Deleterious Mutation Accumulation in Asexual *Timema* Stick Insects

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Abstract

Sexual reproduction is extremely widespread in spite of its presumed costs relative to asexual reproduction, indicating that it must provide significant advantages. One postulated benefit of sex and recombination is that they facilitate the purging of mildly deleterious mutations, which would accumulate in asexual lineages and contribute to their short evolutionary life span. To test this prediction, we estimated the accumulation rate of coding (nonsynonymous) mutations, which are expected to be deleterious, in parts of one mitochondrial (*COI*) and two nuclear (*Actin* and *Hsp70*) genes in six independently derived asexual lineages and related sexual species of *Timema* stick insects. We found signatures of increased coding mutation accumulation in all six asexual *Timema* and for each of the three analyzed genes, with 3.6- to 13.4-fold higher rates in the asexuals as compared with the sexuals. In addition, because coding mutations in the asexuals often resulted in considerable hydrophobicity changes at the concerned amino acid positions, coding mutations in the asexuals are likely associated with more strongly deleterious effects than in the sexuals. Our results demonstrate that deleterious mutation accumulation can differentially affect sexual and asexual lineages and support the idea that deleterious mutation accumulation plays an important role in limiting the long-term persistence of all-female lineages.

Key words: asexual reproduction, parthenogenesis, Hill–Robertson effect, *Timema* stick insects.

Introduction

One of the greatest challenges for evolutionary biology is explaining the widespread occurrence of sexual reproduction, given its multifaceted costs compared with asexuality (Bell 1982; Otto 2009). A large body of theoretical work, encompassing ecological and genetic approaches, has presented possible explanations for the evolutionary maintenance of sexual reproduction and recombination despite apparent advantages of asexuality (e.g., Hamilton 1980; Bell 1982; Kondrashov 1993; Otto and Lenormand 2002; Otto 2009; Schön et al. 2009; Lively 2010).

Genetically based arguments for a long-term advantage of sex typically suggest that sexual reproduction accelerates the rate of adaptation and reduces the accumulation of deleterious mutations (Kondrashov 1993; Barton and Charlesworth 1998). These advantages of sex are caused by recombination and segregation facilitating the ability of natural selection to act independently on different genetic loci (Kondrashov 1988; Barton and Charlesworth 1998; Otto and Lenormand 2002). In a finite population, genetic drift creates random regions of linkage disequilibrium, but recombination and segregation break down these disequilibria. Linkage disequilibria reduce the efficiency of selection because of possible interference among loci (the "Hill–Robertson effect"; Hill and Robertson 1966; Gillespie 2000), so that selection becomes less efficient for larger linkage groups (Hill and Robertson 1966; Barton and Charlesworth 1998; Gillespie 2000). This prediction of less efficient selection under linkage disequilibrium is largely supported by empirical evidence for increased accumulation of deleterious mutations in genomic regions with low levels of recombination and in nonrecombining chromosomes (e.g., Lynch 1997; Lynch and Blanchard 1998; Bachtrog 2004).

The accumulation of deleterious mutations is expected to be especially severe in asexual all-female lineages, where all nuclear and organelle genes form a single linkage group. It is widely believed that mutation accumulation plays an important role in limiting the long-term persistence of all-female lineages (Muller 1964; Bell 1982; Lynch et al. 1993); deleterious mutations may increase the extinction rate of asexual lineages relative to their sexual congeners, thereby contributing to a presumed "twiggy" distribution of asexuality in phylogenetic trees (Bell 1982; Schwander and Crespi 2009b). The empirical evidence supporting a role for deleterious mutation accumulation in the maintenance of asexual lineages remains mixed. A higher rate of presumably nonadaptive mutation accumulation in asexual than sexual lineages has been documented for mitochondrial genes of asexual *Daphnia* (Paland and Lynch 2006b) and different freshwater snail lineages (Johnson and Howard 2007; Neiman et al. 2010). Note that predictions of mutation accumulation under asexuality also apply for the (normally) clonally reproducing mitochondria because the mitochondrial genome segregates from the nuclear

Table 1. Independently Derived Asexual *Timema* Lineages and Their Sexual Sister Species.

