**Amino Acid**	**Synonymous**	**Nonsynonymous**	**Radical**	**Conservative**	**Radical**	**Conservative**	**Radical**	**Conservative**	**Radical**	**Conservative**	**Radical**	**Conservative**	**Radical**	**Conservative**	**Radical**	**Conservative**
TTT	F	0.33	2.67	0.00	2.67	1.00	1.67	2.33	0.33	2.33	0.33	2.33	0.33	1.00	1.67	1.00	1.67
TTC	F	0.33	2.67	0.00	2.67	1.00	1.67	2.33	0.33	2.33	0.33	2.33	0.33	1.00	1.67	1.00	1.67
TTA	L	0.67	2.33	0.33	2.00	0.67	1.67	1.67	0.67	1.67	0.67	1.33	1.00	0.67	1.67	0.67	1.67
TTG	L	0.67	2.33	0.33	2.00	0.67	1.67	1.67	0.67	1.67	0.67	1.33	1.00	0.67	1.67	0.67	1.67
TTG	M†	0.67	2.33	0.33	2.00	0.67	1.67	1.67	0.67	1.67	0.67	1.33	1.00	0.67	1.67	0.67	1.67
TCT	S	1.00	2.00	0.00	2.00	1.00	1.00	1.00	1.00	0.67	1.33	0.67	1.33	1.00	1.00	1.00	1.00
TCC	S	1.00	2.00	0.00	2.00	1.00	1.00	1.00	1.00	0.67	1.33	0.67	1.33	1.00	1.00	1.00	1.00
TCA	S	1.00	2.00	0.33	1.67	1.67	0.33	1.00	1.00	1.00	1.00	0.67	1.33	1.67	0.33	1.67	0.33
TCG	S	1.00	2.00	0.33	1.67	1.67	0.33	1.00	1.00	1.00	1.00	0.67	1.33	1.67	0.33	1.67	0.33
TAT	Y	0.33	2.67	1.33	1.33	1.00	1.67	2.33	0.33	2.00	0.67	2.33	0.33	1.67	1.00	1.67	1.00
TAC	Y	0.33	2.67	1.33	1.33	1.00	1.67	2.33	0.33	2.00	0.67	2.33	0.33	1.67	1.00	1.67	1.00
TAA	*	0.33	2.67	2.67	0.00	2.67	0.00	2.67	0.00	2.67	0.00	2.67	0.00	2.67	0.00	2.67	0.00
TAG	*	0.33	2.67	2.67	0.00	2.67	0.00	2.67	0.00	2.67	0.00	2.67	0.00	2.67	0.00	2.67	0.00
TGT	C	0.33	2.67	0.33	2.33	1.00	1.67	2.67	0.00	1.67	1.00	1.67	1.00	1.67	1.00	1.33	1.33
TGC	C	0.33	2.67	0.33	2.33	1.00	1.67	2.67	0.00	1.67	1.00	1.67	1.00	1.67	1.00	1.33	1.33
TGA	W	0.33	2.67	0.67	2.00	2.33	0.33	2.67	0.00	2.33	0.33	2.67	0.00	2.00	0.67	2.33	0.33
TGG	W	0.33	2.67	0.67	2.00	2.33	0.33	2.67	0.00	2.33	0.33	2.67	0.00	2.00	0.67	2.33	0.33
CTT	L	1.00	2.00	0.67	1.33	0.67	1.33	1.33	0.67	1.33	0.67	1.00	1.00	0.67	1.33	0.67	1.33
CTC	L	1.00	2.00	0.67	1.33	0.67	1.33	1.33	0.67	1.33	0.67	1.00	1.00	0.67	1.33	0.67	1.33
CTA	L	1.33	1.67	0.33	1.33	0.67	1.00	1.00	0.67	1.00	0.67	0.33	1.33	0.67	1.00	0.67	1.00
CTG	L	1.33	1.67	0.33	1.33	0.67	1.00	1.00	0.67	1.00	0.67	0.33	1.33	0.67	1.00	0.67	1.00
CCT	P	1.00	2.00	0.67	1.33	1.33	0.67	1.00	1.00	1.00	1.00	0.67	1.33	1.33	0.67	1.33	0.67
CCC	P	1.00	2.00	0.67	1.33	1.33	0.67	1.00	1.00	1.00	1.00	0.67	1.33	1.33	0.67	1.33	0.67
CCA	P	1.00	2.00	0.33	1.67	1.33	0.67	1.00	1.00	1.00	1.00	0.33	1.67	1.33	0.67	1.33	0.67
