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Report

Molecular Evidence for Ancient Asexuality in *Timema* Stick Insects

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Summary

Asexuality is rare in animals in spite of its apparent advantage relative to sexual reproduction, indicating that it must be associated with profound costs [1–9]. One expectation is that reproductive advantages gained by new asexual lineages will be quickly eroded over time [3, 5–7]. Ancient asexual taxa that have evolved and adapted without sex would be "scandalous" exceptions to this rule, but it is often difficult to exclude the possibility that putative asexuals deploy some form of "cryptic" sex, or have abandoned sex more recently than estimated from divergence times to sexual relatives [10]. Here we provide evidence, from high intraspecific divergence of mitochondrial sequence and nuclear allele divergence patterns, that several independently derived *Timema* stick-insect lineages have persisted without recombination for more than a million generations. Nuclear alleles in the asexual lineages displayed significantly higher intraindividual divergences than in related sexual species. In addition, within two asexuals, nuclear allele phylogenies suggested the presence of two clades, with sequences from the same individual appearing in both clades. These data strongly support ancient asexuality in *Timema* and validate the genus as an exceptional opportunity to attack the question of how asexual reproduction can be maintained over long periods of evolutionary time.

Results and Discussion

High Levels of Intralineage Mitochondrial Divergence in Multiple Transitions to Asexuality

The *Timema* walking-stick genus comprises at least five independently derived asexual lineages (*T. shepardi*, *T. douglasi*, *T. genevievae*, *T. tahoe*, and *T. monikensis*), all of which reproduce by functionally mitotic parthenogenesis [11, 12]. Three of these, *T. genevievae*, *T. tahoe*, and *T. monikensis*, have been hypothesized to be older than the time frame predicted for their extinction under asexual reproduction [11]. To improve available estimates of asexual lineage ages, we first sequenced a 771 bp portion of the mitochondrial *COI* gene in 16–48 individuals of each asexual lineage and its closest sexual relative from geographically widespread locations (see Table S1 available online; GenBank accession numbers HQ184475–HQ184697). This analysis corroborated previous evidence [11] for large-scale variation in asexual lineage age

in *Timema*, ranging from less than 500,000 years to almost 2,000,000 years (Figure 1).

The *COI* data for the asexual *T. douglasi* and its sexual sister *T. poppensis* also revealed that the nominal species *T. douglasi* [13] consists of at least three independently derived asexual lineages, with different geographic distributions. The phylogenetic trees for which we either constrained all *T. douglasi* sequences to be monophyletic, or split them into two independent origins, differed significantly from the best maximum-likelihood (ML) tree in which *T. douglasi* was polyphyletic (Templeton tests, all p < 0.0001; Shimodaira-Hasegawa tests, all p < 0.0001). By contrast, a constrained tree in which we defined three independent monophyletic *T. douglasi* lineages (with individuals from the southern, central, and northern part of the distribution range, respectively) did not differ significantly from the best tree (Templeton test, p = 0.69; Shimodaira-Hasegawa test, p = 0.06). Monophyly of the central and northern *T. douglasi* was also suggested by the best ML tree (Figure 1; Figure S1). Although we did not test for morphological or ecological divergence among independently derived *T. douglasi* lineages, the sampled locations are suggestive of ecological divergence; all northern and central *T. douglasi* were collected on redwood (*Sequoia sempervirens*), whereas the southern *T. douglasi* were almost exclusively found on Douglas fir (*Pseudotsuga menziesii*). Independently of putative ecological divergences among *T. douglasi* lineages, we overall had three asexual lineages of recent origin (*T. shepardi* and "central" and "south" *T. douglasi*), one lineage of intermediate age (*T. douglasi* "north"), and three potentially ancient asexual lineages (*T. genevievae*, *T. tahoe*, and *T. monikensis*) (Figure 1).