Asexual Lineage	Sexual Sister Species	Interspecific Divergence	Estimated Age (years)[a]
Timema shepardi	*Timema californicum/Timema poppensis*[b]	0.008 ± 0.002	400,000
Timema douglasi (central)	*T. poppensis*	0.009 ± 0.003	450,000
T. douglasi (south)	*T. poppensis*	0.012 ± 0.002	600,000
Timema genevievae	*Timema podura*	0.029 ± 0.005	1,450,000
Timema tahoe	*Timema bartmani*	0.031 ± 0.005	1,550,000
Timema monikensis	*Timema cristinae*	0.037 ± 0.006	1,850,000

[a] *Timema* asexuals have been described based on a combination of morphological features, host–plant use, and geographic range (Vickery 1993; Vickery and Sandoval 1999) and are supported by phylogenetic analyses of mitochondrial and nuclear genetic markers (Law and Crespi 2002b; Schwander and Crespi 2009a; Schwander et al. 2011). The estimated age for each asexual lineage is based on its molecular divergence from the closest sexual sister species for the *COI* gene fragment, assuming 2% divergence per million year (Brower 1994; Juan et al. 1996).

[b] Two sexual sister species are indicated for *T. shepardi* because *T. shepardi* mitochondrial haplotypes are most similar to *T. poppensis* haplotypes, whereas the nuclear genome appears to be inherited from *T. californicum* with little, if any, introgression from *T. poppensis* (Schwander and Crespi 2009a).

genome in sexuals but not asexuals (Normark and Moran 2000; Paland and Lynch 2006b). In contrast to the asexual snail and *Daphnia* lineages, studies in two asexual aphid lineages found no evidence for increased mutation accumulation in the mitochondrial genes relative to sexual lineages and evidence for increased mutation accumulation in a nuclear gene in only one of the two asexual lineages (Normark and Moran 2000). In the bdelloid rotifers, the most prominent asexual organisms presumed to have persisted for millions of years, no evidence for increased mutation accumulation was found in nuclear genes (Welch and Meselson 2001). In mitochondrial genes, increased coding mutation accumulation was detected in the short term (within "species") (Barraclough et al. 2007) but not between clades, suggesting that deleterious mutations persist to some degree in asexual rotifers but are removed by selection given enough time (Birky et al. 2005; Barraclough et al. 2007). Similarly, a biologically realistic theoretical estimation of the rate of mutation accumulation in an asexual fish revealed that this lineage is considerably older than the predicted time frame for its extinction as a consequence of the mutational decay (Loewe and Lamatsch 2008). Therefore, whether asexual lineages generally accumulate deleterious mutations more rapidly than their sexual counterparts and whether such mutations contribute to accelerated extinction of asexual lineages remain open questions. Finally, because asexuality is a lineage-level trait, mutational theories of sex should be evaluated by examining multiple, independently derived asexual lineages and related, sexual lineages rather than isolated species pairs (Normark and Moran 2000; Johnson and Howard 2007).

Here, we use molecular sequence information from sexual and asexual *Timema* stick insects to test the prediction that transitions to asexuality are associated with increased accumulation of deleterious mutations in protein-coding genes. *Timema* is a small genus of plant-feeding insects, which comprises at least seven independently derived asexual lineages (Schwander et al. 2011), including recently derived (a few hundred thousand years old) and putatively ancient ones (over 1 My old; table 1; Law and Crespi 2002a; Schwander et al. 2011). We estimated rates of coding mutation accumulation for six of the seven independently derived asexual lineages, their sexual sister species (table 1), and two additional sexual *Timema* species (*T. boharti* and *T. chumash*) in genes a priori expected to be under relatively strong purifying selection: the nuclear genes *Actin* and *Hsp70* and the mitochondrial gene *COI* (Kusakabe et al. 1999; Brocchieri et al. 2008). To infer mutation accumulation in these genes, we used the common approach of comparing the ratio of coding (nonsynonymous) substitutions per nonsynonymous site (d*N*) with synonymous substitutions per synonymous site (d*S*) among the lineages of interest (Lynch 1997; Lynch and Blanchard 1998; Neiman et al. 2010). Because coding mutations change the amino acid sequence, they are most likely deleterious if occurring in such highly conserved genes as the ones we used in our study; without functional tests it is otherwise difficult to discriminate between relatively neutral versus deleterious or beneficial coding mutations. By contrast, synonymous substitutions (which do not affect protein sequence and are presumed to have much smaller, if any, effects) accumulate at rates much closer to the rate at which mutations occur. Thus, d*N*/d*S* can provide a measure of the extent to which harmful mutations are accumulating in a given gene, while controlling for the underlying mutation rate.