CCG	P	1.00	2.00	0.33	1.67	1.33	0.67	1.00	1.00	1.00	1.00	0.33	1.67	1.33	0.67	1.33	0.67
CAT	H	0.33	2.67	2.33	0.33	0.67	2.00	2.33	0.33	1.33	1.33	2.33	0.33	2.33	0.33	2.33	0.33
CAC	H	0.33	2.67	2.33	0.33	0.67	2.00	2.33	0.33	1.33	1.33	2.33	0.33	2.33	0.33	2.33	0.33
CAA	Q	0.33	2.67	2.00	0.67	1.00	1.67	2.33	0.33	1.33	1.33	2.00	0.67	2.67	0.00	2.67	0.00
CAG	Q	0.33	2.67	2.00	0.67	1.00	1.67	2.33	0.33	1.33	1.33	2.00	0.67	2.67	0.00	2.67	0.00
CGT	R	1.00	2.00	1.67	0.33	0.67	1.33	1.67	0.33	1.67	0.33	1.67	0.33	1.67	0.33	1.67	0.33
CGC	R	0.67	2.33	2.00	0.33	0.67	1.33	2.00	0.33	1.67	0.33	1.67	0.33	2.00	0.33	1.67	0.33
CGA	R	1.00	2.00	2.00	0.00	1.00	1.00	2.00	0.00	1.33	0.67	2.00	0.00	2.00	0.00	2.00	0.00
CGG	R	1.00	2.00	2.00	0.00	1.00	1.00	2.00	0.00	1.33	0.67	2.00	0.00	2.00	0.00	2.00	0.00
ATT	I	0.33	2.67	0.00	2.67	1.00	1.67	1.33	1.33	1.33	1.33	0.33	2.33	1.00	1.67	1.00	1.67
ATT	M†	1.00	2.00	0.00	2.00	1.00	1.00	1.33	0.67	1.33	0.67	0.33	1.67	1.00	1.00	1.00	1.00
ATC	I	0.33	2.67	0.00	2.67	1.00	1.67	1.33	1.33	1.33	1.33	0.33	2.33	1.00	1.67	1.00	1.67
ATC	M†	1.00	2.00	0.00	2.00	1.00	1.00	1.33	0.67	1.33	0.67	0.33	1.67	1.00	1.00	1.00	1.00
ATA	M	0.33	2.67	0.33	2.33	1.00	1.67	1.00	1.67	1.00	1.67	0.33	2.33	1.00	1.67	1.00	1.67
ATA	M†	1.00	2.00	0.33	1.67	1.00	1.00	1.00	1.00	1.00	1.00	0.33	1.67	1.00	1.00	1.00	1.00
ATG	M	0.33	2.67	0.33	2.33	1.00	1.67	1.00	1.67	1.00	1.67	0.33	2.33	1.00	1.67	1.00	1.67
ATG	M†	1.67	1.33	0.33	1.00	1.00	0.33	1.00	0.33	1.00	0.33	0.33	1.00	1.00	0.33	1.00	0.33
ACT	T	1.00	2.00	0.00	2.00	1.00	1.00	0.67	1.33	0.33	1.67	0.00	2.00	1.00	1.00	1.00	1.00
ACC	T	1.00	2.00	0.00	2.00	1.00	1.00	0.67	1.33	0.33	1.67	0.00	2.00	1.00	1.00	1.00	1.00
ACA	T	1.00	2.00	0.33	1.67	1.00	1.00	0.67	1.33	0.67	1.33	0.33	1.67	1.33	0.67	1.33	0.67
ACG	T	1.00	2.00	0.33	1.67	1.00	1.00	0.67	1.33	0.67	1.33	0.33	1.67	1.33	0.67	1.33	0.67
AAT	N	0.33	2.67	1.33	1.33	0.33	2.33	2.33	0.33	2.00	0.67	1.67	1.00	1.67	1.00	1.67	1.00
AAC	N	0.33	2.67	1.33	1.33	0.33	2.33	2.33	0.33	2.00	0.67	1.67	1.00	1.67	1.00	1.67	1.00
AAA	K	0.33	2.67	2.67	0.00	0.67	2.00	2.67	0.00	2.33	1.30	2.67	0.00	2.67	0.00	2.67	0.00
AAG	K	0.33	2.67	2.67	0.00	0.67	2.00	2.67	0.00	2.33	1.30	2.67	0.00	2.67	0.00	2.67	0.00
AGT	S	1.00	2.00	0.33	1.67	0.33	1.67	1.33	0.67	0.67	1.33	0.33	1.67	1.00	1.00	0.67	1.33
AGC	S	1.00	2.00	0.33	1.67	0.33	1.67	1.33	0.67	0.67	1.33	0.33	1.67	1.00	1.00	0.67	1.33
