TITLE:

Radical amino acid changes persist longer in the absence of sex

AUTHORS:

Joel Sharbrough[1,2], Meagan Luse[1], Jeffrey L. Boore[3,4], John M. Logsdon, Jr.[1], Maurine Neiman[1]

[1] Department of Biology, University of Iowa, Iowa City, IA 52242, USA
[2] Department of Biology, Colorado State University, Fort Collins, CO 80523, USA
[3] Department of Integrative Biology, University of California, Berkeley, Berkeley CA 94720, USA
[4] Providence St. Joseph Health and Institute for Systems Biology, 401 Terry Avenue N, Seattle, WA 98109, USA

CORRESPONDING AUTHOR:

Joel Sharbrough
jsharbro@rams.colostate.edu
(970) 491-22256
440 Biology Building
Colorado State University
Fort Collins, CO 80523

KEYWORDS:

asexual reproduction, Hill-Robertson effect, mitochondrial genome, *Potamopyrgus antipodarum,* purifying selection, sexual reproduction

DATA ARCHIVAL LOCATION:

Sequence data – GenBank
Python scripts – https://github.com/jsharbrough

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 sexual reproduction should facilitate mutational clearance by reducing selective interference between sites and by allowing the production of offspring with different mutational complements than their parents. Here, we compared the rate of removal of conservative and radical nonsynonymous mutations from mitochondrial genomes of sexual vs. asexual populations of *Potamopyrgus antipodarum*, a New Zealand freshwater snail species with 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. 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.

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). Although this LD disruption should be most apparent in the nuclear genome, biparental inheritance and meiotic recombination in the nuclear genome should also decrease LD between the nuclear genome and the mitochondrial genome (i.e., mitonuclear LD). Reduced mitonuclear LD should therefore decrease the extent to which selection against deleterious mitochondrial mutations is impeded by simultaneous selection on nuclear variants (Normark and Moran 2000; Neiman and Taylor 2009). The evolutionary consequences of sexual reproduction (i.e., elevated efficacy of natural selection) should therefore extend beyond the nuclear genome to cytoplasmic genomes, which are typically uniparentally transmitted and often lack recombination (Barr et al. 2005). Accordingly, the uniparental inheritance of both mitochondrial and nuclear genomes and reduced or absent meiotic recombination that characterize asexual reproduction should result in effectively complete mitonuclear LD, such that nuclear and mitochondrial genomes are co-transmitted from mother to daughter. This elevated mitonuclear LD, combined with simultaneous selection on nuclear variants, should impede selection in the cytoplasmic genomes of asexual lineages (Normark and Moran 2000). Reduced efficacy of selection in the cytoplasmic genomes of asexual lineages is most obviously linked to accumulation of *slightly* deleterious mutations over time (Gabriel et al. 1993; Neiman and Taylor 2009); however, the Hill-Robertson effect (i.e., smaller N_e*s) is expected to reduce the efficacy of selection with respect to *all* mutational changes. The

implications are that although most strongly deleterious mutations should eventually be purged from asexual populations, these mutations will tend to remain polymorphic for a longer period of time than similar mutations in sexual populations. This means that even severely deleterious changes will have a higher probability of fixation by random drift in asexual vs. sexual lineages.

The co-transmission of nuclear and cytoplasmic genomes might also confer benefits. In particular, the heritability of epistatic variation should be elevated in asexual lineages, with the implication that multilocus genotypes (e.g., genes encoding multi-subunit enzymes) harbored in asexuals might experience relatively effective coevolution (Neiman and Linksvayer 2006). The transition to permanent linkage of nuclear and cytoplasmic genomes could therefore result in particularly rapid coevolution of cytoplasmic genomes and their corresponding nuclear-encoded interacting partners following the loss of sex. Because both (1) accelerated accumulation of harmful mutations and (2) elevated rates of epistatic coevolution in asexual lineages should result in increased evolutionary rates at nonsynonymous sites in the mitochondrial genomes of asexual lineages compared to those of sexual lineages, it is critical to distinguish between the types of nonsynonymous mutations that are accumulating in asexual lineages.

The prediction that asexual lineages should experience a higher rate of accumulation of harmful mutations has found support from animal (Neiman et al. 2010; Henry et al. 2012) and plant (Voigt-Zielinski et al. 2012; Hollister et al. 2015; Lovell et al. 2017) taxa. While these results represent important steps towards understanding the genomic consequences of asexuality, the evolutionary mechanisms underlying these

64 observations remain unclear, in large part because the extent to which accumulated
65 mutations are actually deleterious in asexuals has not been evaluated. This type of
66 information is especially important in light of substantial evidence that nonsynonymous
67 mutations are likely to vary widely in fitness effects (Eyre-Walker and Keightley 2007;
68 Boyko et al. 2008). Predicting and characterizing the fitness effects of these
69 nonsynonymous mutations remains a central area of focus in evolutionary biology
70 (Keightley and Charlesworth 2005; Xue et al. 2008; Eyre-Walker and Keightley 2009;
71 Andolfatto et al. 2011; Halligan et al. 2011). Here, we take advantage of the fact that the
72 biochemical properties of amino acids represent useful heuristics for inferring effects of
73 mutations on protein phenotype (Zhang 2000; Hanada et al. 2007; Popadin et al. 2007)
74 to take critical steps towards assessing whether mutations likely to influence fitness
75 accumulate differently in asexual than in sexual lineages.

76 The removal of deleterious mutations (and the fixation of beneficial mutations)
77 depends upon the efficacy of selection as well as the fitness effect of mutations, such
78 that elevated N_e and reduced LD in sexual vs. asexual populations should result in more
79 rapid removal of deleterious mutations for the former (Birky and Walsh 1988). As such,
80 comparing rates and patterns of evolution across reproductive modes while
81 incorporating information about mutational severity will provide a powerful glimpse into
82 the evolutionary dynamics governing the removal of deleterious mutations. The New
83 Zealand freshwater snail *Potamopyrgus antipodarum* is ideally suited to evaluate this
84 critically important evolutionary process because otherwise similar obligately sexual and
85 obligately asexual *P. antipodarum* frequently coexist within New Zealand lake
86 populations (Lively 1987; Jokela et al. 1997), enabling direct comparisons across

reproductive modes, and, thereby, across genomes that experience predictable variation in the efficacy of selection.

Asexual lineages of *P. antipodarum* are the product of multiple distinct transitions from sexual *P. antipodarum* (Neiman and Lively 2004; Neiman et al. 2011), meaning that these asexual lineages represent separate natural experiments into the consequences of the absence of sex. Neiman et al. (2010) showed that asexual lineages of *P. antipodarum* experience a higher rate of nonsynonymous substitution in their mitochondrial genomes than sexual lineages. Here, we qualitatively extend these studies by using whole mitochondrial genomes to evaluate whether sexual lineages distinguish between radical and conservative changes more effectively than asexual lineages. This approach allowed us to evaluate whether harmful mutation accumulation is detectable within species and, if so, whether this phenomenon is driven by more effective selection in sexual lineages vs. relatively rapid mitonuclear coevolution in asexual lineages. The outcome of these analyses emphasizes fundamental differences in the rate of accumulation of conservative vs. radical nonsynonymous mutations and suggests that radical mutations persist longer in mitochondrial genomes of asexual lineages of *P. antipodarum* compared to sexual counterparts, likely as a consequence of reduced efficacy of purifying selection.

MATERIALS & METHODS

Sequencing. We analyzed 31 whole mitochondrial genomes from eight sexual lineages and 23 asexual lineages of *P. antipodarum* and 1 whole mitochondrial genome from the closely related *Potamopyrgus estuarinus*, representing the wide-ranging mitochondrial

genetic diversity of this species in New Zealand along with several invasive lineages (European, North American, see Table S1, Figure 1, Neiman and Lively 2004; Neiman et al. 2011; Paczesniak et al. 2013). We obtained 19 publicly available mitochondrial genomes (4 sexual, 14 asexual, 1 *P. estuarinus,* Accession Nos.: GQ996415 – GQ996433, Neiman et al. 2010), sequenced eight mitochondrial genomes (2 sexual, 6 asexual) using bi-directional Sanger sequencing on an ABI 3730 (Applied Biosystems, Foster City, CA), and sequenced and assembled five mitochondrial genomes (2 sexual, 3 asexual) using 2x100 bp paired-end sequencing on an Illumina HiSeq 2500 (Illumina, Inc., San Diego, CA) as part of the ongoing *P. antipodarum* nuclear genome project. For all newly sequenced lineages, we determined ploidy and thus reproductive mode (2x – sexual; ≥ 3x – asexual) using flow cytometry following the protocol outlined in (Neiman et al. 2011; Neiman et al. 2012; Paczesniak et al. 2013; Krist et al. 2014) and extracted total genomic DNA following a mollusk-adapted phenol-chloroform extraction protocol (Fukami et al. 2004). We cleaned DNA extractions using the Zymo Clean and Concentrate Kit (Zymo Research, Irvine, CA), re-suspended DNA in 30-100 µL of T-low-E buffer (10mM Tris pH8.0, 0.1mM EDTA), and determined DNA concentration and purity for each sample on a NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA). For the eight Sanger-sequenced samples, we amplified mitochondrial genomes 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). Sanger-sequenced mitochondrial genomes were assembled and manually edited in Sequencher 5.0 (Gene Codes Corporation, Ann Arbor, MI). Only unambiguous sites

supported by ≥ 2 reads were included in the assemblies. For the remaining five DNA samples, we constructed sequencing libraries using the Illumina Nextera DNA Library Prep Kit and sequenced 2x100 bp paired-end reads on a single lane of an Illumina HiSeq 2500. Following read trimming and quality control (≥ 30 Phred quality score) using the Fastx-toolkit (Gordon and Hannon 2010), we assembled mitochondrial genomes *de novo* using the CLC Genomics Work Bench (Qiagen, Hilden, Germany). Consensus base calls were then determined for sites with >10x coverage and Phred-scaled quality scores >30. Sites with >1 read supporting a minor allele (i.e., heteroplasmies) were not used in this analysis. All newly sequenced mitochondrial genomes have been deposited in GenBank (Accession Nos.: *sequences will be uploaded to GenBank upon acceptance*).

Phylogenetic analysis. We aligned whole mitochondrial genome sequences from the 31 *P. antipodarum* lineages and one *P. estuarinus* sample using MAFFTv v7.305b (Katoh and Standley 2013) and manually edited the alignments in MEGA v5.2.2 (Kumar et al. 2008), Supplementary File S1). We used jModelTest 2, which uses a corrected Akaike Information Criterion (AICc), Bayesian Information Criterion (BIC) and performance-based decision theory (DT) in a Maximum Likelihood (ML) framework, (Darriba et al. 2012), to select appropriate models of molecular evolution. Of the 1,624 possible models evaluated by jModelTest 2, two models produced the highest log-likelihood scores across all three model estimators: TIM3 (Posada 2003) and GTR (Tavaré 1986), both with invariant sites (I) and a Gamma distribution of site classes (G). We therefore used both models (TIM3+I+G, GTR+I+G) to infer ML-based phylogenies

using PAUP*4.0 (Swofford and Sullivan 2009) with 1,000 bootstrap replicates each and assuming the following priors: TIM3+I+G: lset – base = (0.2587, 0.1736, 0.1748), nst = 6, rmat = (2.7926, 49.1045, 1.0000, 2.7926, 37.8686), rates = gamma, shape = 0.8150, ncat = 4, pinvar = 0.4550; GTR+I+G: lset – base = (0.2597, 0.1736, 0.1738), nst = 6, rmat = (1.5661, 37.1103, 0.5913, 2.9024, 28.5161), rates = gamma, shape = 0.8380, ncat = 4, pinvar = 0.4610. We also inferred the phylogeny for these mitochondrial genomes under a Bayesian statistical framework using MrBayes v3.2 (Ronquist et al. 2012) assuming the following priors: nucmodel = 4by4, nst = mixed, ploidy = haploid, rates = invgamma, ngammacat = 4, number of generations = 10^6, relative burn-in = 25%, number of chains = 4, heating parameter = 0.5, revmat = all GTR submodels have equal probabilities, statefreq = dirichlet, shape = exponential (1.0), pinvar = uniform (0.0, 1.0), ratemultiplier = fixed (1.0), topology = all topologies equally probable, brlens = unconstrained: gammaDir (1.0, 0.1, 1.0, 1.0). After ensuring that the number of generations (10^6 generations) and burn-in (2.5×10^5 generations) were sufficient for the log probability of trees to plateau, trees were sampled every 500 generations, resulting in 24,016 trees of which 2,045 contributed to the 95% credible tree set. We visualized ML majority rule trees and the consensus Bayesian tree in FigTree v1.4 (Rambaut 2007); only nodes with bootstrap values ≥ 60 and posterior probabilities ≥ 75 were used in subsequent tests of molecular evolution (Figure 1).