Materials and Methods

We used sequence information that we generated for a previous study (Schwander et al. 2011; for GenBank accession numbers, see table 2) for coding portions of the nuclear genes *Actin* (825 bp) and *Hsp70* (1,107 bp) and the mitochondrial gene *COI* (771 bp), with 2–12 sequences available for each species for the nuclear genes and 4–48 for the mitochondrial gene (table 2). The mean pairwise divergence for these sequences was 12.0% for *COI* and 1.2% and 2.9% for *Actin* and *Hsp70*, respectively (Schwander et al. 2011).

We first evaluated the assumption that the vast majority of coding mutations in the three studied genes are deleterious. To this end, we estimated the ratios of coding to synonymous mutations (d*N*/d*S*) in a phylogeny comprising only the seven sexual *Timema* species included in this study (table 2) using the maximum likelihood (ML) methods implemented in the program "codeml" in PAML 4.4b (Yang 2007). Ratio estimates close to 1 indicate selective neutrality, whereas values converging on 0 suggest strong purifying selection. Similar to previous studies (Paland and Lynch 2006b; Neiman et al. 2010), we also evaluated whether coding mutations tended to be "short-lived," that is, whether there is a time lag between

Table 2. Number of Sequences Used per Species and Gene with the Corresponding GenBank Accession Numbers.

Species	Number of Sequences (haplotypes)			Accession Numbers
	Actin	*Hsp70*	*COI*	*Actin/Hsp70/COI*
Asexuals				
Timema shepardi	8 (5)	6 (6)	20 (6)	HQ198136–143/HQ198236–241/HQ184658–678
Timema douglasi (central)	2 (2)	2 (2)	5 (5)	HQ198083–084/HQ198188–189/HQ184543–547
T. douglasi (south)	10 (7)	6 (3)	8 (6)	HQ198089–098/HQ198190–195/HQ184552–559
Timema genevievae	8 (8)	12 (10)	22 (13)	HQ198099–106/HQ198196–207/HQ184560–580
Timema tahoe	10 (10)	6 (6)	19 (16)	HQ198144–153/HQ198242–247/HQ184679–697
Timema monikensis	8 (7)	8 (7)	15 (3)	HQ198107–114/HQ198208–215/HQ184581–596
Sexuals				
Timema californicum	10 (9)	8 (4)	29 (20)	HQ198061–070/HQ198164–171/HQ184492–518
Timema poppensis	11 (4)	12 (6)	48 (45)	HQ198125–135/HQ198224–235/HQ184611–657
Timema. podura	10 (6)	8 (7)	14 (7)	HQ198115–124/HQ198216–223/HQ184597–610
Timema bartmani	10 (3)	6 (4)	13 (8)	HQ198047–056/HQ198154–159/HQ184475–487
Timema cristinae	8 (6)	12 (9)	14 (13)	HQ198075–082/HQ198176–187/HQ184530–542
Timema boharti	4 (3)	4 (2)	4 (4)	HQ198057–060/HQ198160–163/HQ184488–491
Timema chumash	4 (4)	4 (3)	11 (9)	HQ198071–074/HQ198172–175/HQ184519–529

the appearance of new mutations and their clearance via purifying selection. In the phylogeny, such a process would be indicated by higher d*N*/d*S* ratios in terminal as compared with internal branches (Paland and Lynch 2006b). To test whether different ratios for internal and terminal branches provided a better fit to the data than only a single ratio for all branches, we first fit a model to the phylogenies that estimated a single d*N*/d*S* value for all branches. We then ran a two-ratio model where internal and terminal branches were allowed to have different ratios and used a likelihood ratio test to compare the two models.