Divergence of Nuclear Alleles under Asexuality

Sexual and asexual reproduction are expected to generate different patterns of nuclear allele divergences and tree topologies [14–16] (Figure 2). In a sexually reproducing diploid species, recombination and genetic exchange between individuals should tend to homogenize alleles within a population [14, 17]. Under asexuality, alleles should accumulate mutations independently of each other, a pattern also known as the "Meselson effect" [14–16] (Figure 2). However, neutral allelic divergence cannot always be used as an indicator of ancient asexuality. For example, empirical studies revealed that alleles in asexual *Daphnia* converge due to mitotic recombination and that the rate of conversion may be faster than divergence due to mutation accumulation [18]. Conversely, Meselson effect-like allele divergence patterns may not necessarily indicate ancient asexuality. Most prominently, recent hybridization between sexual species will generate allele divergences that mimic the patterns expected under long-term asexuality, and many asexual lineages are known to derive from interspecific hybrids [3, 19–21]. Similarly, polyploidy and gene duplications could also generate Meselson effect-like patterns, if paralogous loci instead of alleles at a single locus are analyzed.

To develop insights into nuclear allele divergences under asexuality and to test for "ancient asexuality" in *Timema*, we analyzed allelic variation at *Actin* and *Hsp70* in each of the

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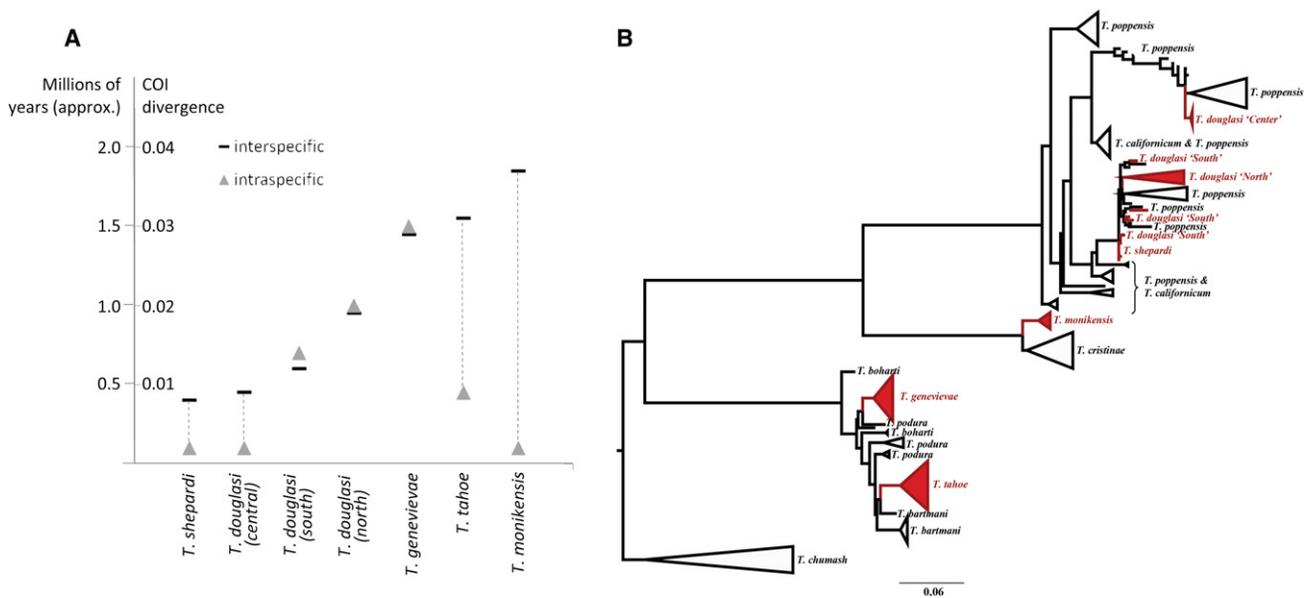
Figure 1. High Levels of Intralineage Mitochondrial Sequence Divergence in Asexual *Timema*

(A) Asexual lineage age estimates; sequence divergences were converted to ages with the approximation of 2% divergence per million years in insect mtDNA [39, 40]. The upper limits (horizontal bars) are based on the divergences (simple p-distances) from their sexual sister species, lower limits (triangles) on intraspecific divergence. The interspecific divergences are based, in each case, on the closest sexual haplotype(s), not the entire sexual sister species. Note that the similar levels of intra- and interspecific divergences in *T. genevievae* and *T. douglasi* (north), in combination with the monophyly of these groups in the best ML tree indicate that their ages are unlikely to be overestimated because of a missing sexual sister population. In contrast, intralineage divergence in *T. douglasi* (south) may be overestimated if this group comprises multiple, independently derived asexual lineages.
(B) Simplified best ML phylogeny; for the complete phylogeny with branch support values, see Figure S1. The sizes of the triangles are proportional to the sequence diversity in a given clade.

