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MULTIPLE DIRECT TRANSITIONS FROM SEXUAL REPRODUCTION TO APOMICTIC PARTHENOGENESIS IN *TIMEMA* STICK INSECTS

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Transitions from sexual reproduction to parthenogenesis may occur along multiple evolutionary pathways and involve various cytological mechanisms to produce diploid eggs. Here, we investigate routes to parthenogenesis in *Timema* stick insects, a genus comprising five obligate parthenogens. By combining information from microsatellites and karyotypes with a previously published mitochondrial phylogeny, we show that all five parthenogens likely evolved spontaneously from sexually reproducing species, and that the sexual ancestor of one of the five parthenogens was probably of hybrid origin. The complete maintenance of heterozygosity between generations in the five parthenogens strongly suggests that eggs are produced by apomixis. Virgin females of the sexual species were also able to produce parthenogenetic offspring, but these females produced eggs by automixis. High heterozygosity levels stemming from conserved ancestral alleles in the parthenogens suggest, however, that automixis has not generated the current parthenogenetic *Timema* lineages but that apomixis appeared abruptly in several sexual species. A direct transition from sexual reproduction to (at least functional) apomixis results in a relatively high level of allelic diversity and high efficiency for parthenogenesis. Because both of these traits should positively affect the demographic success of asexual lineages, spontaneous apomixis may have contributed to the origin and maintenance of asexuality in *Timema*.

KEY WORDS: **Asexual reproduction, hybridization, stick insect, tychoparthenogenesis.**

The maintenance of sex is paradoxical because asexual lineages can, in principle, reproduce at twice the rate of sexual lineages and asexual females transmit twice as many genes to each offspring as compared to sexually reproducing females (Williams 1975; Maynard Smith 1978; Bell 1982). Yet sex is the predominant mode of reproduction in nearly all multicellular taxa. A large body of theory seeks to explain the rarity of asexually reproducing lineages, given their theoretical reproductive advantage (Williams 1975; Maynard Smith 1978; Bell 1982; Hamilton et al. 1990; Kondrashov 1993; Otto and Gerstein 2006). Most hypotheses explain the advantage of sex and recombination as an effect of genetic variance reshuffling, that accelerates the production of advantageous new genotypes and facilitates adaptation, limits the accumulation of deleterious mutations, or provides both forms of long-term benefit (e.g., Bell 1982; Kondrashov 1993; Lynch et al. 1993; Hurst and Peck 1996; West et al. 1999). It is typically assumed that most asexual lineages, even if initially successful, suffer early extinction (White 1973; Bell 1982). Under this view, both reproductive modes persist in the long term as a result of a dynamic equilibrium between the origin of new asexual lineages and their extinction (Simon et al. 2003).

There are at least three ways in which parthenogenetic lineages may arise from sexual species (Innes and Hebert 1988; Stouthammer 1993; Vrijenhoek 1998; Simon et al. 2003) and the mode of origin of parthenogens has important consequences for their level of genetic diversity, ecological adaptability, and the outcome of competition with their sexual relatives (Bell 1982). First, a spontaneous transition to asexuality may occur through

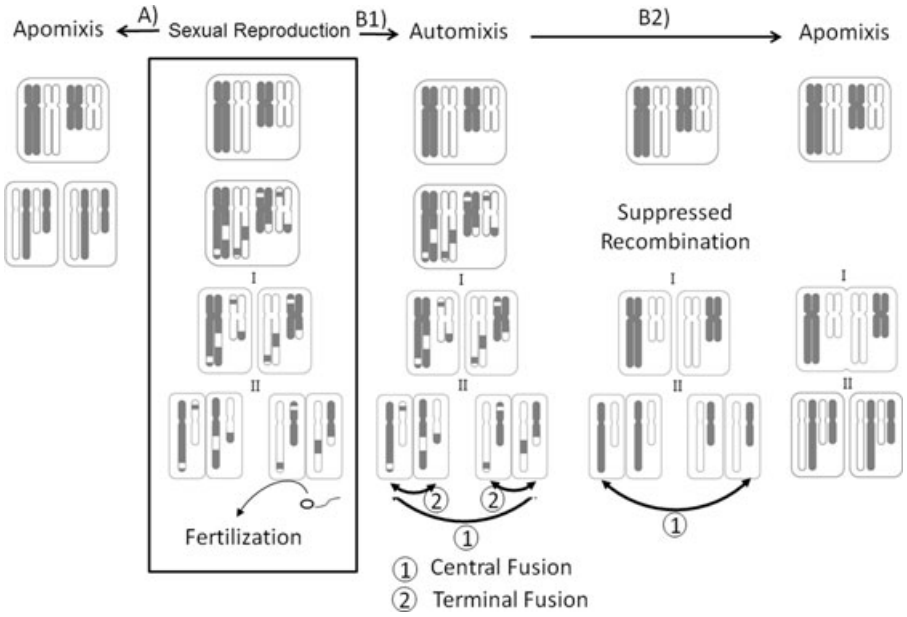

Figure 1. **Possible transitions to apomixis. Route (A): Direct transition from sexual reproduction involving major cytological changes for oogenesis (White 1964, 1973). Route (B): stepwise transition via automictic parthenogenesis (B1): with recombination suppression and an increase of the relative proportion of oocytes produced by central as compared to terminal fusion and suppression of the meiotic division I (B2): as a final step. I, first meiotic division; II, second meiotic division.**