AGA	S	1.00	2.00	0.67	1.33	0.67	1.33	1.33	0.67	1.33	0.67	1.00	1.00	1.67	0.33	1.33	0.67
AGG	S	1.00	2.00	0.67	1.33	0.67	1.33	1.33	0.67	1.33	0.67	1.00	1.00	1.67	0.33	1.33	0.67
GTT	V	1.00	2.00	0.33	1.67	0.67	1.33	1.33	0.67	1.33	0.67	0.67	1.33	0.33	1.67	0.67	1.33
GTC	V	1.00	2.00	0.33	1.67	0.67	1.33	1.33	0.67	1.33	0.67	0.67	1.33	0.33	1.67	0.67	1.33
GTA	V	1.00	2.00	0.33	1.67	0.67	1.33	1.00	1.00	1.00	1.00	0.33	1.67	0.33	1.67	0.67	1.33
GTG	V	1.00	2.00	0.33	1.67	0.67	1.33	1.00	1.00	1.00	1.00	0.33	1.67	0.33	1.67	0.67	1.33
GTG	M†	0.67	2.33	0.33	2.00	0.67	1.67	1.00	1.33	1.00	1.33	0.33	2.00	0.33	2.00	0.67	1.67
GCT	A	1.00	2.00	0.33	1.67	1.33	0.67	0.67	1.33	0.67	1.33	0.33	1.67	1.00	1.00	1.33	0.67
GCC	A	1.00	2.00	0.33	1.67	1.33	0.67	0.67	1.33	0.67	1.33	0.33	1.67	1.00	1.00	1.33	0.67
GCA	A	1.00	2.00	0.33	1.67	1.33	0.67	0.67	1.33	0.67	1.33	0.33	1.67	1.00	1.00	1.33	0.67
GCG	A	1.00	2.00	0.33	1.67	1.33	0.67	0.67	1.33	0.67	1.33	0.33	1.67	1.00	1.00	1.33	0.67
GAT	D	0.33	2.67	2.00	0.67	0.67	2.00	1.67	1.00	2.00	0.67	2.00	0.67	2.00	0.67	2.00	0.67
GAC	D	0.33	2.67	2.00	0.67	0.67	2.00	1.67	1.00	2.00	0.67	2.00	0.67	2.00	0.67	2.00	0.67
GAA	E	0.33	2.67	2.00	0.67	1.00	1.67	1.67	1.00	2.00	0.67	2.00	0.67	2.00	0.67	2.00	0.67
GAG	E	0.33	2.67	2.00	0.67	1.00	1.67	1.67	1.00	2.00	0.67	2.00	0.67	2.00	0.67	2.00	0.67
GGT	G	1.00	2.00	0.67	1.33	0.67	1.33	1.33	0.67	1.00	1.00	0.67	1.33	1.33	0.67	1.33	0.67
GGC	G	1.00	2.00	0.67	1.33	0.67	1.33	1.33	0.67	1.00	1.00	0.67	1.33	1.33	0.67	1.33	0.67
GGA	G	1.00	2.00	0.67	1.33	1.00	1.00	1.33	0.67	1.33	0.67	1.00	1.00	1.00	1.00	1.67	0.33
GGG	G	1.00	2.00	0.67	1.33	1.00	1.00	1.33	0.67	1.33	0.67	1.00	1.00	1.00	1.00	1.67	0.33
	Mean (SD)	0.75 (+/- 0.35)	2.25 (+/- 0.35)	0.82 (+/- 0.82)	1.43 (+/- 0.71)	0.99 (+/- 0.47)	1.26 (+/- 0.54)	1.54 (+/- 0.65)	0.71 (+/- 0.45)	1.36 (+/- 0.57)	0.91 (+/- 0.40)	1.15 (+/- 0.85)	1.10 (+/- 0.66)	1.37 (+/- 0.64)	0.88 (+/- 0.53)	1.41 (+/- 0.61)	0.84 (+/- 0.52)
	Total	52.33	157.67	57.67	100.00	69.33	88.00	107.67	50.00	95.33	63.93	80.33	77.00	96.00	61.67	98.67	58.67

* -- Indicates a stop codon

† -- Indicates case in which a codon is used as a start codon. Codons used as start codons have fewer nonsynonymous sites (mean = 2.00 +/- 0.41) than when they are used at any other time in a given amino acid sequence (mean = 2.50 +/- 0.28) .