seven asexual lineages (for a total of 25 individuals for *Actin* and 20 for *Hsp70*). For comparison, we also analyzed allele divergence patterns in the sexual sister species of the asexual lineages (*T. poppensis*, *T. californicum*, *T. cristinae*, *T. podura*, and *T. bartmani*) as well as two additional sexual species in the genus (*T. boharti* and *T. chumash*; total number of individuals, 28 for *Actin* and 27 for *Hsp70*). The *Actin* and *Hsp70* sequences were intron-free and displayed low levels of variability, with only 1.2% (*Actin*) and 2.9% (*Hsp70*) mean pairwise divergence overall in the genus (GenBank accession numbers HQ198047–HQ198153 and HQ198154–HQ198247). Although both genes are known to typically occur as multiple copies (e.g., [22, 23]), we found only one individual of the sexual species *T. poppensis* with three different *Actin* "alleles." In the remaining 52 individuals (46 for *Hsp70*), we detected only one or two versions of each gene, despite sequencing at least 6 and up to 12 clones per individual (Figure 3).

First, we compared intraindividual allele differences between sexual and asexual *Timema* by using species-level averages. As expected under long-term asexual reproduction, intraindividual allele differences were significantly higher in the asexuals as compared to the sexual species (Wilcoxon rank sum tests; *Actin*, W = 42, p = 0.003; *Hsp70*, W = 35.5, p = 0.045; Figure S2). Consistent with the prediction that old asexuals should have particularly large allelic divergences, one of the putatively ancient asexuals, *T. tahoe*, had exceptionally high divergences for both *Actin* (6–17 differences) and *Hsp70* (8–11 differences) (Figure S2). The two other putatively old asexuals, *T. genevievae* and *T. monikensis,* showed large allelic divergences only for *Actin* and not for *Hsp70* (Figure S2). By using the four gametes test [24], we also did not detect a single recombination event in any of the asexual species.

In contrast, and in spite of the low sequence variability, we found evidence for recombination in three sexual species: *T. cristinae* (at least one recombination event for both *Actin* and *Hsp70*), *T. californicum* (*Actin*, one event), and *T. podura* (*Hsp70,* six events).

The lack of recombination in asexuals can generate high intraindividual allele divergences by allowing for the independent accumulation of mutations in each allele (Figure 2). In addition, the genome-wide lack of recombination should result in less efficient selection against deleterious mutations [25, 26], which could also generate high intraindividual allele divergences in young asexuals. To disentangle the two processes, we also compared intraindividual allele divergences between sexuals and asexuals using only the synonymous (supposedly neutral) mutations in each gene. Even after excluding all nonsynonymous mutations, asexual *Timema* still had significantly higher intraindividual allele divergences than sexual *Timema* for *Actin* (mean ± SD: sexual, 0.74 ± 0.41; asexual, 3.17 ± 2.69, W = 40.5, p = 0.006) though not for *Hsp70* (sexual, 1.71 ± 1.47; asexual, 1.88 ± 1.09, W = 24.5, p = 0.67).