mutations in genes underlying the production of sexual forms (Simon et al. 2003) or in genes affecting mating behavior and successful egg fertilization (Carson et al. 1982). Such mutations could directly result in obligate asexuality or be initially maintained as genetic variation for facultative parthenogenesis in the sexual population. The smallest step from sexual to asexual reproduction (i.e., "apomixis") is presumed to involve the preservation of meiotic oocyte production (White 1964, 1973; Bell 1982) whereby the diploidy of the zygote is restored secondarily by either fusion or duplication of haploid meiotic products (i.e., "automictic parthenogenesis," White 1973; Bell 1982; Suomalainen et al. 1987, Fig. 1).

Second, parthenogenesis may be induced through infection by microorganisms, the best known being *Wolbachia* (Stouthamer et al. 1993; Werren 1997), a member of the proteobacteria, and *Cardinium* (Zchori-Fein et al. 2001; Weeks et al. 2003; Zchori-Fein and Perlman 2004; Provencher et al. 2005) a member of *Bacteroidetes*-group. Parthenogenesis-inducing *Wolbachia* are known in species with haplo–diploid sex determination, where the presence of *Wolbachia* causes diploidization of the unfertilized haploid eggs, which develop as females. This process usually occurs through different forms of chromosome duplication in the early stages of meiosis and results in the production of fully homozygous progeny (Suomalainen et al. 1987; Stouthamer and Kazmer 1994; Pannebakker et al. 2004).

Third, many described asexual lineages, and notably all vertebrate asexuals, are of hybrid origin (Bell 1982; Lynch 1984; Dawley and Bogart 1989; Bullini and Nascetti 1990; Avise et al. 1992; Bullini 1994). Because different species frequently display different chromosomal rearrangements, chromosome pairing during meiosis is typically inefficient in interspecific hybrids, which results in low offspring viability (e.g., White 1973; Barton and Gale 1993; Rieseberg 1997). Interspecific hybrids are also often polyploid, which can result in similar problems during meiosis (White 1973; Barton and Gale 1993; Rieseberg 1997). As a consequence, interspecific hybridization creates opportunities for the selection of cytological processes that rescue viable egg production, such as egg production through apomictic parthenogenesis.

The mechanism whereby asexual lineages originate can be inferred by analyzing strictly maternally inherited genetic markers located on the mitochondrial genome in conjunction with nuclear, biparentally inherited genetic markers (Simon et al. 2003). Under spontaneous and infectious origins of asexuality, the most likely sexual ancestor inferred from maternally inherited and nuclear markers should correspond perfectly. Indeed, because genetic exchange does not occur between asexual individuals, nuclear and mitochondrial genomes are inherited as one unit. An association of endosymbiotic strains with parthenogenesis would further support the idea of an infectious origin. In contrast, if unisexual lineages result from hybridization between sexual relatives, the origins inferred from nuclear and mitochondrial genetic markers should be incongruent. Karyotypes, if they vary across species, can give additional information on potential parental species and reveal cases of auto- and allopolyploidy (White 1973).

The aim of the present study is to investigate possible routes to parthenogenesis in *Timema* stick insects. *Timema* is a small genus of wingless, plant-feeding insects which mostly inhabit chaparral vegetation (Vickery 1993; Sandoval et al. 1998), and the group is considered the sister group to other phasmids (Terry and Whiting 2005; Zompro 2005). This genus is particularly interesting for investigating the origin of parthenogenesis, because it comprises a notably large proportion of obligate parthenogens (five out of the 21 described species; Sandoval et al. 1998; Vickery and Sandoval 1999, 2001) and because three of the five parthenogens are among the rare candidates for "old" (> 1 MYA) asexuality (Law and Crespi 2002b; Normark et al. 2003). A phylogeny of the genus based on a 416-bp portion of the mitochondrial gene COI (Law and Crespi 2002a) revealed that at least four of the five all-female lineages represent independent transitions to obligate parthenogenesis, but the use of a single genetic marker did not allow addressing potential routes to parthenogenesis. We infer the origin of each parthenogen by determining the karyotype of the majority of the species in the genus and by combining the COI phylogeny with microsatellite genotyping at newly developed markers.