Once the deleterious effect of the coding mutations was established (see Results), we tested whether independently derived asexual *Timema* lineages were characterized by higher rates of coding mutation accumulation than their sexual counterparts. To do so, we estimated d*N*/d*S* ratios independently for each sexual:asexual species pair, by using separate ML phylogenies for each pair and gene (supplementary figs. 1 and 2, Supplementary Material online). The phylogenies were inferred using ML methods implemented in PAUP*4.0b10 (Swofford 1993, 2000), with heuristic searches, on the freely available Bioportal (www.bioportal.uio.no). The appropriate model of nucleotide substitution was determined in each case using the Akaike information criterion for model selection as implemented in jMODELTEST 0.1.1 (Posada 2008). For each species-pair phylogeny, we applied the ML methods implemented in codeml to fit a model in which branches leading to sexual clades and individuals were allowed to have a different d*N*/d*S* ratio than branches leading to asexual clades and individuals (for branch identities, see supplementary figs. 3 and 4, Supplementary Material online). Because the transition from sexuality to asexuality may have occurred partway down a given "asexual branch," the differences between the estimated d*N*/d*S* ratios on sexual versus asexual branches should be underestimated, making our comparisons conservative. We then compared these d*N*/d*S* estimates between sexual and asexual *Timema* lineages for the different genes using an analysis of variance (ANOVA). Because the residuals in the linear model were not normally distributed, statistical significance was obtained using a permutation scheme with 5,000 replicates to determine the random distributions of the mean squares (Manly 1997). The indicated *P* values for the different effects (gene, reproductive mode, and interaction) represent the proportion of random mean squares, which were equal to or larger than the observed one.

Note that *T. poppensis* is the closest sister species for more than one independently derived asexual, notably for 2 asexuals (*T. douglasi* "central" and *T. douglasi* "south") regarding the nuclear genome and for 3 asexuals (*T. douglasi* central, *T. douglasi* south, and *T. shepardi*) regarding the mitochondrial genome (table 1). In the ANOVA, we used a single d*N*/d*S* value per gene for *T. poppensis*, which was the average d*N*/d*S* value across the two pairwise phylogenies (*T. poppensis*–*T. douglasi* central and *T. poppensis*–*T. douglasi* south) for the nuclear genes *Actin* and *Hsp70*, and the average d*N*/d*S* value across the three pairwise phylogenies (*T. poppensis*–*T. douglasi* central, *T. poppensis*–*T. douglasi* south, and *T. poppensis*–*T. shepardi*) for *COI*.

For each of the three genes, we also conducted a global test for whether asexual *Timema* lineages were characterized by higher rates of deleterious mutation accumulation than their sexual counterparts. To this end, we used previously published phylogenies (Schwander et al. 2011) including all sexual and asexual lineages for each gene (supplementary figs. 3 and 4, Supplementary Material online). Using codeml, we first estimated the goodness of fit of a model where the d*N*/d*S* ratio was the same for all the branches in the tree. Next, we estimated the goodness of fit for a model with two different ratios for branches, one for branches in sexual clades and another for branches in asexual clades. We then applied a likelihood ratio test to assess whether the two-ratio model was a significantly better fit to the data than the single-ratio model. Finally, we evaluated a third model with four different ratios. This four-ratio model allowed for different rates on terminal sexual, terminal asexual, internal

sexual, and internal asexual branches, to account for possible time lags between the appearance of new deleterious mutations and their clearance via purifying selection (for branch identities, see supplementary figs. 4 and 5, Supplementary Material online). This model was not evaluated for the phylogeny subsets with one sexual and one asexual *Timema* species because these phylogenies only have a very small number of internal branches.