Next, we tested for signatures of asexuality by analyzing the topologies of the nuclear allele phylogenies in the species for which we sequenced the alleles of at least three individuals, which was the case for five asexuals and their five sexual sister species (Table 1). For each of the species, we compared the best ML phylogenies with ML phylogenies in which we constrained alleles sampled from the same population to be monophyletic (the tree topology expected under sexual reproduction and in the absence of recent hybridization between individuals from diverged populations). The results of these comparisons were also broadly consistent with the patterns expected for each reproductive mode, although within the
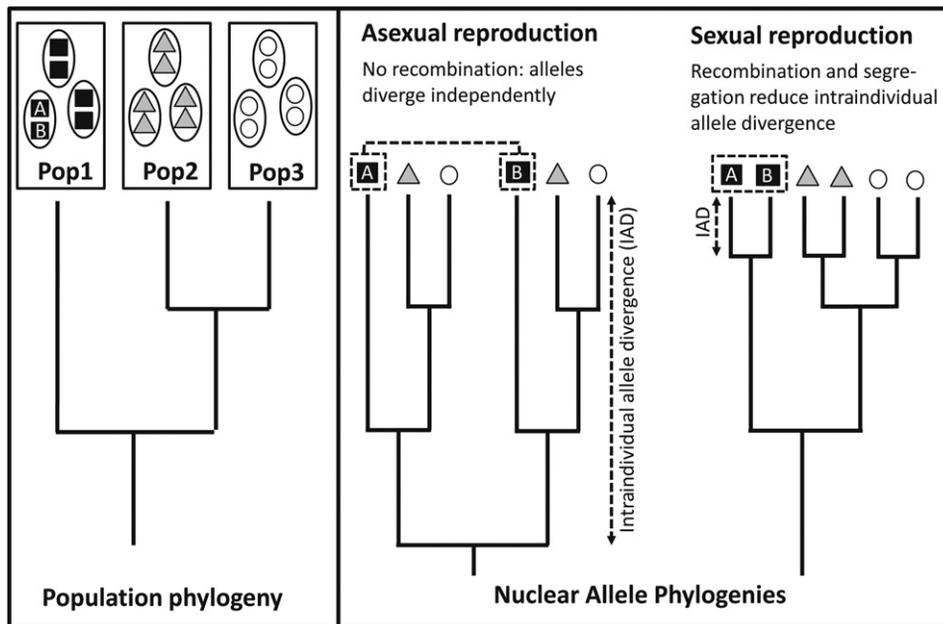
Figure 2. Expected Nuclear Allele Phylogenies under Asexual and Sexual Reproduction

Expected allele phylogenies are indicated for individuals from three differentiated populations (pop1, pop2, pop3). In a sexually reproducing diploid species, population genetic theory predicts that the neutral sequence divergence between two alleles of an individual's gene will be low, with the exact level depending on the effective population size and the mutation rate. In addition, recombination should tend to homogenize alleles within a population and reduce their level of divergence [14, 17]. This process would result in relatively little divergence between alleles of the same individual and allele phylogenies that match the population phylogeny. Under asexuality, the two alleles at a given locus should accumulate mutations independently from each other and evolve as independent lineages [14–16]. Given enough time, this process will result in high levels of intraindividual allele divergence (IAD). In addition, a focal allele should be more closely related to the corresponding allele found in a different individual (possibly from a very distant population) than to its sister allele, a pattern also known as the Meselson effect [14–16], and illustrated by the alleles A and B from a typical individual of population 1.

asexuals there was considerable variation among species and markers. For each of the five sexual species, the best trees for *Actin* and *Hsp70* never differed significantly from the trees in which alleles sampled from the same population were constrained to be monophyletic (Table 1), and for 6 out of the 10 tests, the best and constrained trees were even identical. In contrast, for the three asexuals inferred as old (*T. genevievae*, *T. tahoe,* and *T. monikensis*), the constrained tree was a significantly worse fit to the data than the unconstrained tree for at least one of the two markers (Table 1). In the young asexuals *T. shepardi* and *T. douglasi* south, the topology tests were nonsignificant, suggesting that the loss of sexual reproduction in these lineages may have occurred too recently to generate typical allele divergence patterns.

Nuclear allele phylogenies in an ancient asexual species are also expected to display a split into two independently evolving branches, with the alleles from a given individual appearing in both branches (Figure 2) [14, 16]. This pattern occurred for a subset of individuals in the three old asexuals. Thus, the *Actin* alleles for *T. monikensis* and four of the five *T. tahoe* individuals indeed segregate into two distinct branches (Figure 3A). For *T. genevievae,* the phylogenies are characterized by long branches with no indication of a split into two clades, but the mechanisms underlying this pattern require further investigation. Importantly, however, neither the *Actin* nor the *Hsp70* phylogenies suggested that *Timema* asexuals evolved from historical hybridization between sexual species, corroborating previous evidence [12] for a spontaneous origin of these lineages. The topologies of the allele phylogenies and high intraindividual allele divergences in the

Timema asexuals are thus unlikely to derive from interspecific hybridization.