To further investigate whether the evolution of new parthenogenetic lineages may be facilitated in the genus *Timema* because of an existing faculty for parthenogenetic offspring production among females of the sexual species, we determined the extent of spontaneous parthenogenesis among several sexual species in the genus. We inferred the mechanism of parthenogenetic egg production occurring in normally sexual and asexual females to investigate whether facultative and obligate parthenogenesis proceed along the same cytological mechanisms and to investigate whether obligate asexuality evolves from spontaneous automictic parthenogenesis. This was achieved by genotyping offspring produced by virgin females at multiple microsatellite loci and analyzing the segregation of maternal alleles among offspring.

Materials and Methods

SAMPLING

To distinguish between a spontaneous and hybrid origin for each of the five parthenogenetic lineages (*T. douglasi, T. shepardi, T. monikensis, T. tahoe,* and *T. genevievae*), we compared its most likely sexual ancestor in the COI phylogeny from Law and Crespi (2002a) to the most likely sexual ancestor inferred from nuclear allele frequency distributions and karyotype structures. Although the COI phylogeny revealed four independent transitions to parthenogenesis in *Timema*, the molecular evidence alone allowed identifying a maternal, sexual ancestor for only one of the five parthenogens (*T. monikensis*; sexual ancestor *T. cristinae*). Two additional parthenogens (*T. tahoe* and *T. genevievae*) formed a monophyletic clade together with two sexual species (*T. bartmani* and *T. podura*). In this case, *T. bartmani* was inferred to be the sexual ancestor for *T. tahoe*, because the COI sequence divergence was relatively smaller between *T. tahoe* and *T. bartmani* than between *T. tahoe* and *T. podura* (2.93 $\pm$ 0.36% and 3.89 $\pm$ 0.82%, respectively) and because both, *T. tahoe* and *T. bartmani* occur on white fir (*Abies concolor*), a host plant not used by either *T. podura* or *T. genevieve* (or any other species in the genus, see also Table 1). *Timema tahoe* females also closely resemble *T. bartmani* and are morphologically very distinct from *T. podura* females. Similarly, *T. podura* was inferred to be the sexual ancestor for *T. genevievae*, because of sequence divergence levels (*T. genevievae– T. podura*: 4.53 $\pm$ 0.91%; *T. genevievae: T. bartmani*: 4.79 $\pm$ 0.73%), female morphology, and used host plants (*T. genevievae* only occurs on *Adenostoma*, a host plant also used by *T. podura*, but not by *T. bartmani* or *T. tahoe*, Table 1). The two remaining parthenogens, *T. douglasi* and *T. shepardi* were both very closely related to the sexual species *T. poppensis* (Table 1), with which they even shared several haplotypes. However, the resolution of the phylogeny did not provide statistical support for any species relationships within the clade comprising *T. douglasi*, *T. shepardi, T. poppensis,* and four additional sexual species (*T. californicum, T. petita, T. knulli,* and *T. landelsensis*).

We determined allele frequencies and karyotypes for all five obligately parthenogenetic *Timema* lineages, and nine of the 10 sexual species that we considered to be plausible parental species for one or several parthenogens. The six remaining sexual species of the genus are very unlikely parents for any of the parthenogens because they are genetically highly divergent (estimated age of the split within the genus: 20 MYA; Sandoval et al. 1998) and because most of these species use host plants not used by any of the parthenogens (Vickery and Sandoval 1999, 2001).Thus, in addition to the four most likely maternal ancestors inferred from the COI phylogeny (*T. cristinae, T. bartmani, T. podura*, and *T. poppensis*), we included *T. californicum, T. petita, T. knulli,* and *T. landelsensis*, because these four species were closely related to *T. shepardi* and *T. douglasi* in the COI phylogeny and because they occur in close geographic proximity with the parthenogens (Fig. 2). Each of these species also shares one or several host plants with at least one of the parthenogens (Table 1). Special attention was given to *T. californicum*, because the parthenogen *T. shepardi* is morphologically more similar to *T. californicum* than to its closest relative in the COI phylogeny, *T. poppensis* (Vickery and Sandoval 1999; 2001; Law and Crespi 2002a). Finally, we also included *T. chumash*, because this species occurs on both host plants used by the parthenogen *T. monikensis* and because the taxonomic description of *T. monikensis* mentioned behavioral and morphological similarities with *T. chumash* (Vickery and Sandoval 1998). The 10th candidate species, *T. boharti*, was not included because no samples could be obtained. For each of the nine sexual and five asexual species, 5–20 individuals were