After extracting and concatenating the 13 protein-coding regions from the alignment (~11.2 kbp, 3,738 amino acids) and for all 32 lineages, we mapped all mutational changes in protein-coding regions to the fixed tree topology (described above) according to the rules of parsimony. Only non-homoplasious changes were

179 considered in subsequent phylogenetic analyses of molecular evolution. In our
180 population genetic analyses, we considered all sites that were variable within *P.*
181 *antipodarum* (including homoplasious sites) to be polymorphic and all sites that were
182 distinct between *P. antipodarum* and *P. estuarinus* to be substitutions.
183
184 **Identifying conservative and radical amino acid changes.** To compare mitochondrial
185 mutation accumulation across mutational types, we classified nonsynonymous changes
186 as “conservative” changes when the derived amino acid had similar biochemical
187 properties to the ancestral amino acid and “radical” changes when the derived amino
188 acid had markedly different biochemical properties compared to the ancestral amino
189 acid. We used seven different amino acid classification schemes, three drawn from
190 (Zhang 2000), three drawn from Hanada et al. (2007), and a modified Grantham
191 scheme based on amino acid composition, polarity, and volume (Grantham 1974) to
192 evaluate rates and patterns of radical and conservative amino acid evolution (Table 1).
193 While there is some overlap between different classification schemes, each scheme
194 highlights different amino acid properties that are likely to shape protein evolution. For
195 example, amino acid charge is a major determinant of protein folding (Perutz et al.
196 1965; Anfinsen 1973; Nakashima et al. 1986; Bashford et al. 1987; Wright et al. 2005)
197 and three-dimensional structure (Lesk and Chothia 1980; Geisler and Weber 1982;
198 Doms et al. 1988; Rumbley et al. 2001), changes between polar and non-polar amino
199 acids may expose or bury key interaction residues in membrane-associated proteins
200 (von Heijne 1992), and volume and aromaticity can both affect protein folding and play a
201 role in protein-protein interactions (Burley and Petsko 1985). Classification schemes 4

and 7 (Table 1) are unique in that they are based on evolutionary information (although classification scheme 7 largely fits with charge and polarity classifications), meaning that these schemes incorporate aspects of other amino acid characteristics into their classifications. Using these seven amino acid classification schemes, we developed an overall index of the degree of amino acid change that we termed Conservative-Radical Index (CRI). For each possible amino acid change, we calculated CRI by averaging across the seven amino acid classification schemes (radical changes assigned a value = 1, conservative changes assigned a value = 0), such that CRI = 1.0 indicates that the amino acid change in question was radical in all amino acid classification schemes (e.g., V ←→ E) whereas CRI = 0.0 indicates that the amino acid change in question was conservative in all amino acid classification schemes (e.g., V ←→ I). Amino acid changes with CRI ≤ 0.5 were treated as conservative changes and amino acid changes with CRI > 0.5 were treated as radical changes (Table S3). All analyses of molecular evolution and population genetics were repeated for each amino acid scheme individually as well as for the classification scheme-averaged classifier. All mutational types were defined relative to the invertebrate mitochondrial genetic code (http://www.ncbi.nlm.nih.gov/Taxonomy/Utils/wprintgc.cgi#SG5).

We then calculated the number of mutational target sites per codon for each different type of mutational change (i.e., synonymous, nonsynonymous, conservative nonsynonymous, and radical nonsynonymous) to ensure that we properly accounted for the different probabilities of different types of mutational changes (Table S4). To confirm that the number of each of type of site was properly calculated, we checked that the number of nonsynonymous sites and the number of synonymous sites per codon

summed to three and that the number of conservative nonsynonymous sites and the number of radical nonsynonymous sites per codon summed to the number of nonsynonymous sites per codon (Zhang 2000). Of particular note is that the “GTG”, “TTG”, “ATT”, “ATC”, and “ATA” codons can all be used as alternative start codons in invertebrate mitochondrial genomes (only the GTG alternative start codon was observed in the present dataset), which we accounted for in our site calculations.

Molecular evolution and population genetic analyses. To evaluate the relative intensity of selection acting on different types of mutational changes, we estimated rates of substitution between *P. antipodarum* and *P. estuarinus* (Li et al. 1985), ratios of polymorphism to divergence (Mcdonald and Kreitman 1991), nucleotide diversity (Watterson 1975; Nei and Li 1979; Fu and Li 1993), and site frequency spectra (SFS) in protein-coding regions of the mitochondrial genome for each mutational type using all seven amino acid classification schemes and the scheme-averaged classifier with a custom python tool (available at https://github.com/jsharbrough/PolymorphismsSubstitutions). For codons with multiple hits, we assumed the fewest number of nonsynonymous changes necessary to explain the data.

To compare rates of molecular evolution in *P. antipodarum* relative to *P. estuarinus*, we first calculated substitution rates for each mutational type in *P. antipodarum* as in Li et al. (1985), corrected these estimates for multiple hits using the Jukes-Cantor model of molecular evolution (Jukes and Cantor 1969), and estimated variance as in Jukes and Cantor (1969). We then compared 95% confidence intervals

(CIs) of substitution rates between *P. antipodarum* and *P. estuarinus*, estimated as 95% CI = $2\sqrt{(\text{variance})}$ for each substitution type, such that non-overlapping CIs indicate statistically different rates of substitution. We also took advantage of the non-homoplasious changes mapped to our fixed tree topology (see above) to compare branch lengths for synonymous, nonsynonymous, conservative, and radical changes relative to *P. estuarinus*. We estimated branch lengths by summing the mapped changes for each lineage and mutational type and dividing the number of changes by the number of mutational target sites (Table S4). We then used pairwise Wilcoxon Signed-Rank (WSR) tests and the Holm procedure for correcting for multiple comparisons to determine whether branch lengths differed across mutational types (Holm 1979). We next used McDonald-Kreitman (MK) tests to compare molecular evolution in conservative vs. radical changes using a series of Fisher's Exact Tests (FET): (1) synonymous changes to conservative changes, (2) synonymous changes to radical changes, and (3) conservative changes to radical changes. All statistical tests were performed in R v3.2.4 (R Core Team 2016).

To compare patterns of polymorphism within species, we estimated nucleotide diversity using θ_π – hereafter π – (Nei and Li 1979) and θ_W – hereafter θ – (Watterson 1975), and their respective variances (as in Durrett 2008) within *P. antipodarum*. We compared levels of nucleotide diversity across mutational types by constructing 95% CIs for each mutational type using 10,000 bootstrap replicates. For each bootstrap replicate, we randomly selected 3,738 codons with replacement and recalculated all population genetic statistics for each replicate using a custom Python tool (available at https://github.com/jsharbrough/popGenBootstrapping). We also performed an estimate

of θ using only private polymorphisms – hereafter θ_U – because private polymorphisms represent relatively recent changes and are therefore not as phylogenetically constrained as older polymorphisms (Fu and Li 1993). We compared levels of these private polymorphisms across mutational types using the same bootstrapping approach as with π and θ using a custom Python tool to compare CIs for each mutational type and compute *p* values (available at https://github.com/jsharbrough/bootstrapPvalues), which rank-orders bootstrap replicates and tests the degree to which bootstrap distributions overlap (i.e., perfect overlap of bootstrap distributions: $p > 0.9999$, no overlap of bootstrap distributions: $p < 0.0001$). Because deleterious changes should be found at lower frequencies than relatively neutral changes, we also compared the SFS across mutational types using a series of Goodness-of-Fit (GoF) analyses assuming a χ^2 distribution, first for nonsynonymous, conservative, and radical polymorphisms vs. synonymous polymorphisms, and second for conservative vs. radical polymorphisms. We performed these GoF analyses with the chisq() function in R v3.2.4 (R Core Team 2016) and visualized species-wide polymorphism levels using the R package ggplot2 (Wickham 2016).

After establishing that the substitution rate for radical amino acid changes is lower than for conservative amino acid changes and that radical changes exhibit lower nucleotide diversity within species than conservative changes (see Results, Figure 2), we compared rates of mutation accumulation of conservative and radical amino acid changes across reproductive modes. To compare rates of molecular evolution in sexual vs. asexual lineages, we first used the mapped changes to estimate synonymous-corrected branch lengths for conservative and radical changes separately using WSR

294 tests. We also performed these same comparisons on internal branches only and on
295 branch tips only to test whether patterns of mutation accumulation across reproductive
296 modes were robust across evolutionary time.

297 We next compared polymorphism levels for conservative and radical changes
298 across reproductive modes by dividing our sample into sexual (n = 8) and asexual (n =
299 23) groups and separately calculating π, θ, and θ_U for each mutational type. Asexual *P.*
300 *antipodarum* have already been shown to accumulate nonsynonymous changes more
301 rapidly than their sexual counterparts (Neiman et al. 2010), leading to the *a priori*
302 expectation that asexual lineages should exhibit higher levels of nucleotide diversity at
303 nonsynonymous sites than sexual lineages. To address whether this prediction was met,
304 we calculated a test statistic, D_{AS}, in which $D_{AS} = \bar{x}_{Asex} - \bar{x}_{Sex}$ where $\bar{x}$ represents the
305 diversity-related population genetic statistic under consideration. We then constructed a
306 null distribution for each of these statistics using a custom-built python program
307 (available at https://github.com/jsharbrough/conRadNullDistribution), which randomly
308 assigned lineages without replacement to one of two groups: group 1 (n = 23,
309 representing the asexual lineage sample size) and group 2 (n = 8, representing the
310 sexual lineage sample size), calculated the difference between the two groups, D_{12},
311 where $D_{12} = \bar{x}_1 - \bar{x}_2$, for each population genetic statistic, and repeated this process
312 10,000 times. Because we expected asexual lineages to exhibit higher levels of
313 nonsynonymous polymorphism than sexual lineages, we conducted one-tailed tests of
314 polymorphism across reproductive modes (H_O: $D_{AS} = 0$; H_A: $D_{AS} > 0$) by comparing D_{AS}
315 to the null distribution for each population genetic statistic (i.e., π, θ, and θ_U) for
316 synonymous sites, synonymous-corrected conservative sites, and synonymous-

317 corrected radical sites. We inferred *p* values directly from these comparisons using a
318 custom python tool (available at https://github.com/jsharbrough/distributionPValues) and
319 visualized null distributions and test statistics using the ggplot2 package for R (Wickham
320 2016).
321
322 **RESULTS**
323 **Phylogenetic analysis of mitochondrial genetic diversity in *P. antipodarum*.** We
324 used 32 whole mitochondrial genomes to infer a phylogeny of sexual and asexual *P.*
325 *antipodarum* relative to a *P. estuarinus* outgroup using Maximum Likelihood and
326 Bayesian methods (Figure 1). We found evidence for substantial intraspecific genetic
327 variation within *P. antipodarum* (mean pairwise genetic distance ≈ 0.025 changes/site).
328 This analysis also revealed relatively deep divergence for one entirely asexual clade
329 (clade A; mean pairwise distance between clade A vs. clades B/C ≈ 0.035 changes/site),
330 especially relative to the more minor divergence within clades B and C (mean pairwise
331 distance within B/C ≈ 0.013). Clade A is composed mostly of lineages from the North
332 Island of New Zealand (Lakes Waikaremoana and Tarawera), but also includes one
333 lineage from Wales as well as two lineages collected from lakes on the South Island of
334 New Zealand (Lakes Brunner and Kaniere). Clades B/C are predominantly composed of
335 lineages collected from the South Island of New Zealand (Lakes Alexandrina, Brunner,
336 Grasmere, Gunn, Heron, Ianthe, Kaniere, Lady, McGregor, Poerua, Rotoiti, and
337 Rotoroa); however, two invasive lineages (collected from Lake Superior in Duluth, MN,
338 USA and Denmark), one North Island lineage (collected from Lake Waikaremoana), and

a lineage collected from Lake Alexandrina form a monophyletic, all-asexual clade within clade C.