Our dN/dS ratio comparisons between sexual and asexual lineages assume that noncoding mutations are similarly representative of the mutation rate in each group. To verify this assumption, we tested whether the two groups differed in their base composition at silent sites, by estimating the GC content at synonymous third codon positions using DNAsp (Librado and Rozas 2009) and by comparing the estimates between sexuals and asexuals for each gene.

If purifying selection was less efficient in asexual than sexual organisms, one would also expect that coding mutations with more strongly deleterious effects persist in asexuals than sexuals. As an indication for the "deleteriousness" of the coding mutations in the *Hsp70*, *Actin*, and *COI* genes, we compared the hydrophobic properties of the current amino acid with the properties of the ancestral amino acid at the same position. Given that hydrophobic interactions between amino acids are fundamental for protein folding (Murphy et al. 1990) and stabilizing protein structure (Privalov and Gill 1988), a mutation coding for an amino acid whose hydrophobic properties differ greatly from the ancestral amino acid should be more deleterious than a mutation coding for a similar amino acid. The similarity between pairs of amino acids can be summarized by the "hydrophobicity scoring matrix value" (HS), ranging from 0 for completely different to 100 for identical amino acid properties (Riek et al. 1995). We identified all amino acid transitions by reconstructing ancestral amino acid sequences at the nodes of the nucleotide ML phylogenies using the Bayesian framework implemented in the program ANCESTOR (Zhang and Nei 1997). Parsimony- and distance-based methods gave identical reconstructions (results not shown), most likely because the studied genes are highly conserved within the genus and because the majority of coding mutations occur along the terminal branches (see Results). HS values were then compared between transitions on branches of sexual versus asexual clades (for branch identities, see supplementary figs. 3 and 4, Supplementary Material online).

Results and Discussion

The ML estimates of dN/dS ratios for the sexual *Timema* species supported the assumption that coding mutations in the three analyzed genes are deleterious. The ratios on the internal branches of the phylogenies comprising only the sexual species were extremely low and very similar for the three genes (*Actin*: 0.027; *Hsp70*: 0.029; and *COI*: 0.027). The estimates for the terminal branches were also low but with considerable variation among genes. Consistent with the prediction that linkage between mitochondrial genes should result in less efficient selection on the mitochondrial as compared with the nuclear genome in sexual species (Lynch and Blanchard 1998), the dN/dS estimate for terminal branches in the *COI* phylogeny (0.192, 95% confidence interval [CI] 0.151–0.233) was considerably higher than the estimates for the two nuclear genes (*Actin*: 0.085, CI 0.036–0.146 and *Hsp70*: 0.091, CI 0.045–0.137). For *COI* and *Hsp70*, the model allowing for different ratios for internal and terminal branches provided a significantly better fit to the data than a model where all branches were constrained to have the same ratio (likelihood ratio tests; *COI*: $\chi^2 = 83.2$, degrees of freedom [df] = 1, $P < 0.0001$; *Hsp70*: $\chi^2 = 4.3$, df = 1, $P = 0.039$). For *Actin*, the two-ratio model did not differ significantly from the single-ratio model (global dN/dS estimate = 0.052; $\chi^2 = 5.2$, df = 1, $P = 0.113$), most likely because there was little variation in the *Actin* gene. Independently of the variation among genes for the detailed patterns of negative selection, these results indicate that the majority of extant deleterious mutations are found on terminal branches because they will be cleared by purifying selection before they contribute to divergence in internal branches.