Table 1. **Species included in the study and the most likely maternal ancestor for each parthenogen inferred from the COI phylogeny, with the estimated age for the transition (Law and Crespi 2002a,b). Parthenogenetic species names are in bold. Clade refers to the distinction of three major groups of related species in the COI phylogeny (N, Northern clade; SB, Santa Barbara clade; S, Southern clade). Chromosome numbers refer to female karyotypes. Host plants are indicated in brackets after species names: D, Douglas fir (*Pseudotsuga menziesii*); R, Redwood (*Sequoia sempervirens*); M, Manzanita (*Arctostaphylos* spp.); CC, *Cercocarpus* spp.; C, *Ceanothus* spp.; A, *Adenostoma fasiculatum*; W, White Fir (*Abies concolor*); O, Oak (*Quercus* spp.); T, Toyon *(Heteromeles arbutifolia).***

Clade	Karyotype	Analyzed species	Closest sexual relative (for parthenogens only)	Sequence divergence to closest sexual relative	Estimated age of parthenogen [MYA]
N	2*n*=24	***T. douglasi*** (D)	*T. poppensis* (D, R)	1.03±0.63%	<0.5
N	2*n*=24	***T. shepardi*** (M)	*T. poppensis* (D, R)	0.98±0.64%	<0.5
N	2*n*=24	*T. poppensis* (D, R)			
N	2*n*=24	*T. knulli* (R, C)			
N	2*n*=24	*T. landelsensis* (M)			
N	2*n*=24	*T. petita* (C)			
N	2*n*=24	*T. californicum* (M, C, O, CC, T)			
SB	2*n*=26	***T. monikensis*** (CC, C)	*T. cristinae* (C, A, T)	3.89±0.59%	0.3–1.9
SB	2*n*=26	*T. cristinae* (C, A, T)			
S	2*n*=28	***T. tahoe*** (W)	*T. bartmani* (W)	2.93±0.36%	0.3–1.5
S	2*n*=27 or 21	***T. genevievae*** (A)	*T. podura* (A, C, O, CC)	4.53±0.91%	1.0–2.2
S	2*n*=28	*T. bartmani* (W)			
S	2*n*=28	*T. podura* (A, C, O, CC)			
S	2*n*=22, 24 or 26	*T. chumash* (C, O, CC)			

collected from each of two to five localities (see Appendix 1). Most populations were documented in the Law and Crespi (2002a) study, and additional populations were sampled for species with only one or two populations included in the previous study: *T. tahoe* (one additional population) and *T. monikensis* (three populations) and the sexuals *T. petita* (one population) and *T. knulli* (two populations). For *T. knulli* the two additional populations comprised one on the *Ceanothus* host plant and one on the *Sequoia sempervirens* host plant, because the study by Law and Crespi (2002a) showed that the *T. knulli* populations on the two host plants were relatively genetically divergent (4.13 ± 0.34% sequence divergence). Although obligate parthenogenetic reproduction in *T. monikensis* (and the four other all-female *Timema* lineages) was confirmed by laboratory experiments, a small proportion of males of unknown function and viability have been occasionally found in *T. monikensis* populations (Vickery and Sandoval 1998, 2001). Because these males have never been studied in detail, we included collected males in our genetic and karyotypic analyses.

KARYOTYPING

We determined the karyotypes for a subset (5–12) of the collected individuals of each species, including individuals from different populations whenever possible. To identify potential sex chromosomes and sex determination mechanism, karyotypes were determined separately for males and females in the nine sexual species. Karyotypes were identified from cells in metaphase in testes and ovarioles. Adult anesthetized animals were injected with 0.1 mL of a 0.05% colchicine solution 1 h before dissecting the gonads. The tissues were then processed through a modified Crozier (1968) technique. The samples were treated with a hypotonic (1%) solution of Na-citrate for 20 min, and fixed in ethanol: acetic acid (3:1), for 30 min at room temperature. Small tissue pieces were transferred into a few drops of 60% acetic acid on a slide and macerated to ensure a maximum spread of the cells. The slides were air dried and stained with a 2% Giemsa solution in 0. I M phosphate buffer, at pH 7.0, for 20 min.

MICROSATELLITE GENOTYPING AND ANALYSES

To infer the most likely sexual ancestor based on nuclear genetic markers, we optimized nine microsatellites for interspecific comparisons. The loci were isolated from tri- and tetranucleotide microsatellite-enriched genomic libraries developed for *T. cristinae* by Genetic Identification Services (GIS, http://www.genetic-id-services.com/). Twenty micrograms of DNA were extracted from *T. cristinae* legs using a standard phenol–chloroform protocol, precipitated and brought up to 100 μL of 10 mm Tris, pH 8.0, for shipping to GIS. GIS provided four libraries, created using biotin- $(AAT)_{15,}$ $(ATG)_{15,}$ $(TACA)_{15}$ and $(TAGA)_{15}$ as capture molecules. Primer sets were designed for 27 microsatellite-containing sequences, using the software Primer3, ver. 0.4.0 (Rozen and Skaletsky 2000). Eight individuals (belonging to six