We next used the rules of parsimony to map the 1,524 non-homoplasious changes (out of 1,711 total variable sites) present in the ~11 kbp protein-coding region of the mitochondrial genome onto this tree topology (Figure 1). In all, there were 814 synonymous substitutions and 35 nonsynonymous substitutions between *P. antipodarum* and *P. estuarinus*. Of these 35 nonsynonymous substitutions, 30 were considered conservative amino acid changes and 5 were considered radical amino acid changes by our scheme-averaged classifier index, CRI (see Materials and Methods). Within *P. antipodarum*, we observed 651 synonymous polymorphisms and 211 nonsynonymous polymorphisms; CRI classified 151 of these sites as conservative amino acid polymorphisms and 60 as radical amino acid polymorphisms (Figure 2b).

Substitution rates and polymorphism levels for conservative vs. radical amino acid changes. Using the mapped changes to calculate branch-specific estimates of Jukes-Cantor-corrected substitution rate (K), we found that branch-specific estimates of K_S (mean +/- standard deviation = 0.48 +/- 0.012 changes/site) were significantly higher than branch-specific estimates of K_A (mean +/- standard deviation = 0.0077 +/- 8.0×10^{-4} changes/site; WSR $V = 496$, $p = 1.2 \times 10^{-6}$), K_C (mean +/- standard deviation = 0.013 +/- 1.1×10^{-3} changes/site; WSR $V = 496$, $p = 1.2 \times 10^{-6}$), and K_R (mean +/- standard deviation = 0.0029 +/- 6.8×10^{-4} changes/site; WSR $V = 496$, $p = 1.2 \times 10^{-6}$), indicating that nonsynonymous changes evolve at slower rates than do synonymous changes (Figure 2a). Branch-specific estimates of K_C were also significantly higher than branch-

specific estimates of K_R (WSR V = 496, p = 1.2 x 10^{-6}), indicating that among amino acid-changing mutations, radical amino acid changes evolve particularly slowly (Figure 2a). In accordance with branch-specific estimates, species-wide estimates of substitution rates that only use fixed differences between *P. antipodarum* and *P. estuarinus* revealed that K_S was significantly greater than K_A ($p < 0.05$), K_C ($p < 0.05$), and K_R ($p < 0.05$), and that K_C was significantly greater than K_R ($p < 0.05$; Table 2). In summary, branch-specific and species-wide estimates of substitution rate indicate that nonsynonymous sites have lower rates of substitution than synonymous sites and that radical nonsynonymous sites have lower rates of substitution than conservative nonsynonymous sites.

To determine the probability of fixation of conservative vs. radical nonsynonymous polymorphisms, we performed pairwise MK tests of selection across all mutational types. We found that nonsynonymous (FET: $p < 2.2 \times 10^{-16}$), conservative (FET: $p < 2.2 \times 10^{-16}$), and radical (FET: $p < 2.2 \times 10^{-16}$) amino acid polymorphisms were significantly less likely to reach fixation than synonymous polymorphisms (Table 3, Figure 2b). There was a trend for conservative polymorphisms to become fixed within *P. antipodarum* at higher rates than radical polymorphisms, but this difference was not significant (FET: p = 0.098). These data indicate that nonsynonymous changes have a lower probability of fixation than synonymous changes, but that there is no difference in fixation probabilities across different types of nonsynonymous changes.

To investigate how conservative and radical changes evolve within species, we estimated nucleotide diversity for synonymous, nonsynonymous, conservative nonsynonymous, and radical nonsynonymous changes using the frequency-dependent

385 measure π, the frequency-independent measure θ, and a measure of private nucleotide
386 diversity θ_U, treating all lineages as a single population. Comparisons of π revealed that
387 nonsynonymous, conservative, and radical sites exhibited lower levels of nucleotide
388 diversity ($p_{\pi S\text{-}\pi A} < 0.0002$; $p_{\pi S\text{-}\pi C} < 0.0002$; $p_{\pi S\text{-}\pi R} < 0.0002$), as did comparisons of θ
389 ($p_{\theta S\text{-}\theta A} < 0.0002$; $p_{\theta S\text{-}\theta C} < 0.0002$; $p_{\theta S\text{-}\theta R} < 0.0002$), and θ_U ($p_{\theta u\text{-}S\text{-}\theta u\text{-}A} < 0.0002$; $p_{\theta u\text{-}S\text{-}\theta u\text{-}C} <$
390 0.0002; $p_{\theta u\text{-}S\text{-}\theta u\text{-}R} < 0.0002$) than synonymous sites, indicating that all types of
391 nonsynonymous changes are eliminated from *P. antipodarum* genomes more rapidly
392 than synonymous changes (Table 2, Figure 2c). We also found that π_C and θ_C were
393 significantly greater than π_R ($p = 0.0004$) and θ_R ($p < 0.0002$), respectively (Table 2,
394 Figure 2c); however, $\theta_{U\text{-}C}$ and $\theta_{U\text{-}R}$ are statistically indistinguishable within *P.*
395 *antipodarum* ($p = 0.072$, Table 2, Figure S3). Because asexual lineages likely
396 experience distinct demographic forces that sexual lineages do not (Charlesworth and
397 Wright 2001; Kaiser and Charlesworth 2009) we also compared these population
398 genetic statistics using only sexual lineages and found evidence of similarly elevated
399 intensity of selection against radical vs. conservative polymorphisms as in analyses
400 incorporating all *P. antipodarum* mitochondrial genome sequences (Figure S1).
401 Finally, we compared allele frequencies of different mutational types by
402 comparing their SFS (Figure 2d, Table 2). We found that nonsynonymous ($\chi^2 = 56.14$,
403 df = 7, $p = 8.9 \times 10^{-10}$), conservative ($\chi^2 = 28.57$, df = 7, $p = 1.7 \times 10^{-4}$), and radical SFS
404 ($\chi^2 = 35.04$, df = 7, $p = 1.1 \times 10^{-5}$) are significantly left-skewed (i.e., excess of rare
405 variants) relative to synonymous changes. We did not detect any differences in
406 conservative vs. radical SFS ($\chi^2 = 6.36$, df = 4, $p = 0.17$). Together, these data indicate
407 that all types of nonsynonymous changes are found in lower numbers and at lower

408 frequencies than synonymous changes and that radical amino acid polymorphisms tend
409 to be found in lower numbers and at lower frequencies than conservative amino acid
410 polymorphisms.
411
412 **Efficacy of purifying selection in sexual vs. asexual lineages.** To evaluate the
413 degree to which the efficacy of selection is reduced in asexual vs. sexual lineages of *P.*
414 *antipodarum*, we used a set of 1,524 synapomorphic and autapomorphic changes to
415 estimate sexual and asexual lineage branch lengths relative to outgroup *P. estuarinus*
416 and compare relative rates of conservative and radical amino acid molecular evolution.
417 After accounting for the number of mutational target sites (Table S4) and correcting for
418 multiple hits (Jukes and Cantor 1969), we found that asexual lineages exhibited higher
419 rates of evolution at synonymous sites (mean K_S +/- SD = 0.48 +/- 0.013 synonymous
420 substitutions/ synonymous site) than sexual lineages (mean K_S +/- SD = 0.47 +/- 4.4 x
421 10^{-3} synonymous substitutions/ synonymous site, MWU W = 142, p = 0.025), potentially
422 indicating that the mitochondrial genomes of asexual lineages of *P. antipodarum*
423 experience higher mutation rates than their sexual counterparts. We therefore corrected
424 nonsynonymous rates of molecular evolution with synonymous rates to ensure that we
425 could adequately compare efficacy of selection acting on conservative and radical
426 changes across reproductive modes. We did not detect a difference in the rate of
427 molecular evolution at conservative sites in sexual vs. asexual lineages (MWU: W = 95,
428 p = 0.91, Figure 3a, Table 4). By contrast, we did find that asexual lineages exhibit
429 significantly higher rates of molecular evolution at radical sites than sexual lineages
430 (MWU: W = 168, p = 6.5 x 10^{-4}, Figure 3b, Table 4), indicating that asexual lineages

accumulate radical amino acid changes more rapidly than sexual lineages. We obtained a similar pattern of accelerated accumulation of radical amino acid changes in asexual vs. sexual lineages when excluding branch tips from the analysis (K_C/K_S – MWU: W = 84, p = 0.74; K_R/K_S – MWU: W = 168, p = 6.5 x 10^{-4}), indicating that this pattern is robust across evolutionary time.

We next compared patterns of polymorphism across reproductive modes and mutational types using π *and* θ. For each mutational type, we calculated an estimate of nucleotide diversity for asexuals and sexuals separately, using the difference D as a test statistic to compare against a null distribution (see Methods for a description on how this null distribution was constructed). Asexuals exhibited slightly higher levels of synonymous polymorphism (mean π_S +- variance = 0.066 +/- 0.025 synonymous changes/ synonymous site; mean θ_S +/- variance = 0.053 +/- 0.015 synonymous changes/ synonymous site) than sexual lineages (mean π_S +- variance = 0.043 +/- 0.019 synonymous changes/ synonymous site; mean θ_S +/- variance = 0.040 +/- 0.016 synonymous changes/ synonymous site, D_θ = 0.013, p = 0.15), but the differences were not significant (Figure S3). Although sexual and asexual lineages do not appear to differ in terms of synonymous polymorphism levels, K_S, which is robust to shifts in demography, was elevated in asexual compared to sexual lineages and the possibility that differential effects of neutral processes might change in response to the transition to asexuality (Charlesworth and Wright 2001; Kaiser and Charlesworth 2009) meant that accounting for levels of neutral polymorphism across reproductive modes was still warranted. We therefore corrected nonsynonymous estimates of nucleotide diversity with the corresponding synonymous rate (Table 4, Figure 4b). For both π and θ, there

454 was no evidence that sexual lineages differed from asexual lineages in the level of
455 conservative amino acid polymorphism (D_π = -8.6 x 10^{-3}, p = 0.91; D_θ = 0.014, p = 0.70).
456 The same analyses applied to radical amino acid polymorphisms revealed significantly
457 higher levels in asexual vs. sexual lineages (D_π = 0.012, p = 0.049; D_θ = 0.035, p = 1.5 x
458 10^{-3}).

459 Clade A asexuals appear to be genetically distinct relative to the rest of the *P.*
460 *antipodarum* dataset (Figure 1), raising the question of whether this group might be
461 contributing disproportionately to our observations of elevated radical amino acid
462 polymorphism levels in asexual *P. antipodarum*. We therefore repeated the
463 comparisons of π and θ with clade A excluded, using the clade B sexuals as an
464 outgroup to clade C (Table 4, Figure 4b). In this more limited analysis, we again found
465 that asexuals harbored higher levels of radical (D_π = 0.023, p = 0.0038; D_θ = 0.050, p <
466 1.0 x 10^{-4}) but not conservative amino acid polymorphism (D_π = -5.7 x 10^{-4}, p = 0.60; D_θ
467 = 4.0 x 10^{-3}, p = 0.74), indicating that the clade A asexuals were not entirely responsible
468 for the patterns of elevated radical amino acid polymorphism in *P. antipodarum*
469 asexuals.