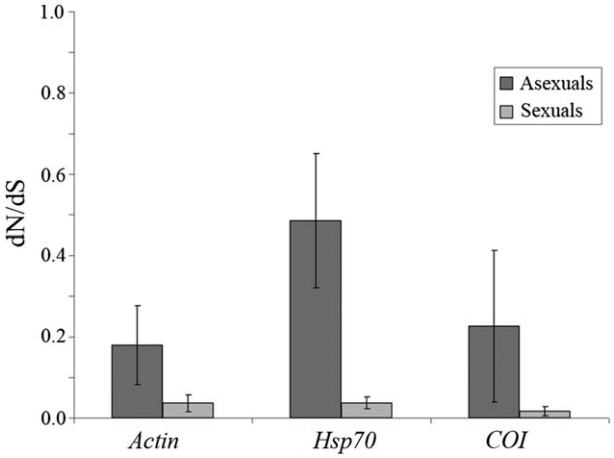

FIG. 1. The per codon dN/dS rates (±standard deviation) for sexual and asexual *Timema* lineages for the three analyzed genes.

Given the likely deleterious effects of coding mutations in *Actin*, *Hsp70*, and *COI*, we tested the prediction that transitions to asexuality are associated with less efficient purifying selection on these genes. Consistent with this prediction, the ML estimates for the (per codon) ratio of nonsynonymous to synonymous mutations in the asexual lineages were substantially higher than the estimates for their sexual sister species—typically about seven times higher for the asexuals. This pattern was found for all three genes and for each of the six independently derived asexuals, resulting in overall significantly higher rates of coding mutation accumulation in asexual than sexual *Timema* lineages (permutation ANOVA: gene effect $P = 0.091$, reproductive mode effect $P = 0.0002$, interaction $P = 0.173$; fig. 1).

Higher rates of coding mutation accumulation in the asexual than sexual *Timema* lineages were also supported, for the three genes, by dN/dS estimates for the phylogeny including all seven sexual and six asexual species. In each case, a model where branches in asexual clades were

Table 3. Per Codon dN/dS Rates Estimated for the Different Models on the Full Phylogenies Comprising Seven Sexual and Six Asexual *Timema* Species.

Model	Estimated Rate (s)	dN/dS	ln *L*	Likelihood Ratio Tests[a]
Actin				
1 rate	Global	0.117	−2145.2	NA
2 rates	Sexual/asexual	0.052/0.189	−2139.9	$\chi^2 = 10.6$, df = 1, $P = 0.001$
4 rates	Sexual (internal, terminal)/asexual (internal, terminal)	(0.032, 0.087)/(0.187, 0.192)	−2138.9	$\chi^2 = 2.0$, df = 2, $P = 0.158$
Hsp70				
1 rate	Global	0.122	−3269.0	NA
2 rates	Sexual/asexual	0.042/0.561	−3236.8	$\chi^2 = 64.5$, df = 1, $P < 0.0001$
4 rates	Sexual (internal, terminal)/asexual (internal, terminal)	(0.030, 0.094)/(0.310, 0.728)	−3233.3	$\chi^2 = 7.0$, df = 2, $P = 0.008$
COI				
1 rate	Global	0.037	−7453.6	NA
2 rates	Sexual/asexual	0.027/0.192	−7412.0	$\chi^2 = 83.2$, df = 1, $P < 0.0001$
4 rates	Sexual (internal, terminal)/asexual (internal, terminal)	(0.012, 0.065)/(0.106, 0.324)	−7373.3	$\chi^2 = 77.4$, df = 2, $P < 0.0001$

NOTE.—NA, not applicable.

[a] The indicated likelihood ratio tests are relative to the next simpler model.

allowed to have different dN/dS ratios than branches in sexual clades provided a significantly better fit to the data than a model were the ratios were the same for all branches (table 3; supplementary figs. 3 and 4, Supplementary Material online). Depending on the gene, estimates were between 3.6 and 13.4 times higher for the asexuals than sexuals.