Overall, the strongest evidence for antiquity, from nuclear allele divergence and phylogenies in conjunction with sequence variation at *COI*, is for the asexual *T. tahoe* (Figure 1, Table 1; Figure S2). For the two other putatively old asexual lineages, the allele phylogenies for only one of the two nuclear markers (*Hsp70* for *T. genevievae* and *Actin* for *T. monikensis*) provided support for ancient asexuality (Table 1). For *T. monikensis*, this inconsistency, in combination with the low level of *COI* variation (Figure 1), may indicate that this lineage represents a recent asexual and that the most closely related populations of the sexual sister species *T. cristinae* are either extinct or have yet to be sampled. In contrast, for *T. genevievae*, the high intralineage *COI* divergence and large differentiation between nuclear alleles (Figure 1; Figures S1 and S2) are still best explained by ancient asexuality. In addition, *T. genevievae*'s karyotype is characterized by chromosomes without homologs [12]. Such karyotypes support asexuality [10, 27] because the absence of meiosis should result in reduced selective constraints on chromosome pairing [28, 29]. The genus *Timema* is thus unique, in comprising at least two, possibly three, independently derived asexual "scandals."

Although studies in several putatively ancient asexuals have tested for the Meselson effect, they either found no evidence for the expected nuclear allele-divergence patterns, or if they did, the patterns could be explained by recent hybridization between sexual species rather than by ancient asexuality. Thus, tests for the signature of ancient asexuality in darwinulid
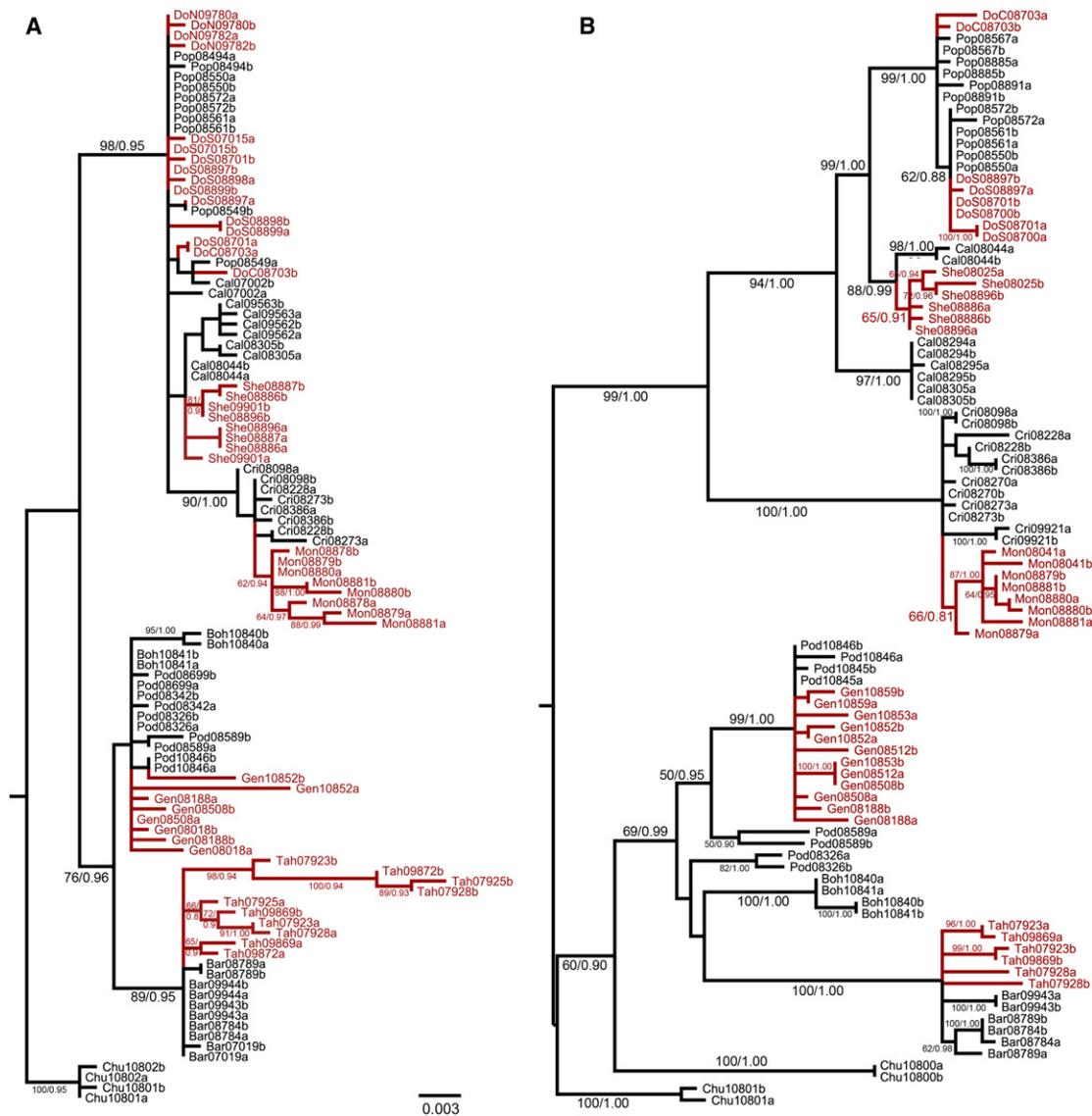
Figure 3. *Actin* and *Hsp70* Allele Phylogenies for Sexual and Asexual *Timema* Species