470 To account for the phylogenetic non-independence of asexual lineages in *P.*
471 *antipodarum* in our population genetic analyses, we also compared levels of private
472 polymorphism across reproductive modes and mutational types (Figure 4c, Table 4).
473 Because private polymorphisms should often represent relatively young mutations,
474 these polymorphisms can be used to estimate rates of mutation accumulation since the
475 transition to asexuality. Sexual lineages exhibited slightly higher levels of synonymous
476 private polymorphism ($\theta_{U\text{-}S}$ +/- variance = 6.5 x 10^{-3} +/- 2.5 x 10^{-3} synonymous changes/

477 synonymous site) compared to asexual lineages ($\theta_{U\text{-}S}$ +/- variance = 4.8 x 10^{-3} +/- 1.3 x
478 10^{-3} synonymous changes/ synonymous site), but the difference was not significant ($D_{\theta U}$
479 = -1.7 x 10^{-3}, p = 0.057). After correcting for private synonymous polymorphism levels,
480 we found that asexual lineages (asex $\theta_{U\text{-}C} / \theta_{U\text{-}S}$ +/- variance = 0.53 +/- 0.18
481 conservative nonsynonymous changes/ conservative nonsynonymous site) exhibited
482 significantly higher levels of both private conservative amino acid polymorphism (sex $\theta_{U\text{-}C} / \theta_{U\text{-}S}$ +/- variance = 0.25 +/- 0.11 conservative nonsynonymous changes/ conservative
483
484 nonsynonymous site; $D_{\theta U}$ = 0.28, p = 3.1 x 10^{-3}) and private radical amino acid
485 polymorphism (asex $\theta_{U\text{-}R} / \theta_{U\text{-}S}$ +/- variance = 0.33 +/- 0.10 radical nonsynonymous
486 changes/ radical nonsynonymous site) than sexual lineages (sex $\theta_{U\text{-}R} / \theta_{U\text{-}S}$ +/- variance
487 = 0.055 +/- 0.022 radical nonsynonymous changes/ radical nonsynonymous site; $D_{\theta U}$ =
488 0.27, p = 0.5.9 x 10^{-3}). The lower levels of private polymorphism in sexual compared to
489 asexual lineages for both conservative and radical mutational types indicates that
490 selection is less effective in asexual lineages, and that this effect is detectable even
491 over relatively short time scales.
492
493 **DISCUSSION**
494 **Radical amino acid changes accumulate in the absence of sex**
495 Together, these results are consistent with the expectation that radical mutations are
496 usually more harmful than conservative mutations (Freudenberg-Hua et al. 2003; Smith
497 2003), and demonstrate that radical and conservative mutations appear to experience
498 very different histories of selection in natural populations. In particular, we provide
499 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. Indeed, comparisons of both substitution and polymorphism reveal that radical changes experience a higher intensity of purifying selection than do conservative changes in *P. antipodarum* (Figure 2). This result indicates that radical amino acid changes usually impart substantially more severe fitness effects than conservative changes and are therefore expected return to mutation-selection-drift equilibrium more rapidly than conservative amino acid changes (Fisher 1930).

We also found that asexual *P. antipodarum* lineages experience higher rates of radical amino acid substitution and exhibit higher incidences (θ) and frequencies (π) of radical amino acid polymorphisms than sexual counterparts, suggesting that asexual *P. antipodarum* experience a relatively high rate of accumulation of likely harmful mutations in their mitochondrial genomes. We take this result as evidence that mutations in asexual lineages experience smaller N_e*s than in sexual lineages, meaning that the elimination of even strongly deleterious mutations should occur more slowly in asexual than in sexual lineages. Theoretical (Ohta 1987; Charlesworth et al. 1993; Charlesworth and Wright 2001) and empirical (Eyre-Walker and Keightley 2007; Wright et al. 2008; Katju et al. 2015) work support this conclusion, suggesting that populations with low N_e should harbor a larger proportion of “effectively neutral” mutations than populations with large N_e. The somewhat surprising upshot of this prediction is that even severely deleterious mutations become more effectively neutral and thus more likely to be subject to fixation by drift when N_e is low. The absence of any detectable differences in sexual and asexual lineages in their rate of removal of conservative

523 changes would seem to suggest that the conservative changes observed in this dataset
524 are evolving via neutral processes in both sexual and asexual lineages. Only radical
525 amino acid changes exhibit severe enough effects on fitness to be differentially
526 maintained in asexual vs. sexual lineages, highlighting the utility of distinguishing
527 between mutations with different effects on fitness when comparing the efficacy of
528 selection across lineages.

529 The observations that radical mutations are more likely to be deleterious than
530 conservative mutations and that asexual *P. antipodarum* accumulate radical
531 nonsynonymous mutations more rapidly than sexual *P. antipodarum* raise the intriguing
532 possibility that asexual *P. antipodarum* might exhibit decreased mitochondrial function
533 compared to sexual counterparts. The presumed severity of some mutations in these
534 genomes (e.g., a nonsense mutation in *nad2* of one asexual lineage that would truncate
535 the NAD2 protein by three amino acids) suggests that either mitochondrial function is
536 decreased in at least some asexual lineages or that asexuals possess one or more
537 mechanisms to compensate for deleterious mutation load (e.g., RNA editing). RNA
538 editing of mitochondrially encoded transcripts has been observed in a variety of plant
539 taxa (Covello and Gray 1989; Gualberto et al. 1989) and in the mitochondrially encoded
540 tRNAs of land snails (Yokobori and Paabo 1995); whether *P. antipodarum* employs
541 similar strategies has not been addressed. Asexual lineages of *P. antipodarum* do
542 exhibit substantial phenotyic variation for mitochondrial function, variation that has been
543 linked to mitonuclear genotype (Sharbrough et al. 2017). Future work evaluating
544 mitochondrial function at the organelle and organismal levels in *P. antipodarum* will be
545 essential to understanding how the efficacy of selection influences the relative success

546 of sexual vs. asexual lineages and the maintenance and distribution of sexual *P.*
547 *antipodarum*.

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

569 Ultimately, inefficient removal of deleterious mutations in asexual lineages is only
570 important to the maintenance of sex and/or the persistence of asexual lineages if those
571 mutations, in fact, negatively affect fitness. Recent empirical evidence suggests that
572 harmful mutations indeed play a role in asexual lineage deterioration: Tucker et al.
573 (2013) found that obligately asexual *Daphnia* suffer from gene conversion-type
574 processes that decrease heterozygosity and subsequently expose deleterious recessive
575 alleles, leading to lineage deterioration. By contrast, our data indicate that asexual *P.*
576 *antipodarum* harbor elevated numbers of deleterious mutations due to relatively
577 ineffective removal of existing mutations despite being visible to selection rather than
578 exposure of recessive deleterious alleles that can accumulate in nuclear genomes.
579 Because mitochondrial genotype is critically important to organismal function and fitness
580 (Ellison and Burton 2006; Hoekstra et al. 2013; Pichaud et al. 2013), this increased load
581 of likely harmful mutations could potentially contribute to negative phenotypic
582 consequences in asexuals, though the same phenomenon would need to also apply to
583 asexual nuclear genomes in order to provide the short-term advantages necessary to
584 maintain sexual reproduction within a population (Maynard Smith 1978). Given that the
585 nuclear genome is the site of the vast majority of gene content and recombination in
586 sexual lineages, our observation of elevated retention of deleterious mutations in
587 mitochondrial genomes of asexual *P. antipodarum* leads us to predict that the nuclear
588 genome is also likely to exhibit substantial differences in deleterious mutational load and
589 efficacy of selection across reproductive modes.
590
591 **Mitonuclear LD and the efficacy of purifying selection in mitochondrial genomes**

For decades, deleterious mutation accumulation has been thought to be an unavoidable hallmark of mitochondrial genome evolution (Gabriel et al. 1993; Neiman and Taylor 2009); however, this widely held assumption that has been recently called into question by several empirical examples of relatively effective natural selection operating in mitochondrial genomes compared to nuclear genomes (Cooper et al. 2015; Konrad et al. 2017). The extent to which mitochondrial genomes actually undergo mutational meltdown has profound implications for a number of downstream evolutionary hypotheses including the compensatory model of mitonuclear coevolution (Rand et al. 2004; Dowling et al. 2008; Niehuis et al. 2008; Osada and Akashi 2012; Havird et al. 2015), the evolution of mitonuclear reproductive incompatibilities that could contribute to speciation (Harrison and Burton 2006; Niehuis et al. 2008; Burton and Barreto 2012; Hill 2016), introgressive mitochondrial replacement (Toews and Brelsford 2012; Sloan et al. 2017), and rates of extinction (Lynch et al. 1993; Havird et al. 2016), among others (Sloan et al. 2017). If natural selection is indeed effective in mitochondrial genomes (especially purifying selection), selection to alleviate deleterious epistatic interactions between nuclear and mitochondrial genomes may not play as central a role in the generation of eukaryotic diversity as previously thought (Montooth et al. 2010; Adrion et al. 2016).

While the results presented here are restricted to mitochondrial genomes, they do provide empirical evidence into the efficacy of selection operating in mitochondrial genomes in conditions of low vs. high N_e. In conditions of low N_e (i.e., asexual lineages), elevated selective interference appears to increase rates of deleterious mutation accumulation in mitochondrial genomes. That is, as expected under the Hill-Robertson

615 effect, the higher mitonuclear LD experienced by mitochondrial genomes "trapped" in
616 asexual lineages is associated with mitochondrial mutation accumulation. It follows that
617 mitochondrial genomes that appear to experience relatively effective purifying selection
618 (e.g., the mitochondrial genomes in sexual lineages of *P. antipodarum*), there must be
619 some mechanism (likely sexual reproduction) whereby the Hill-Robertson effect is
620 ameliorated. Based on this logic, we predict that taxa with relatively low rates of
621 mitochondrial mutation accumulation (e.g., *Caenorhabditis*, *Drosophila*) would also
622 exhibit low levels of mitonuclear LD compared to taxa with relatively high rates of
623 mitochondrial mutation accumulation (e.g., *Nasonia*, *Tigriopus*). Contrary to these
624 expectations, the taxa in which some of the most powerful evidence for effective
625 selection in mitochondrial genomes has been presented also exhibit perturbations to
626 sexual reproduction that would be expected to *increase* mitonuclear LD: male
627 *Drosophila* rarely undergo recombination during meiosis (Vazquez et al. 2002), and *C.*
628 *elegans* are predominantly selfing (Felix and Braendle 2010). Clearly, investigation into
629 the genetic mechanisms that promote and restrict mitochondrial mutation accumulation
630 in some lineages but not others is necessary if we are to understand the role
631 mitonuclear coevolution has played in eukaryotic evolution.
632

633 **TABLES**

Table 1. Amino acid classification schemes.

1	2	3	4	5	6	7
Zhang (2000)	Zhang (2000)	Zhang (2000)	Hanada et al. (2007)	Hanada et al. (2007)	Hanada et al. (2007)	Grantham (1974)
Charge	**Polarity**	**Polarity & Volume**	**Correlation with K_A/K_S**	**Charge & Aromaticity**	**Polarity & Volume**	**Charge & Polarity**
A, C, F, G, I, L, M, N, P, Q, S, T, V, W, Y	C, D, E, G, H, K, N, Q, R, S, T, Y	A, G, P, S, T	A, C, G, N, P, S, T	A, C, G, I, L, M, N, P, Q, S, T, V	A, F, G, I, L, M, P, V, W	A, F, I, L, M, P, V, W
		D, E, N, Q	F, H, K, Q, R, W, Y	F, W, Y	C, N, Q, S, T, Y	C, G, N, Q, S, T, Y
H, K, R		I, L, M, V				
	A, F, I, L, M, P, V, W	H, K, R	I, L, M, V	H, K, R	H, K, R	H, K, R
D, E		F, W, Y	D, E	D, E	D, E	D, E

634

Table 2. Substitution rate and nucleotide diversity across mutational types in *P. antipodarum* mitochondrial genomes.