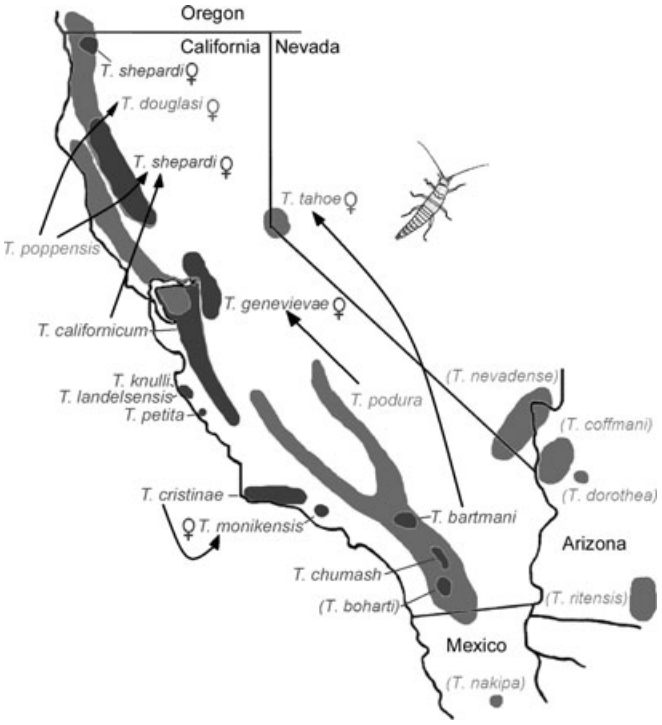

Figure 2. Distributions for 20 of the described *Timema* species (figure adapted from Law and Crespi 2002a). The light and dark shadings are only used to distinguish species ranges. Parthenogenetic lineages are labeled with a female sign and arrows indicate sexual ancestor(s) for each parthenogen. Species in brackets are not included in the present study. The full distribution of the recently described and not indicated *T. morongensis,* (Vickery and Sandoval 2001) is not known.

different species) were used for a preliminary test of amplification success and polymorphism following optimization of PCR conditions for each locus. Among the 27 primer sets, six did not produce an amplified product in all six tested species and 12 were not scorable (i.e., gave no specific products). The nine remaining microsatellites (Tim1–Tim9, Table 2) produced repeatable allele sizes in all eight individuals although depending on the species, considerable numbers of weaker, nonrepeatable products coamplified along with the microsatellite. One marker (Tim9) amplified three sets of specific alleles in each individual with 200-bp intervals between sets. We included the smallest set in each species. Excluding this marker for statistical analyses did not qualitatively affect our results.

PCR amplification of microsatellites was conducted on a Veriti Thermal Cycler (Applied Biosystems, Foster City, CA) in a 10 μL reaction volume containing approximately 10–20 ng genomic DNA, 1× PCR buffer (Invitrogen, Carlsbad, CA), 20 mM $MgCl_2$, 3 μM of each primer (Table 2), 1.5 mM dNTP, and 1.0 U *Taq* DNA polymerase. PCR conditions were 95°C for 5 min, 35 cycles consisting of 94°C for 45 sec, a primer-specific annealing temperature (Table 2) for 45 sec, 72°C for 45 sec, with a final extension at 72°C for 7–10 min. Fragment analysis was carried out on a Li-Cor 4300 DNA Analyzer (Li-Cor Biosciences, Lincoln, NE) and the alleles generated were sized relative to molecular weight marker IRDye700 and 800 (Li-Cor, Biosciences), using the Gene ImagIR software (version 4.05, Scanalytics, Inc, Rockville, MD).

Because our aim was to infer the sexual ancestor of each parthenogenetic lineage (i.e., assign "populations" of parthenogens to the sexual species) rather than perform a population genetic analysis, we pooled individuals from all sexual populations within a species to compute representative species-level allele frequencies at the nine microsatellite loci. *Timema* taxonomy relies on interspecific variation in male genitalia (Vickery 1993; Vickery and Sandoval 2001), and all individual males from a given sampling location were examined to verify their species identity. Treating sexual populations individually did not qualitatively affect our results, but resulted in overall low power for assignments because only a moderate number of individuals were genotyped in each population. We then used three different methods to infer the most likely sexual ancestor for each parthenogenetic population. First, we used pairwise genetic distances (Cavalli-Sforza chord distance, Cavalli-Sforza and Edwards 1967) and neighbor-joining methods implemented in the program Populations (Langella 1999) to assess possible associations of asexual populations with specific sexual species.

Table 2. PCR temperatures and forward (F) and reverse (R) primer sequences (5′-3′) for microsatellite markers Tim1–Tim9.