The higher dN/dS ratios in asexual than sexual *Timema* cannot be explained by different codon usage. We found no systematic differences in base composition between the sexual and the asexual *Timema* for either of the two nuclear genes (Wilcoxon tests; *Actin*: $W = 1241$, $P = 0.245$; *Hsp70*: $W = 1287$, $P = 0.113$) or in the mitochondrial gene *COI* ($W = 6786$, $P = 0.127$). Most animal mitochondrial genomes are characterized by a significant base composition bias (a marked excess of A+T nucleotides). Such unequal frequencies of the four bases in a genome may be maintained by selection, stem from a mutation bias, or result from a balance between these two forces (Bulmer 1991). Since selection appears to be less efficient in the asexual than sexual *Timema*, the asexuals should display a change in base composition if the composition bias was mostly maintained by selection (Butlin 2006). Similar to our study, no evidence for base composition differences between sexual and asexual lineages was also reported for mitochondrial genomes in sexual and asexual *Daphnia* (Butlin 2006; Paland and Lynch 2006a). These negative results may thus indicate that selection is not the predominant force determining base-composition bias in these lineages, although the asexual lineages may be of too recent origin for a base-composition shift to be detected (Butlin 2006; Paland and Lynch 2006a).

In addition to accumulating coding mutations at a higher rate than the sexual *Timema*, the asexuals most likely also harbor coding mutations with more strongly deleterious effects. This was indicated by the hydrophobicity of the ancestral and replacement amino acids being overall more different for the coding mutations in the asexuals than the sexuals (as measured by the HS value; Wilcoxon rank sum test $W = 4948$, $P = 0.029$; fig. 2). In the sexual *Timema*, over 70% of coding mutations involved transitions between amino acids with similar hydrophobicity (HS $\geq$ 90). In the asexuals, such transitions accounted for only 54% of the coding mutations. Although we did not directly evaluate the consequence of the different coding mutations on the tertiary structure of the proteins, given the importance of hydrophobic interactions between amino acids for protein folding and structure stabilization (Murphy et al. 1990), these mutations are unlikely to be neutral. For example, the *COI* portion we analyzed in our study covers the second half of the gene and comprises 6 membrane-spanning helices; 5 of these appear to be highly conserved across insects most likely as a consequence of functional constraints (Lunt et al. 1996). Hydrophobicity changes at such functionally significant protein sequence positions are thus expected to result in deleterious phenotypic effects.

Although the asexual *Timema* lineages appear to accumulate coding mutations with more severe effects and at

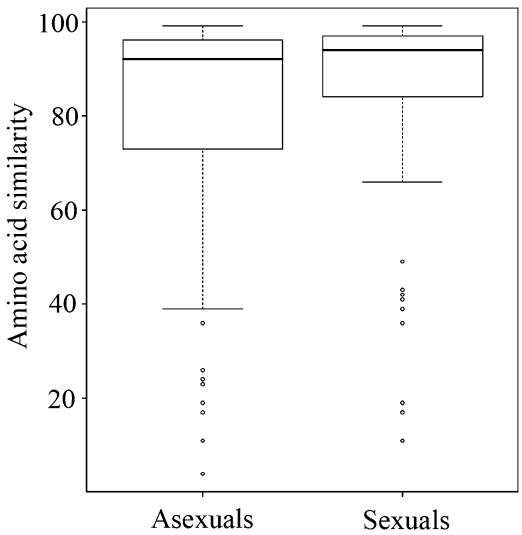

FIG. 2. Hydrophobicity changes between ancestral and replacement amino acids in asexual and sexual *Timema*. Values of 100 indicate identical levels of hydrophobicity (Riek et al. 1995). Boxes delimit the upper and lower quartiles, black horizontal lines indicate the median. Whiskers include 95% of all observations.

a higher rate than the sexual lineages, the dN/dS ratios indicate that the three analyzed genes are still under selection (fig. 1 and table 3). In addition, there seems to be a time lag between the appearance of new coding mutations and their removal by selection. This process was suggested for the *Hsp70* and *COI* genes by a model in which different dN/dS ratios were allowed for internal and external branches within each reproductive mode (i.e., four different rates in the phylogeny, supplementary figs. 3 and 4, Supplementary Material online). In both cases, the model distinguishing internal and terminal branches was a significantly better fit to the data than the simpler model (table 3). The dN/dS ratios for terminal branches in the asexual clades were approximately 2 times higher than the estimates for the internal branches for *Hsp70* and 3 times higher for *COI*. The contrast between terminal and internal branches was somewhat stronger for the sexual clades, with approximately three times higher estimates for terminal than internal branches for *Hsp70* and six times higher estimates for *COI* (table 3). On the contrary, there was no evidence for differences between internal and terminal branches in the asexual clades for *Actin*; similarly to the analysis of sexual species only, this was most likely due to the low sequence variability for this gene.