Mutational Type	K[a] (95% CI)	π[b] (95% CI)	θ[b] (95% CI)	θ_U[b] (95% CI)
Syn	0.41 [d] (0.38 - 0.44)	0.064 [d] (0.058 - 0.069)	0.063 [d] (0.058 - 0.067)	8.7×10^{-3} [d] (6.9×10^{-3} - 0.011)
Nsyn	4.1×10^{-3} (2.7×10^{-3} - 5.5×10^{-3})	4.6×10^{-3} (3.8×10^{-3} - 5.3×10^{-3})	6.1×10^{-3} (5.2×10^{-3} - 6.9×10^{-3})	2.5×10^{-3} (2.0×10^{-3} - 3.1×10^{-3})
Con[c]	6.6×10^{-3} (4.2×10^{-3} - 9.0×10^{-3})	6.5×10^{-3} (5.0×10^{-3} - 7.5×10^{-3})	8.3×10^{-3} (6.7×10^{-3} - 9.4×10^{-3})	3.4×10^{-3} (2.2×10^{-3} - 3.6×10^{-3})
Rad[c]	1.2×10^{-3} [e] (1.3×10^{-4} - 2.4×10^{-3})	2.5×10^{-3} [e] (1.8×10^{-3} - 3.4×10^{-3})	3.7×10^{-3} [e] (2.8×10^{-3} - 4.7×10^{-3})	1.7×10^{-3} (7.6×10^{-4} - 2.4×10^{-3})
Con-1	5.4×10^{-3} (3.6×10^{-3} - 7.3×10^{-3})	5.8×10^{-3} (4.7×10^{-3} - 6.6×10^{-3})	7.4×10^{-3} (6.2×10^{-3} - 8.4×10^{-3})	2.9×10^{-3} (1.9×10^{-3} - 3.4×10^{-3})
Rad-1	4.3×10^{-4} [e] (0 - 1.3×10^{-3})	1.5×10^{-3} [e] (7.8×10^{-4} - 2.4×10^{-3})	2.6×10^{-3} [e] (1.6×10^{-3} - 3.7×10^{-3})	1.5×10^{-3} (1.6×10^{-3} - 3.3×10^{-3})
Con-2	5.9×10^{-3} (3.8×10^{-3} - 8.1×10^{-3})	5.5×10^{-3} (4.0×10^{-3} - 6.1×10^{-3})	6.5×10^{-3} (5.3×10^{-3} - 7.5×10^{-3})	2.6×10^{-3} (2.2×10^{-3} - 4.2×10^{-3})
Rad-2	1.4×10^{-3} [e] (1.5×10^{-4} - 2.7×10^{-3})	3.7×10^{-3} (2.8×10^{-3} - 4.8×10^{-3})	5.5×10^{-3} (4.4×10^{-3} - 6.8×10^{-3})	2.4×10^{-3} (1.6×10^{-3} - 2.8×10^{-3})
Con-3	8.6×10^{-3} (5.1×10^{-3} - 0.012)	7.7×10^{-3} (6.0×10^{-3} - 9.4×10^{-3})	9.4×10^{-3} (7.6×10^{-3} - 0.011)	3.2×10^{-3} (1.8×10^{-3} - 3.6×10^{-3})
Rad-3	1.8×10^{-3} [e] (6.5×10^{-4} - 2.9×10^{-3})	3.0×10^{-3} [e] (2.2×10^{-3} - 3.6×10^{-3})	4.5×10^{-3} [e] (3.5×10^{-3} - 5.2×10^{-3})	2.2×10^{-3} (1.7×10^{-3} - 3.1×10^{-3})
Con-4	7.4×10^{-3} (4.4×10^{-3} - 0.010)	6.9×10^{-3} (5.4×10^{-3} - 8.4×10^{-3})	8.4×10^{-3} (6.9×10^{-3} - 0.010)	2.7×10^{-3} (2.5×10^{-3} - 4.2×10^{-3})
Rad-4	1.9×10^{-3} [e] (7.1×10^{-4} - 3.1×10^{-3})	3.1×10^{-3} [e] (2.2×10^{-3} - 3.7×10^{-3})	4.6×10^{-3} [e] (3.6×10^{-3} - 5.4×10^{-3})	2.4×10^{-3} [e] (1.1×10^{-3} - 2.4×10^{-3})
Con-5	7.1×10^{-3} (4.6×10^{-3} - 9.7×10^{-3})	7.5×10^{-3} (6.1×10^{-3} - 8.8×10^{-3})	9.3×10^{-3} (7.8×10^{-3} - 0.011)	3.3×10^{-3} (2.0×10^{-3} - 3.7×10^{-3})
Rad-5	9.4×10^{-4} [e] (0 - 1.9×10^{-3})	1.7×10^{-3} [e] (9.5×10^{-4} - 2.2×10^{-3})	2.8×10^{-3} [e] (2.0×10^{-3} - 3.5×10^{-3})	1.7×10^{-3} (1.6×10^{-3} - 2.9×10^{-3})
Con-6	7.1×10^{-3} (4.5×10^{-3} - 9.7×10^{-3})	5.5×10^{-3} (4.0×10^{-3} - 6.4×10^{-3})	6.7×10^{-3} (5.3×10^{-3} - 7.8×10^{-3})	2.9×10^{-3} (2.0×10^{-3} - 3.8×10^{-3})
Rad-6	1.3×10^{-3} [e] (2.4×10^{-4} - 2.4×10^{-3})	3.9×10^{-3} (3.0×10^{-3} - 4.8×10^{-3})	5.6×10^{-3} (4.6×10^{-3} - 6.7×10^{-3})	2.2×10^{-3} (1.6×10^{-3} – 3.0×10^{-3})
Con-7	7.6×10^{-3} (4.7×10^{-3} - 0.010)	6.2×10^{-3} (4.6×10^{-3} - 7.3×10^{-3})	7.5×10^{-3} (5.9×10^{-3} - 8.7×10^{-3})	2.9×10^{-3} (2.4×10^{-3} - 4.0×10^{-3})
Rad-7	1.3×10^{-3} [e] (2.3×10^{-4} - 2.3×10^{-3})	3.4×10^{-3} [e] (2.6×10^{-3} - 4.3×10^{-3})	5.0×10^{-3} (4.1×10^{-3} - 6.0×10^{-3})	2.3×10^{-3} (1.2×10^{-3} - 2.5×10^{-3})

[a] - 95% CIs estimated from equation (1) in Methods
[b] - 95% CIs estimated from 10,000 bootstrap replicates
[c] - Mutational type defined by CRI score
[d] - All nonsynonymous mutational types had significantly lower values ($p < 0.05$) than synonymous sites
[e] - Value significantly lower than corresponding value for conservative sites, with $p < 0.05$

635

636 **Table 2. Substitution rate and nucleotide diversity across mutational types in *P.***
637 ***antipodarum* mitochondrial genomes.** For each mutational type, we estimated
638 substitution rates (K), nucleotide diversity (π and θ), and private allele diversity (θ_U)
639 using seven distinct amino acid classification schemes plus a model-averaged classifier
640 (CRI). We used 95% CIs (parenthetical listed below population statistic) to compare
641 evolutionary rates for different types of mutations.
642

Table 3. McDonald-Kreitman tests of selection across mutational types in *P. antipodarum* mitochondrial genomes.

	Syn	Nsyn (Nsyn/Syn)	Con[a] (Con/Syn)	Rad[a] (Rad/Syn)
Polymorphism	651	211 (0.043)	151 (0.037)	60 (6.1×10^{-3})
Divergence	814	35 (0.32)	30 (0.23)	5 (0.092)
FET vs. Syn	-	$p < 2.2 \times 10^{-16}$	$p < 2.2 \times 10^{-16}$	$p < 2.2 \times 10^{-16}$
FET vs. Con	-	$p = 0.59$	-	$p = 0.098$

[a] – Mutational type defined by CRI score

643

Table 4. Population genetic comparisons of conservative and radical mutations in sexual vs. asexual lineages of *P. antipodarum*.

	Con[a]			Rad[a]		
	Sex	Asex	p^b	Sex	Asex	p^b
K/K_S (variance)	0.026 (0.024 - 0.029)	0.026 (0.022 - 0.030)	0.95	4.77×10^{-3} (3.60×10^{-3} - 5.94×10^{-3})	6.32×10^{-3} (3.90×10^{-3} - 8.73×10^{-3})	6.5×10^{-4}

	Con[a]				Rad[a]			
	Sex	Asex	D	p^c	Sex	Asex	D	p^c
Polymorphisms	53	113	-	-	13	54	-	-
π/π_S (variance)	0.11 (0.058 - 0.16)	0.099 (0.036 - 0.061)	-7.5×10^{-3}	0.91	0.030 (0.017 - 0.043)	0.043 (0.027 - 0.059)	0.013	0.049
θ/θ_S (variance)	0.12 (0.070 - 0.17)	0.13 (0.061 - 0.14)	7.0×10^{-3}	0.70	0.029 (0.018 - 0.041)	0.068 (0.049 - 0.087)	0.039	1.5×10^{-3}
$\theta_U/\theta_{U\text{-}S}$ (variance)	0.25 (0.14 - 0.36)	0.53 (0.35 - 0.70)	0.28	3.1×10^{-3}	0.055 (0.033 - 0.077)	0.33 (0.23 - 0.43)	0.27	5.9×10^{-3}

[a] - Mutational type defined by CRI score
[b] - Mann-Whitney U test
[c] - One-tailed test of significance (H_A: $D > 0$)

644 **FIGURE LEGENDS**

645 **Figure 1. Whole-mitochondrial genome phylogeny for *P. antipodarum* and an**
646 **outgroup, *P. estuarinus*.** Consensus phylogenetic tree depicting evolutionary
647 relationships of 23 asexual (red) and eight sexual (blue) *P. antipodarum* mitochondrial
648 genomes. Tree topology was inferred using Maximum Likelihood (ML, performed in
649 PAUP*) and Bayesian (performed in MrBayes) methods. Two models of molecular
650 evolution identified by jModelTest v 2.0 (GTR + I + G, TIM3 + I + G) were used to infer
651 ML-based trees, while the model of molecular evolution was directly inferred from the
652 alignment over 1.0 x 10^6 generations for the Bayesian (MrBayes) tree. Branch support
653 from the GTR + I + G ML approach and from the Bayesian consensus tree is listed
654 below branches (bootstrap support/posterior probability). Asterisks indicate nodes for
655 which both ML models exhibited > 90 bootstrap support. Numbers above each line
656 represent the numbers of non-homoplasious mutational changes, with the ratio of
657 synonymous: nonsynonymous changes in plain font and the ratio of conservative:
658 radical changes in bold font and parentheses. Clade A represents a particularly distinct
659 group of asexual lineages.

660

661 **Figure 2. Molecular evolution and population genetics of synonymous,**
662 **nonsynonymous, conservative, and radical mutations in *P. antipodarum***
663 **mitochondrial genomes.** a) Branch-specific estimates of synonymous-corrected
664 substitution rates for all nonsynonymous changes (black), conservative nonsynonymous
665 changes (blue), and radical nonsynonymous changes (orange), as identified by the
666 model-averaged amino acid classification scheme (CRI). Error bars represent standard

667 deviations of branch-specific substitution rates. Lower-case letters reflect statistical
668 groupings based on pairwise Mann-Whitney U tests ($p < 0.017$). b) Ratios of
669 polymorphism to divergence for synonymous, all nonsynonymous, conservative
670 nonsynonymous, and radical nonsynonymous changes. Lower-case letters reflect
671 statistical groupings based on pairwise Fisher's Exact Tests ($p < 0.013$). c) Nucleotide
672 diversity estimated using π (circles) and θ (triangles) for all nonsynonymous (black),
673 conservative nonsynonymous (blue), and radical nonsynonymous (orange) sites. Error
674 bars reflect variance calculated as in Durrett (2008). Radical nonsynonymous sites
675 exhibit significantly lower levels of nucleotide diversity than conservative sites ($p <$
676 0.0002) for both measures of polymorphism. d) Site frequency spectra of synonymous
677 (white), nonsynonymous (black), conservative nonsynonymous (blue), and radical
678 nonsynonymous (orange) polymorphisms within *P. antipodarum*.
679
680 **Figure 3. Molecular evolution of conservative and radical amino acid**
681 **substitutions in *P. antipodarum*.** Branch-specific estimates of a) synonymous-
682 corrected conservative nonsynonymous substitution rates did not differ across sexual
683 (blue) and asexual (red) lineages ($W = 95$, $p = 0.91$), but b) synonymous-corrected
684 radical nonsynonymous substitution rates were significantly higher in asexual lineages
685 than in sexual lineages ($W = 168$, $p = 6.5 \times 10^{-4}$).
686
687 **Figure 4. Population genetic comparisons of conservative and radical amino acid**
688 **polymorphism in sexual (blue) vs. asexual (red) lineages of *P. antipodarum*.** a)
689 π_N/π_S (circles) and θ_N/θ_S (triangles) in sexual vs. asexual lineages for conservative and

690 radical sites. b) π_N/π_S (circles) and θ_N/θ_S (triangles) in sexual vs. asexual lineages for
691 conservative and radical sites. Only lineages from clade C were used in this analysis. c)
692 Private polymorphism ($\theta_{U\text{-}N}/\theta_{U\text{-}S}$) in sexual vs. asexual lineages for conservative and
693 radical sites. Error bars reflect variance calculated as in Durrett (2008). For all
694 comparisons, one-tailed *p* values (i.e., Asex > Sex) are indicated by asterisks (* – $p <$
695 0.05, ** – $p < 0.01$, *** – $p < 0.001$).