Locus	Forward primer	Reverse primer	Repeat	Th [°C]	GenBank no.
Tim1	CCCCCTTCATGTAACACC	CGCGTTTGTATGTGCAGT	ATT	57	EU860204
Tim2	ATTCCACGTTAAACTGTAGGTC	GCGTCATAGTAAGTGTTTCATG	TAA	57	EU860205
Tim3	CCGAACGGGTAGTAAGACG	TCCTCATAAGACCACACACTTG	TAA	57	EU860206
Tim4	CGTCAAGTCATGGGATCTG	AAATGCGACCAAAATTGG	TAA	55	EU860207
Tim5	TTAGGTGCCCAACTACGAG	CCTCCATTTCATCTTACAATCA	ATT	57	EU860208
Tim6	TGGGGGTAGTTTTTCTTAGATG	TCCTCCTATCAGAGCGTTATG	TAA	52	EU860209
Tim7	TTAGCGTAAATGCGAGTAGC	CGTAGCCAAATAAACATCAGA	TAA	55	EU860210
Tim8	TTTTGGAGCGACACATTTG	CCGAACGGGTAGTAAGACG	ATT	55	EU860211
Tim9	GTAGGTCCTTCATTTCATCTC	GGTAGTTGTCACCATCACTC	TAA	60	EU860212

Second, we used Bayesian methods implemented in the population assignment software Structure 2.1 (Pritchard et al. 2000) to compute the relative assignment probabilities of each asexual female to each of the nine sexual species. For these analyses, the multilocus genotypes of the nine sexual species were used to develop genetic profiles for each species. All parthenogenetic females were then analyzed as unknowns under an admixture ancestry model. Under this model, each individual is assigned a relative probability of belonging to each of the nine sexual species, with all probabilities summing to 1. Burn-in and run lengths were set to 50,000; increasing or decreasing these values by 20,000 did not alter any of the population assignments.

Finally, we pooled all populations of a given parthenogen and computed an allele-sharing index between each asexual and all sexual species. This index simply represents the per-locus proportion of alleles found in an asexual lineage that also occur in a given sexual species, averaged over loci. The most likely sexual ancestor of an asexual lineage would be characterized by a relatively high allele-sharing index.

Because two parthenogens, *T. shepardi* and *T. douglasi*, were both closely related to *T. poppensis* (Table 1) but not reciprocally monophyletic in the COI phylogeny (Law and Crespi 2002a,b) it was not possible to infer, using the COI sequences, whether the two asexual lineages represented two independent origins of asexuality from *T. poppensis* or whether *T. shepardi* derived from *T. douglasi* (Law and Crespi 2002a,b). To distinguish between the two alternatives, we quantified, using the microsatellite markers, the relative contributions of *T. poppensis* and *T. douglasi* to *T. shepardi*. To this end, we computed admixture proportions of *T. shepardi* using the maximum-likelihood methods implemented in LEADMIX2 (Wang 2003) with 500 integration points for the likelihood function and accounting for differentiation between parental populations. If *T. poppensis* was a more likely parental population for *T. shepardi* than *T. douglasi*, then we expected to find a significantly larger contribution of *T. poppensis* than *T. douglasi* alleles to the *T. shepardi* lineage. In contrast, the admixture proportion for *T. douglasi* should be significantly higher than the proportion for *T. poppensis* if *T. shepardi* derived from *T. douglasi* rather than from *T. poppensis*. The relative contributions of *T. poppensis* and *T. douglasi* were evaluated by comparing their 95% confidence intervals.

ANALYSES OF PARTHENOGENETICALLY PRODUCED OFFSPRING

To assess the presence of spontaneous parthenogenesis in the nine sexually reproducing species, we collected 22 to 75 juvenile females for each of eight sexual species and raised them individually in petri dishes on *Ceanothus* cuttings. For the ninth sexual species, *T. podura*, only two juvenile females could be obtained. After reaching maturity, the females were maintained for 6–16 days, resulting in 22–84 eggs per female (7703 eggs in total). Eggs were kept at room temperature until hatching. We determined the proportion of females of each species that were able to produce at least one parthenogenetic offspring.

To gain insight into the mechanism of parthenogenesis used by sexual and asexual females, we genotyped all offspring produced by 25 virgin sexual females ($n_{tot} = 96$ offspring; including offspring from at least one female of each of the nine sexual species) and five offspring of 10 females of each asexual species at microsatellite markers ($n_{tot} = 250$ offspring). The offspring were genotyped at the three to five microsatellite markers for which their mother was heterozygous so that we could identify heterozygosity changes between generations. An absence of heterozygosity changes between generations would be consistent with apomictic egg production whereas a decrease in heterozygosity levels from the females to their offspring would be consistent with automictic egg production.