Shown are (A) *Actin* and (B) *Hsp70* allele phylogenies. Asexual species are indicated in red, numbers associated with branches indicate ML bootstrap support (percent) for 500 replicates and Bayesian posterior probabilities (branches with less than 50% support are not labeled). The first three letters of each label indicate the species, followed by a 5-digit individual code; alleles of the same individual are distinguished by the letters "a" and "b." Sampling locations are given in Table S1. Abbreviations: Bar, *Timema bartmani*; Boh, *T. boharti*; Cal, *T. californicum*; Cri, *T. cristinae*; Chu, *T. chumash*; DoS, *T. douglasi* south; DoC, *T. douglasi* center; DoN, *T. douglasi* north; Gen, *T. genevievae*; Mon, *T. monikensis*; Pod, *T. podura*; Pop, *T. poppensis*; She, *T. shepardi*; Tah, *T. tahoe*. See also Figure S3 for amino acid phylogenies for the three sexual:asexual species pairs comprising the putatively ancient asexuals.

ostracod phylogenies revealed no sequence variation at the investigated nuclear markers, possibly because of recent gene conversion events [30–32]. In *Campeloma* snails [33], *Meloidogyne* root knot nematodes [20], the aphid *Rhopalosiphum padi* [34], and *Lasaea* clams [35, 36], divergent alleles in the asexuals derived from distinct sexual species, so that the apparent Meselson effect was not a consequence of evolution without recombination. Finally, the putatively ancient asexual oribatid mites displayed variability and divergence patterns at nuclear markers as expected for sexual rather than asexual populations [37].

Molecular evidence for long-term asexuality via the Meselson effect is thus known only from the bdelloid rotifers [16].

Recent evidence, however, has demonstrated that the bdelloids probably derived from an initially diploid lineage that underwent whole-genome duplication followed by massive gene loss and karyotype restructuring [38]. Thus, the sequence divergence between duplicated chromosome pairs (i.e., paralogous loci) could be misinterpreted as high allelic divergence and the Meselson effect in these lineages [10, 38].

In the *Timema* asexuals, the allele divergences are unlikely to stem from divergences of paralogous loci after whole-genome duplication, given that the asexuals' karyotypes are very similar to the karyotypes of their respective sexual sister species [12] in which intraindividual allele divergences were consistently low. In addition, none of the asexual individuals

Table 1. Topology Tests for *Actin* and *Hsp70* Allele Phylogenies

			Asexuals						Sexuals			
Gene	Species	N	−ln L Best (Diff)	p Value	Length Best (Diff)	p Value	Species	N	-Ln L Best (Diff)	p Value	Length Best (Diff)	p Value
Actin	*T. shepardi*	4	1257 (24)	0.053	18 (4)	0.103	*T. californicum*	5	1307 (11)	0.098	23 (2)	0.157
	T. douglasi	5	1270 (0)	N/A	19 (0)	N/A	*T. poppensis*	5	1236 (0)	N/A	15 (0)	N/A
	T. genevievae	4	1378 (11)	0.118	34 (2)	0.157	*T. podura*	5	1239 (0)	N/A	15 (0)	N/A
	T. tahoe	5	1404 (69)	**<0.001**	37 (12)	**<0.001**	*T. bartmani*	5	1312 (0)	N/A	12 (0)	N/A
	T. monikensis	4	1335 (30)	**0.046**	29 (5)	0.059	*T. cristinae*	4	1266 (3)	0.150	21 (1)	0.317
Hsp70	*T. shepardi*	3	1774 (6)	0.218	20 (1)	0.317	*T. californicum*	4	1787 (0)	N/A	43 (0)	N/A
	T. douglasi	3	1739 (0)	N/A	39 (0)	N/A	*T. poppensis*	6	1772 (9)	0.085	42 (1)	0.317
	T. genevievae	6	1846 (21)	**0.047**	48 (3)	0.083	*T. podura*	4	1863 (0)	N/A	52 (0)	N/A
	T. tahoe	3	1860 (44)	**0.004**	57 (7)	**0.008**	*T. bartmani*	3	1801 (0)	N/A	47 (0)	N/A
	T. monikensis	4	1815 (16)	0.064	48 (3)	0.083	*T. cristinae*	6	1828 (12)	0.092	50 (2)	0.157