CONFLICT OF INTEREST

The authors do not declare a conflict of interest.

ACKNOWLEDGEMENTS

We thank Cindy Toll and Gery Hehman for their assistance with DNA sequencing. We also thank Stephen I. Wright and Aneil F. Agrawal for helpful discussions regarding interpretation of intraspecific data. We thank Samuel J. Fahrner for helpful discussions regarding bootstrapping. We thank Jeremiah W. Busch and several anonymous reviewers who saw previous versions of this manuscript for their helpful comments. Some of the data presented herein were obtained at the Flow Cytometry Facility, which is a Carver College of Medicine / Holden Comprehensive Cancer Center core research facility at the University of Iowa. The Facility is funded through user fees and the generous financial support of the Carver College of Medicine, Holden Comprehensive Cancer Center, and Iowa City Veteran's Administration Medical Center. The National Science Foundation (NSF: MCB – 1122176; DEB – 1310825) and the Iowa Academy of Sciences (ISF #13-10) funded this research.

LITERATURE CITED

Adrion, J. R., P. S. White, and K. L. Montooth. 2016. The roles of compensatory evolution and constraint in aminoacyl tRNA synthetase evolution. Mol Biol Evol 33:152-161.

Andolfatto, P., K. M. Wong, and D. Bachtrog. 2011. Effective population size and the efficacy of selection on the X chromosomes of two closely related *Drosophila* species. Genome Biol Evol 3:114-128.

Anfinsen, C. B. 1973. Principles that govern the folding of protein chains. Science 181:223-230.

Barr, C. M., M. Neiman, and D. R. Taylor. 2005. Inheritance and recombination of mitochondrial genomes in plants, fungi and animals. New Phytol 168:39-50.

Bashford, D., C. Chothia, and A. M. Lesk. 1987. Determinants of a protein fold – unique features of the globin amino acid sequences. J Mol Biol 196:199-216.

Birky, C. W., Jr. and J. B. Walsh. 1988. Effects of linkage on rates of molecular evolution. Proc Natl Acad Sci USA 85:6414-6418.

Blier, P. U., F. Dufresne, and R. S. Burton. 2001. Natural selection and the evolution of mtDNA-encoded peptides: evidence for intergenomic co-adaptation. Trends Genet 17:400-406.

Boyko, A. R., S. H. Williamson, A. R. Indap, J. D. Degenhardt, R. D. Hernandez, K. E. Lohmueller, M. D. Adams, S. Schmidt, J. J. Sninsky, S. R. Sunyaev, T. J. White, R. Nielsen, A. G. Clark, and C. D. Bustamante. 2008. Assessing the evolutionary impact of amino acid mutations in the human genome. PLoS Genet 4:e1000083.

737 Burley, S. K. and G. A. Petsko. 1985. Aromatic-aromatic interaction: a mechanism of
738 protein structure stabilization. Science 229:23-28.
739 Burton, R. S. and F. S. Barreto. 2012. A disproportionate role for mtDNA in
740 Dobzhansky-Muller incompatibilities? Mol Ecol 21:4942-4957.
741 Charlesworth, B., M. T. Morgan, and D. Charlesworth. 1993. The effect of deleterious
742 mutations on neutral molecular variation. Genetics 134:1289-1303.
743 Charlesworth, D. and S. I. Wright. 2001. Breeding systems and genome evolution. Curr
744 Opin Genet Dev 11:685-690.
745 Cooper, B. S., C. R. Burrus, C. Ji, M. W. Hahn, and K. L. Montooth. 2015. Similar
746 efficacies of selection shape mitochondrial and nuclear genes in both *Drosophila*
747 *melanogaster* and *Homo sapiens*. G3 5:2165-2176.
748 Covello, P. S. and M. W. Gray. 1989. RNA editing in plant mitochondria. Nature
749 341:662-666.
750 Darriba, D., G. L. Taboada, R. Doallo, and D. Posada. 2012. jModelTest 2: more
751 models, new heuristics and parallel computing. Nat Methods 9:772.
752 Doms, R. W., A. Ruusala, C. Machamer, J. Helenius, A. Helenius, and J. K. Rose. 1988.
753 Differential effects of mutations in 3 domains on folding, quaternary structure,
754 and intracellular transport of vesicular stomatitis virus G-protein. J Cell Biol
755 107:89-99.
756 Dowling, D. K., U. Friberg, and J. Lindell. 2008. Evolutionary implications of non-neutral
757 mitochondrial genetic variation. Trends Ecol Evol 23:546-554.
758 Durrett, R. 2008. Probability models for DNA sequence evolution. Springer, New York ;
759 London.

760 Ellison, C. K. and R. S. Burton. 2006. Disruption of mitochondrial function in
761 interpopulation hybrids of *Tigriopus californicus*. Evolution 60:1382-1391.
762 Eyre-Walker, A. and P. D. Keightley. 2007. The distribution of fitness effects of new
763 mutations. Nat Rev Genet 8:610-618.
764 Eyre-Walker, A. and P. D. Keightley. 2009. Estimating the rate of adaptive molecular
765 evolution in the presence of slightly deleterious mutations and population size
766 change. Mol Biol Evol 26:2097-2108.
767 Felix, M. A. and C. Braendle. 2010. The natural history of *Caenorhabditis elegans*. Curr
768 Biol 20:R965-969.
769 Fisher, R. A. 1930. The genetical theory of natural selection. The Clarendon Press,
770 Oxford, Eng.
771 Freudenberg-Hua, Y., J. Freudenberg, N. Kluck, S. Cichon, P. Propping, and M. M.
772 Nothen. 2003. Single nucleotide variation analysis in 65 candidate genes for
773 CNS disorders in a representative sample of the European population. Genome
774 Res 13:2271-2276.
775 Fu, Y. X. and W. H. Li. 1993. Statistical tests of neutrality of mutations. Genetics
776 133:693-709.
777 Fukami, H., A. F. Budd, D. R. Levitan, J. Jara, R. Kersanach, and N. Knowlton. 2004.
778 Geographic differences in species boundaries among members of the
779 *Montastraea annularis* complex based on molecular and morphological markers.
780 Evolution 58:324-337.
781 Gabriel, W., M. Lynch, and R. Burger. 1993. Muller's ratchet and mutational meltdowns.
782 Evolution 47:1744-1757.

783 Geisler, N. and K. Weber. 1982. The amino acid sequence of chicken muscle desmin
784 provides a common structural model for intermediate filament proteins. Embo J
785 1:1649-1656.
786 Gordon, A. and G. J. Hannon. 2010. Fastx-toolkit. FASTQ/A short-reads preprocessing
787 tools (unpublished). http://hannonlab.cshl.edu/fastx_toolkit, 5.
788 Grantham, R. 1974. Amino acid difference formula to help explain protein evolution.
789 Science 185:862-864.
790 Gualberto, J. M., L. Lamattina, G. Bonnard, J. H. Weil, and J. M. Grienenberger. 1989.
791 RNA editing in wheat mitochondria results in the conservation of protein
792 sequences. Nature 341:660-662.
793 Halligan, D. L., F. Oliver, J. Guthrie, K. C. Stemshorn, B. Harr, and P. D. Keightley.
794 2011. Positive and negative selection in murine ultraconserved noncoding
795 elements. Mol Biol Evol 28:2651-2660.
796 Harrison, J. S. and R. S. Burton. 2006. Tracing hybrid incompatibilities to single amino
797 acid substitutions. Mol Biol Evol 23:559-564.
798 Havird, J. C., S. W. Fitzpatrick, J. Kronenberger, W. C. Funk, L. M. Angeloni, and D. B.
799 Sloan. 2016. Sex, mitochondria, and genetic rescue. Trends Ecol Evol 31:96-99.
800 Havird, J. C., N. S. Whitehill, C. D. Snow, and D. B. Sloan. 2015. Conservative and
801 compensatory evolution in oxidative phosphorylation complexes of angiosperms
802 with highly divergent rates of mitochondrial genome evolution. Evolution 69:3069-
803 3081.
804 Henry, L., T. Schwander, and B. J. Crespi. 2012. Deleterious mutation accumulation in
805 asexual *Timema* stick insects. Mol Biol Evol 29:401-408.

806 Hill, G. E. 2016. Mitonuclear coevolution as the genesis of speciation and the
807 mitochondrial DNA barcode gap. Ecol Evol 6:5831-5842.
808 Hill, W. G. and A. Robertson. 1966. The effect of linkage on limits to artificial selection.
809 Genet Res 8:269-294.
810 Hoekstra, L. A., M. A. Siddiq, and K. L. Montooth. 2013. Pleiotropic effects of a
811 mitochondrial-nuclear incompatibility depend upon the accelerating effect of
812 temperature in *Drosophila*. Genetics 195:1129-1139.
813 Hollister, J. D., S. Greiner, W. Wang, J. Wang, Y. Zhang, G. K. Wong, S. I. Wright, and
814 M. T. Johnson. 2015. Recurrent loss of sex is associated with accumulation of
815 deleterious mutations in *Oenothera*. Mol Biol Evol 32:896-905.
816 Holm, S. 1979. A simple sequentially rejective multiple test procedure. Scand J Stat
817 6:65-70.
818 Jokela, J., C. M. Lively, M. F. Dybdahl, and J. A. Fox. 1997. Evidence for a cost of sex
819 in the freshwater snail *Potamopyrgus antipodarum*. Ecology 78:452-460.
820 Jukes, T. H., and Cantor, C. R. 1969. Evolution of protein molecules. In '*Mammalian*
821 *Protein Metabolism*'. Ed. HN Munro. pp. 21–132.
822 Kaiser, V. B. and B. Charlesworth. 2009. The effects of deleterious mutations on
823 evolution in non-recombining genomes. Trends Genet 25:9-12.
824 Katju, V., L. B. Packard, L. Bu, P. D. Keightley, and U. Bergthorsson. 2015. Fitness
825 decline in spontaneous mutation accumulation lines of *Caenorhabditis elegans*
826 with varying effective population sizes. Evolution 69:104-116.
827 Katoh, K. and D. M. Standley. 2013. MAFFT multiple sequence alignment software
828 version 7: improvements in performance and usability. Mol Biol Evol 30:772-780.