Results

The chromosome counts revealed XX (female): XO (male) sex determination in all nine analyzed sexually reproducing species. Whatever the number of chromosomes, males had one chromosome fewer than females, and the chromosome with a single copy in males and two copies in females (i.e., the "sex chromosome") was always relatively large and acrocentric (Fig. 3).

We found no evidence for polyploidy in the asexual lineages, or any of the sexual species. Based on the chromosome counts, we identified three groups of species (comprising 12 out of the 14 analyzed species) matching the three divergent clades in the COI phylogeny from Law and Crespi (2002a) (Table 1) with apparent intraspecific karyotype variability in the two remaining species.

The first species group was characterized by $2n = 23$ (males) and $2n = 24$ (females) chromosomes and comprised five of the nine analyzed sexual species (*T. poppensis, T. knulli, T. californicum, T. petita, and T. landelsensis*) and two of the five parthenogens (*T. douglasi and T. shepardi*) (Table 1, Fig. 3A,B). The female karyotype was characterized by one pair of large (~7 μm) submetacentric chromosomes, two pairs of smaller (~5 μm and ~4 μm) metacentric chromosomes and nine pairs of small (~2–3 μm), mostly acrocentric chromosomes (Fig. 3B).

The second species group was characterized by $2n = 25$ (males) and $2n = 26$ (females) chromosomes and comprised the sexual:asexual species pair *T. cristinae* and *T. monikensis* (Table 1, Fig. 3C,D). *Timema cristinae* females had three pairs of large (6–7 μm) telocentric chromosomes, one pair of smaller submetacentric chromosomes (~3–4 μm), and nine pairs of small telocentric chromosomes (~2–3 μm). The karyotype structure of *T. monikensis* was very similar to that of *T. cristinae*, but the centromere was

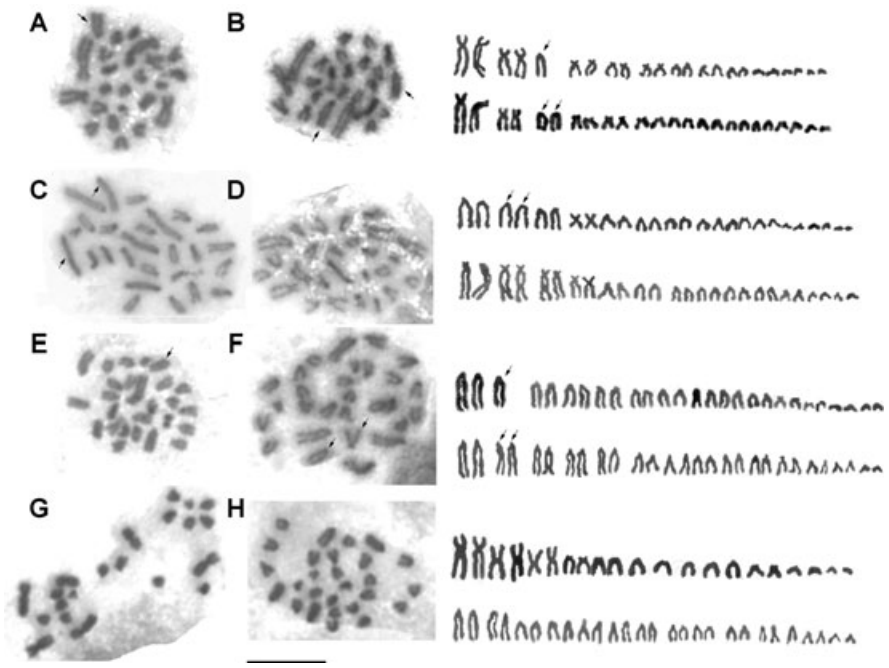

Figure 3. Phase contrast chromosome spread pictures (1000×) and karyotypes of *T. knulli* male (A) and female (B), *T. cristinae* female (C), *T. monikensis* (D), *T. podura* male (E), and female (F), *T. genevievae* from locality "Puerto" (G) and "H20" (H) from Appendix 1. Arrows indicate sex chromosomes. The scale bar is 10 μm.

in a subterminal instead of terminal position in several chromosomes (Fig. 3D).

The third group was characterized by $2n = 27$ (males) and $2n = 28$ chromosomes (females) and comprised the sexual:asexual species pair *T. bartmani* and *T. tahoe* as well as the sexual species *T. podura.* All chromosomes in this group were telo- or subtelocentric and very similar among the three species. The two largest chromosome pairs were ~5 and 4 μm, and the remaining 12 chromosome pairs ~2–3 μm long.