In each case, the best tree (computed by maximum likelihood) was compared to a tree in which alleles from individuals collected at the same locality were constrained to be monophyletic. Test values and associated probabilities refer to the Shimodaira-Hasegawa test with 1000 bootstrap replicates (first test; −ln L and diff are the log likelihood for the best tree and the log likelihood difference for the constrained tree, respectively) and the Templeton signed-rank test (second test; length best and diff indicate the length of the best tree and the number of additional steps required in the constrained tree, respectively). The significance cannot be evaluated in cases where the constrained tree is identical to the best tree (N/A). N, number of individuals analyzed. Bold text indicates significant tests. See Figure S2 for intraindividual allele divergences.

had more than two different alleles at either *Actin* or *Hsp70*. We cannot formally exclude the possibility that the topologies of the allele phylogenies and larger allele divergences in the asexuals than the sexuals are explained by divergence between different gene copies rather than divergences between alleles. However, even if this was the case, the systematically larger intraindividual divergences in the asexuals as compared to the sexuals would still be best explained by the lack of recombination in the asexuals, while concerted evolution would strongly reduce the divergence between the different gene copies in the sexuals. Finally, homologous chromosomes may also diverge in gene copy numbers more easily under asexuality than under sexual reproduction, where recombination should tend to reduce copy number variation between chromosomes. Thus, unless *Timema* asexuals derive from hybrids between different sexual populations that are currently extinct, the best explanation for our results is that *T. tahoe* and *T. genevievae* have reached antiquity in the absence of sexual reproduction and recombination.

The "ancient" *Timema* asexuals, at more than 1,000,000 years old, are still young compared to asexual groups such as the bdelloid rotifers or the darwinulid ostracods, which have apparently persisted for up to a 100,000,000 years [10]. Relative to these very ancient groups, the less extreme divergence between the asexual *Timema* and their sexual sister species may make it easier to identify changes directly associated with the transition to asexuality rather than other adaptive or nonadaptive changes accumulated over extremely long periods. Thus, the allele divergence patterns in *T. tahoe*, *T. genevievae*, and *T. monikensis* may also suggest a new explanation for the complete lack of intraindividual allele divergence found in some asexual groups. For several asexual individuals, the *Actin* and *Hsp70* amino acid sequence of one allele was very similar or even identical to amino acid sequences found in the sexual sister species whereas the second allele comprised many nonsynonymous substitutions (Figure S3). A possible explanation for this pattern is that the first allele to be affected by a nonsynonymous mutation may sometimes tend to accumulate additional such mutations, whereas clone-level selection for a conserved amino acid sequence in the sister allele may become increasingly strong. Thus, in asexual organisms, if only a single copy of a gene is sufficient for its

function and the loss of the other copy does not result in dosage disturbances, parts of the genome may become functionally haploid. Consequently, the complete lack of variation at nuclear alleles in the very old asexual darwinulid ostracods could indicate that these studies have captured only a single allele, the second allele of the locus having changed too much to be picked up by traditional degenerate PCR.

Antiquity of asexuals is in contrast to the idea that asexual lineages are evolutionarily short-lived because of a combination of genetic and ecological factors. Establishing ancient asexuality in *Timema* thus provides exceptional opportunities to elucidate the selective pressures involved in the maintenance of sexual and asexual reproduction and to help resolve the paradox of sex.

Supplemental Information

Acknowledgments

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