829 Keightley, P. D. and B. Charlesworth. 2005. Genetic instability of *C. elegans* comes
830 naturally. Trends Genet 21:67-70.
831 Konrad, A., O. Thompson, R. H. Waterston, D. G. Moerman, P. D. Keightley, U.
832 Bergthorsson, and V. Katju. 2017. Mitochondrial mutation rate, spectrum and
833 heteroplasmy in *Caenorhabditis elegans* spontaneous mutation accumulation
834 lines of differing population size. Mol Biol Evol 34:1319-1334.
835 Krist, A. C., A. D. Kay, K. Larkin, and M. Neiman. 2014. Response to phosphorus
836 limitation varies among lake populations of the freshwater snail *Potamopyrgus*
837 *antipodarum*. PLoS One 9:e85845.
838 Kumar, S., M. Nei, J. Dudley, and K. Tamura. 2008. MEGA: a biologist-centric software
839 for evolutionary analysis of DNA and protein sequences. Brief Bioinform 9:299-
840 306.
841 Lesk, A. M. and C. Chothia. 1980. How different amino acid sequences determine
842 similar protein structures – structure and evolutionary dynamics of the globins. J
843 Mol Biol 136: 225IN1231-230IN2270.
844 Li, W. H., C. I. Wu, and C. C. Luo. 1985. A new method for estimating synonymous and
845 nonsynonymous rates of nucleotide substitution considering the relative
846 likelihood of nucleotide and codon changes. Mol Biol Evol 2:150-174.
847 Lively, C. M. 1987. Evidence from a New Zealand snail for the maintenance of sex by
848 parasitism. Nature 328:519-521.
849 Lovell, J. T., Williamson, R. J., Wright, S. I., McKay, J. K., T. F. Sharbel. 2017. Mutation
850 Accumulation in an Asexual Relative of Arabidopsis. PLoS Genet 13: e1006550.
851

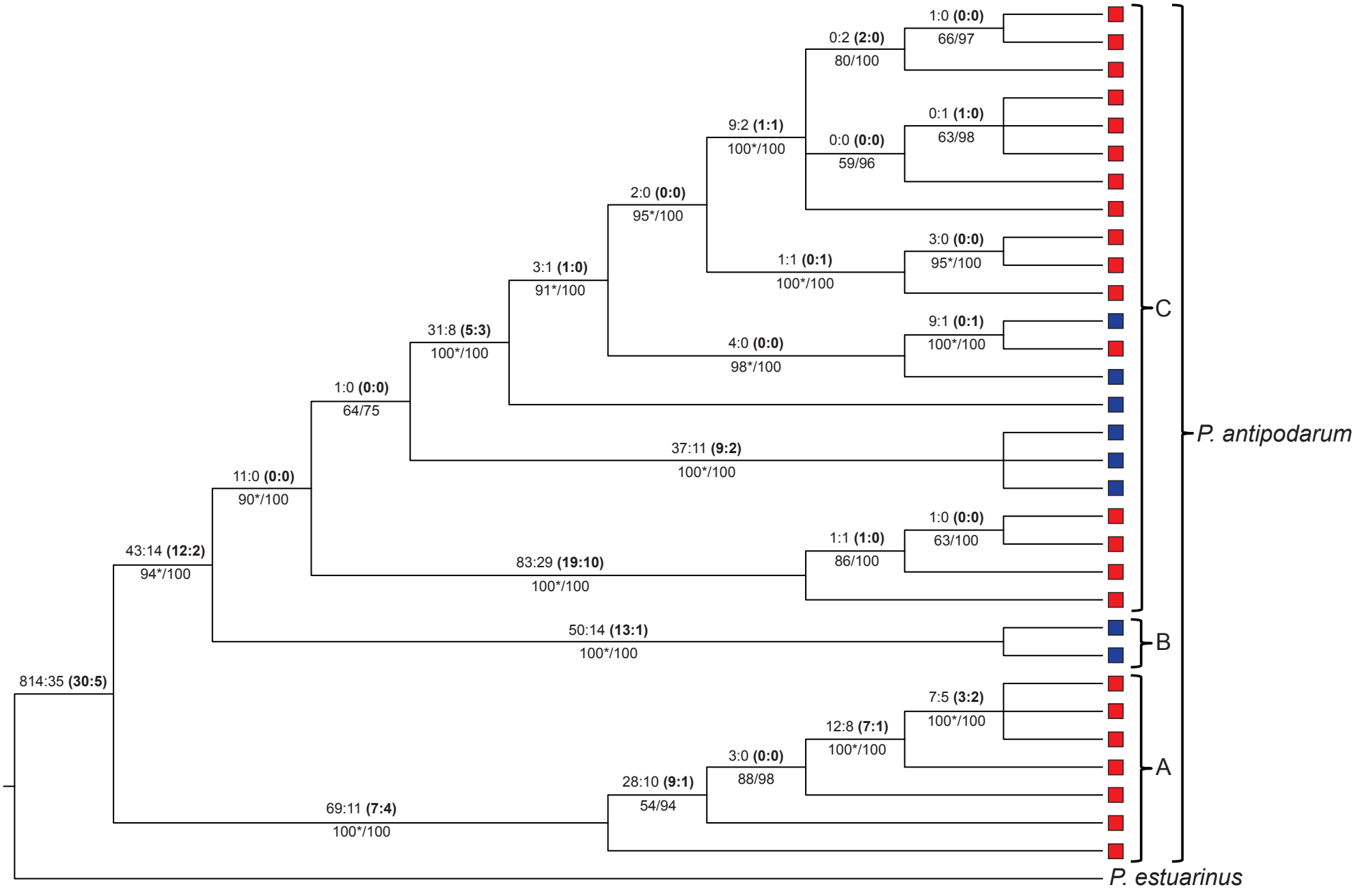
852 Lynch, M., R. Burger, D. Butcher, and W. Gabriel. 1993. The mutational meltdown in asexual
853 populations. J Hered 84:339-344.
854 Maynard Smith, J. 1978. The evolution of sex. Cambridge University Press, Cambridge
855 Eng. ; New York.
856 Mcdonald, J. H. and M. Kreitman. 1991. Adaptive protein evolution at the *Adh* locus in
857 *Drosophila*. Nature 351:652-654.
858 Meiklejohn, C. D., K. L. Montooth, and D. M. Rand. 2007. Positive and negative
859 selection on the mitochondrial genome. Trends Genet 23:259-263.
860 Montooth, K. L., C. D. Meiklejohn, D. N. Abt, and D. M. Rand. 2010. Mitochondrial-
861 nuclear epistasis affects fitness within species but does not contribute to fixed
862 incompatibilities between species of *Drosophila*. Evolution 64:3364-3379.
863 Nakashima, H., K. Nishikawa, and T. Ooi. 1986. The folding type of a protein is relevant
864 to the amino acid composition. J Biochem-Tokyo 99:153-162.
865 Nei, M. and W. H. Li. 1979. Mathematical model for studying genetic variation in terms
866 of restriction endonucleases. Proc Natl Acad Sci USA 76:5269-5273.
867 Neiman, M., G. Hehman, J. T. Miller, J. M. Logsdon, Jr., and D. R. Taylor. 2010.
868 Accelerated mutation accumulation in asexual lineages of a freshwater snail. Mol
869 Biol Evol 27:954-963.
870 Neiman, M., K. Larkin, A. R. Thompson, and P. Wilton. 2012. Male offspring production
871 by asexual *Potamopyrgus antipodarum*, a New Zealand snail. Heredity (Edinb)
872 109:57-62.
873 Neiman, M. and T. A. Linksvayer. 2006. The conversion of variance and the
874 evolutionary potential of restricted recombination. Heredity (Edinb) 96:111-121.

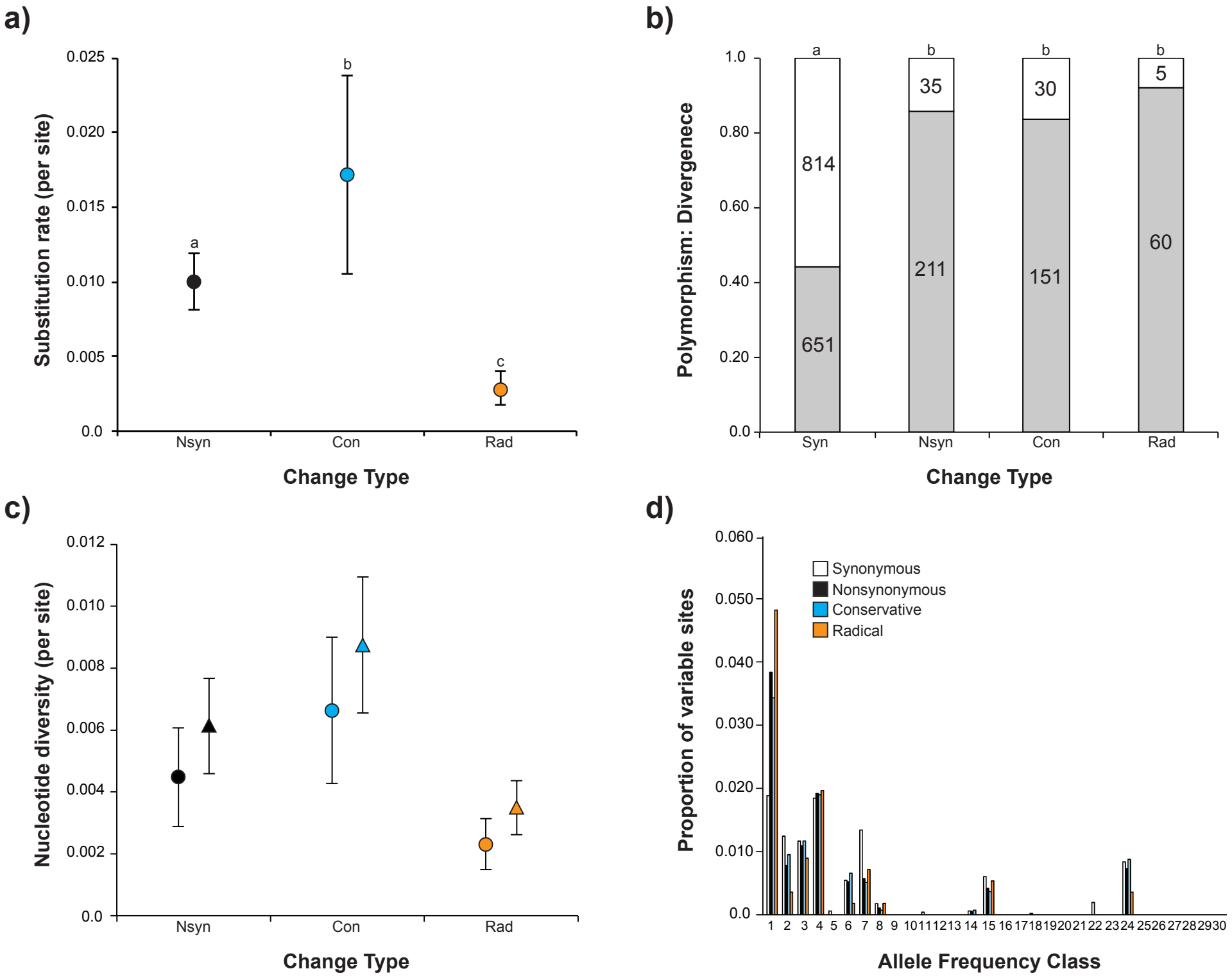
875 Neiman, M. and C. M. Lively. 2004. Pleistocene glaciation is implicated in the
876 phylogeographical structure of *Potamopyrgus antipodarum*, a New Zealand snail.
877 Mol Ecol 13:3085-3098.
878 Neiman, M., D. Paczesniak, D. M. Soper, A. T. Baldwin, and G. Hehman. 2011. Wide
879 variation in ploidy level and genome size in a New Zealand freshwater snail with
880 coexisting sexual and asexual lineages. Evolution 65:3202-3216.
881 Neiman, M. and D. R. Taylor. 2009. The causes of mutation accumulation in
882 mitochondrial genomes. Proc Roy Soc B 276:1201-1209.
883 Niehuis, O., A. K. Judson, and J. Gadau. 2008. Cytonuclear genic incompatibilities
884 cause increased mortality in male F2 hybrids of *Nasonia giraulti* and *N.*
885 *vitripennis*. Genetics 178:413-426.
886 Normark, B. B. and N. A. Moran. 2000. Opinion - Testing for the accumulation of
887 deleterious mutations in asexual eukaryote genomes using molecular sequences.
888 J Nat Hist 34:1719-1729.
889 Ohta, T. 1987. Very slightly deleterious mutations and the molecular clock. J Mol Evol
890 26:1-6.
891 Osada, N. and H. Akashi. 2012. Mitochondrial-nuclear interactions and accelerated
892 compensatory evolution: evidence from the primate cytochrome C oxidase
893 complex. Mol Biol Evol 29:337-346.
894 Paczesniak, D., J. Jokela, K. Larkin, and M. Neiman. 2013. Discordance between
895 nuclear and mitochondrial genomes in sexual and asexual lineages of the
896 freshwater snail *Potamopyrgus antipodarum*. Mol Ecol 22:4695-4710.