Interestingly, the extreme differences between dN/dS ratios for sexual compared with asexual *Timema* lineages appear to be due, in some cases, to an increase in coding mutation accumulation (dN) in combination with a decrease in synonymous mutation accumulation (dS) in the asexuals as compared with the sexuals. For *Hsp70* and *COI*, this pattern was demonstrated by the dS rates being significantly lower for the asexuals than the sexuals for both internal and terminal branches (Welch *t*-tests; *Hsp70* internal branches: $t = 4.3$, df $= 31.01$, $P = 0.0001$, terminal branches: $t = 0.77$, df $= 86.60$, $P = 0.44$; *COI* internal branches: $t = 3.04$, df $= 98.06$, $P = 0.003$, terminal branches: $t = 5.04$, df $= 164.7$, $P < 0.0001$, supplementary fig. 5, Supplementary Material online). By contrast, for *Actin*, there was no evidence for different dS rates in sexual and asexual clades (Welch *t*-test; $t = 0.19$, df $= 122.1$, $P = 0.85$; average $\pm$ standard deviation for branches in sexual clades: 0.0042 ± 0.0052; asexual clades: 0.0044 ± 0.0058). For *Hsp70* and *COI*, the differences between dS rates were much smaller (*Hsp70* internal branches: 4.9×, terminal: 1.3×; *COI* internal branches: 4.9×, terminal: 3.8×) than the corresponding differences between dN/dS rates (*Hsp70* internal branches: 10.3×, terminal: 7.7×; *COI* internal branches: 9.0×, terminal: 5.0×). Thus, the higher dN/dS ratios in the asexuals than sexuals are not only due to lower dS rates but also to higher dN rates. A decrease in dS rates in the asexuals could stem, for example, from overall low mutation rates in these lineages. Such a process would be consistent with hypotheses suggesting selection for reduced mutation rates in asexuals via improved DNA repair (Schön et al. 1998, 2003; Gladyshev and Meselson 2008; Welch et al. 2009), and the presence of "antimutators" (Mukai et al. 1985; Kondrashov 1995; Schaaper 1998) that would slow down the mutational decay of an asexual lineage. The origin of the different dS rates between sexual and asexual *Timema* remains to be investigated.

Taken together, our results indicate that independently derived asexual *Timema* lineages accumulate deleterious mutations at a higher rate than their sexual counterparts. Although this difference between asexual and sexual lineages is likely due to differences in the efficacy of selection because of linkage among the genes in asexual genomes, we cannot exclude a role for other factors associated with asexuality in *Timema*. For example, asexual *Timema* could experience less effective and/or relaxed purifying selection due to factors such as smaller census population sizes or lowered selective constraints. Although prolonged demographic bottlenecks are possible or even likely during the inception of asexuality (Schwander and Crespi 2009b; Schwander et al. 2010), smaller census sizes for populations of established asexual lineages and their sexual sister species are not expected. To the contrary, given that asexual females do not produce sons, asexual lineages are usually assumed to have a 2-fold demographic advantage over sexual lineages (Bell 1982). In *Timema*, comparative census size estimates for sexual and asexual populations are currently not available, but different field collections are not indicative of systematic differences, with considerable spatial fluctuations in population density and size for both asexual (Vickery and Sandoval 1998, 1999, 2001) and sexual (Sandoval 1994; Schwander et al. 2010) species. Thus, the most likely explanation for our results is that sexual reproduction enhances the efficiency of purifying selection, supporting a key assumption of the theory that deleterious mutation accumulation is an important evolutionary force contributing to the extinction of asexual lineages.

Supplementary Material

Supplementary figures 1–5 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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