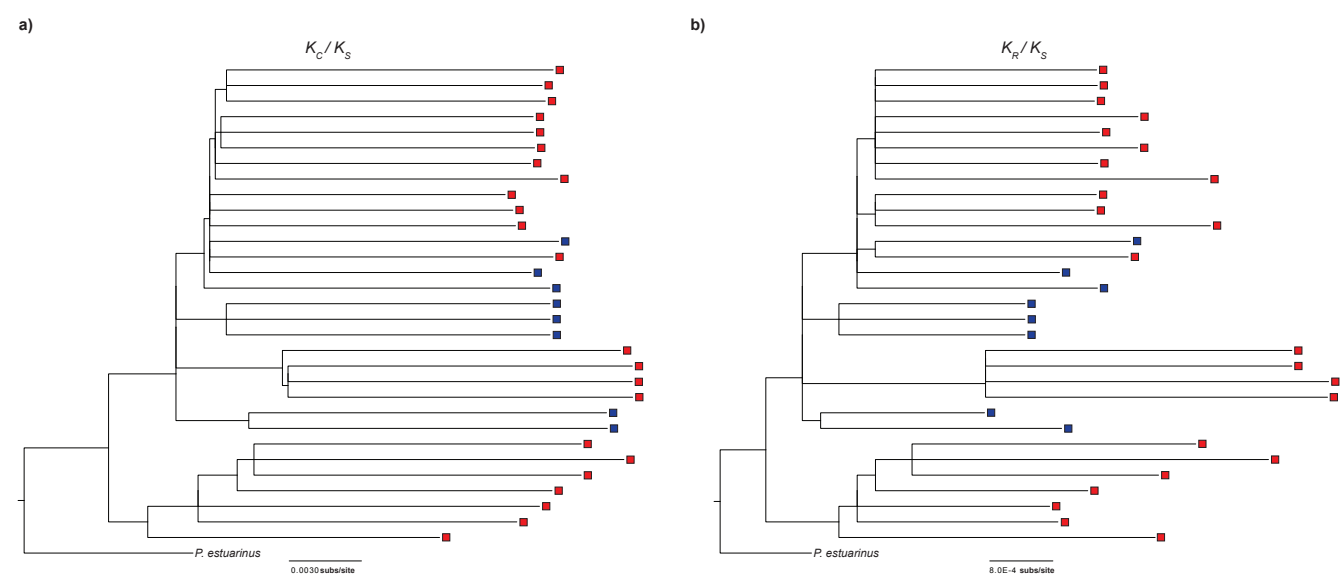
897 Perutz, M. F., J. C. Kendrew, and H. C. Watson. 1965. Structure and function of
898 haemoglobin - 2. Some relations between polypeptide chain configuration and
899 amino acid sequence. J Mol Biol 13:669-678.
900 Pichaud, N., J. W. O. Ballard, R. M. Tanguay, and P. U. Blier. 2013. Mitochondrial
901 haplotype divergences affect specific temperature sensitivity of mitochondrial
902 respiration. J Bioenerg Biomembr 45:25-35.
903 Posada, D. 2003. Using MODELTEST and PAUP* to select a model of nucleotide
904 substitution. Curr Protoc Bioinformatics Chapter 6:Unit 6 5.
905 R Core Team. 2016. R: A language and environment for statistical computing. Vienna,
906 Austria: R Foundation for Statistical Computing, https://www.R-project.org/.
907 Rand, D. M., R. A. Haney, and A. J. Fry. 2004. Cytonuclear coevolution: the genomics
908 of cooperation. Trends Ecol Evol 19:645-653.
909 Rambaut, A. 2007. FigTree, a graphical viewer of phylogenetic trees.
910 http://tree.bio.ed.ac.uk/software/figtree.
911 Ronquist, F., M. Teslenko, P. van der Mark, D. L. Ayres, A. Darling, S. Hohna, B. Larget,
912 L. Liu, M. A. Suchard, and J. P. Huelsenbeck. 2012. MrBayes 3.2: efficient
913 Bayesian phylogenetic inference and model choice across a large model space.
914 Syst Biol 61:539-542.
915 Rumbley, J., L. Hoang, L. Mayne, and S. W. Englander. 2001. An amino acid code for
916 protein folding. Proc Natl Acad Sci USA 98:105-112.
917 Sardiello, M., F. Licciulli, D. Catalano, M. Attimonelli, and C. Caggese. 2003.
918 MitoDrome: a database of *Drosophila melanogaster* nuclear genes encoding
919 proteins targeted to the mitochondrion. Nucleic Acids Res 31:322-324.

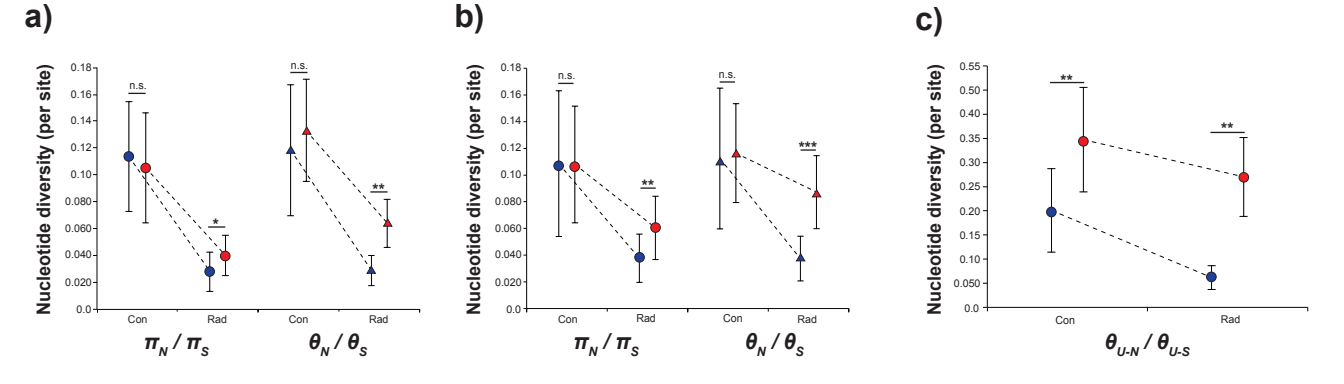
920 Sharbrough, J., J. L. Cruise, M. Beetch, N. M. Enright, and M. Neiman. 2017. Genetic
921 variation for mitochondrial function in the New Zealand freshwater snail
922 *Potamopyrgus antipodarum*. J Hered esx041.
923 Sloan, D. B., J. C. Havird, and J. Sharbrough. 2017. The on-again, off-again relationship
924 between mitochondrial genomes and species boundaries. Mol Ecol 26:2212-
925 2236.
926 Smith, N. G. 2003. Are radical and conservative substitution rates useful statistics in
927 molecular evolution? J Mol Evol 57:467-478.
928 Swofford, D. L. and J. Sullivan. 2009. Phylogeny inference based on parsimony and
929 other methods using PAUP*. Phylogenetic Handbook: A Practical Approach to
930 Phylogenetic Analysis and Hypothesis Testing, 2nd Edition:267-312.
931 Tavaré, S. 1986. Some probabilistic and statistical problems in the analysis of DNA
932 sequences. Lect Mathemat Life Sci 17: 57-86.
933 Toews, D. P. and A. Brelsford. 2012. The biogeography of mitochondrial and nuclear
934 discordance in animals. Mol Ecol 21:3907-3930.
935 Tucker, A. E., M. S. Ackerman, B. D. Eads, S. Xu, and M. Lynch. 2013. Population-
936 genomic insights into the evolutionary origin and fate of obligately asexual
937 *Daphnia pulex*. Proc Natl Acad Sci USA 110:15740-15745.
938 Vazquez, J., A. S. Belmont, and J. W. Sedat. 2002. The dynamics of homologous
939 chromosome pairing during male *Drosophila* meiosis. Curr Biol 12:1473-1483.
940 Voigt-Zielinski, M. L., M. Piwczynski, and T. F. Sharbel. 2012. Differential effects of
941 polyploidy and diploidy on fitness of apomictic *Boechera*. Sex Plant Reprod
942 25:97-109.

943 von Heijne, G. 1992. Membrane protein structure prediction. Hydrophobicity analysis
944 and the positive-inside rule. J Mol Biol 225:487-494.
945 Watterson, G. A. 1975. On the number of segregating sites in genetical models without
946 recombination. Theor Popul Biol 7:256-276.
947 Werle, E., C. Schneider, M. Renner, M. Volker, and W. Fiehn. 1994. Convenient single-
948 step, one tube purification of PCR products for direct sequencing. Nucleic Acids
949 Res 22:4354-4355.
950 Wickham, H. 2016. ggplot2: elegant graphics for data analysis. Springer.
951 Wright, C. F., S. A. Teichmann, J. Clarke, and C. M. Dobson. 2005. The importance of
952 sequence diversity in the aggregation and evolution of proteins. Nature 438:878-
953 881.
954 Wright, S. I., N. Nano, J. P. Foxe, and V. U. Dar. 2008. Effective population size and
955 tests of neutrality at cytoplasmic genes in *Arabidopsis*. Genetics research
956 90:119-128.
957 Xue, D., J. Yin, M. Tan, J. Yue, Y. Wang, and L. Liang. 2008. Prediction of functional
958 nonsynonymous single nucleotide polymorphisms in human G-protein-coupled
959 receptors. J Hum Genet 53:379-389.
960 Yokobori, S. and S. Paabo. 1995. Transfer RNA editing in land snail mitochondria. Proc
961 Natl Acad Sci USA 92:10432-10435.
962 Zhang, J. 2000. Rates of conservative and radical nonsynonymous nucleotide
963 substitutions in mammalian nuclear genes. J Mol Evol 50:56-68.


814:35 (30:5)
43:14 (12:2)
94*/100
11:0 (0:0)
90*/100
1:0 (0:0)
64/75
31:8 (5:3)
100*/100
3:1 (1:0)
91*/100
2:0 (0:0)
95*/100
9:2 (1:1)
100*/100
0:2 (2:0)
80/100
1:0 (0:0)
66/97
0:0 (0:0)
59/96
0:1 (1:0)
63/98
1:1 (0:1)
100*/100
3:0 (0:0)
95*/100
4:0 (0:0)
98*/100
9:1 (0:1)
100*/100
37:11 (9:2)
100*/100
83:29 (19:10)
100*/100
1:1 (1:0)
86/100
1:0 (0:0)
63/100
50:14 (13:1)
100*/100
69:11 (7:4)
100*/100
28:10 (9:1)
54/94
3:0 (0:0)
88/98
12:8 (7:1)
100*/100
7:5 (3:2)
100*/100
C
B
A
P. antipodarum
P. estuarinus


a)
Substitution rate (per site)
0.025
0.020
0.015
0.010
0.005
0.0
a
b
c
Nsyn
Con
Rad
Change Type
b)
Polymorphism: Divergenece
1.0
0.80
0.60
0.40
0.20
0.0
a
b
b
b
814
651
35
211
30
151
5
60
Syn
Nsyn
Con
Rad
Change Type
c)
Nucleotide diversity (per site)
0.012
0.010
0.008
0.006
0.004
0.002
0.0
Nsyn
Con
Rad
Change Type
d)
Proportion of variable sites
0.060
0.050
0.040
0.030
0.020
0.010
0.0
Synonymous
Nonsynonymous
Conservative
Radical
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
Allele Frequency Class


a)
K_C / K_S
P. estuarinus
0.0030 subs/site
b)
K_R / K_S
P. estuarinus
8.0E-4 subs/site


a)
b)
c)
Nucleotide diversity (per site)
0.0
0.020
0.040
0.060
0.080
0.10
0.12
0.14
0.16
0.18
n.s.
*
**
Con
Rad
π_N / π_S
θ_N / θ_S
***
0.050
0.15
0.20
0.25
0.30
0.35
0.40
0.45
0.50
0.55
$\theta_{U\text{-}N} / \theta_{U\text{-}S}$