Finally, the two remaining species (*T. chumash* and the asexual *T. genevievae*) displayed apparent within-species polymorphism for chromosome number and morphology. *Timema chumash* had either $2n = 22$, 24, or 26 chromosomes ($2n = 21$, 23, or 25 in males). The number of individuals analyzed per population (and number of distinct morphs—see Vickery 1993; Sandoval 1994) was too small to characterize groups with identical karyotypes. *Timema genevievae* females had 27 chromosomes in the population "Puerto" (Fig. 3G, Appendix 1) and 21 chromosomes in the population "H20" (Fig. 3G, Appendix 1) as compared to 28 chromosomes in its putative maternal ancestor *T. podura*.

Four of the five parthenogenetic lineages (*T. douglasi*, *T. monikensis*, *T. tahoe*, and *T. genevievae*) most likely represent spontaneous (nonhybrid) transitions to parthenogenesis as revealed by the sexual ancestor inferred from nine microsatellite markers being the same as the most likely sexual ancestor inferred from the mitochondrial marker. All three methods used to infer the sexual ancestor of each parthenogen from microsatellite markers indicated that the most probable parent for *T. douglasi* was *T. poppensis*, *T. cristinae* for *T. monikensis*, *T. bartmani* for *T. tahoe*, and *T. podura* for *T. genevievae* (Table 3, Fig. 4, Appendix 2). In each case, the relative assignment probabilities of the parthenogen to the nine sexual species determined by Structure 2.1 were strongly biased in favor of one sexual species (0.68 ± 0.17, range: 0.48–0.91) and overall low for the others (0.04 ± 0.04, range: 0.01–0.21; Table 2). The majority (0.70–0.89) of microsatellite alleles sampled in each asexual also occurred in its sexual counterpart (Table 3) whereby the proportion of shared alleles was significantly greater than expected by chance (tested using the B-index of skew; Nonacs 2003; *P*-values 0.01–0.05). For comparison, the average proportion of alleles sampled in a sexual species that also occurred in other sexual species of the same mtDNA clade was only 0.34 ± 0.17 (Appendix 2). The association of three parthenogens with their sexual counterpart was highly supported by bootstrap values in the NJ tree (over loci: 88–100%; over individuals: 90–100%, Fig. 4). For the fourth and apparently oldest asexual (*T. genevievae*, Table 1), the association was only supported by bootstraps over individuals (73%), not loci (47%) (Fig. 4). Altogether, our results from the microsatellite allele frequencies also confirm the sexual ancestors inferred with limited statistical support in the COI phylogeny by Law and Crespi (2002a).

For the fifth asexual lineage, *T. shepardi*, the nuclear parent inferred from assignment probabilities, allele sharing, and genetic distance clustering was *T. californicum* (Fig. 4, Table 3), which

Table 3. The most likely sexual ancestor for each parthenogen inferred from microsatellite markers. Chromosome numbers are also indicated (numbers refer to female karyotypes). Assignment probabilities and allele sharing proportions are indicated for each inferred sexual ancestor and the eight remaining sexual species.

Parthenogen (karyotype)	Inferred sexual ancestor	Bayesian assignment probabilities		% Alleles shared: Allele sharing	
		Most likely	Other species	Most likely	Other species
T. douglasi (2*n*=24)	*T. poppensis* (2*n*=24)	0.90 (0.79–0.93)	0.01 (0.01–0.08)	0.87±0.07	0.36±0.14
T. shepardi (2*n*=24)	*T. californicum* (2*n*=24)	0.62 (0.48–0.87)	0.05 (0.01–0.30)	0.70±0.11	0.41±0.09
T. monikensis (2*n*=26)	*T. cristinae* (2*n*=26)	0.73 (0.61–0.84)	0.03 (0.01–0.18)	0.65±0.14	0.19±0.10
T. tahoe (2*n*=28)	*T. bartmani* (2*n*=28)	0.65 (0.59–0.70)	0.04 (0.01–0.16)	0.76±0.12	0.37±0.15
T. genevievae (2*n*=21, 27)	*T. podura* (2*n*=28)	0.62 (0.47–0.78)	0.05 (0.01–0.41)	0.69±0.11	0.35±0.15

does not correspond to either of the possible maternal parents in the COI phylogeny, *T. poppensis* or *T. douglasi* (Table 1). The admixture proportions estimated with LEADMIX2 suggested that *T. poppensis* is much more likely to be the maternal ancestor for *T. shepardi* than is *T. douglasi*. Indeed, a nuclear gene contribution of *T. douglasi* (0.0002; 95% CI: 0.000, 0.082) to *T. shepardi* was not supported and was significantly smaller than the contribution estimated for *T. poppensis* (0.998, 95% CI: 0.918, 1.000) as indicated by nonoverlapping 95% confidence intervals. The discordance between the nuclear and mitochondrial parents for *T. shepardi* is compatible with three processes: (1) a hybrid origin of asexuality in *T. shepardi*, (2) a spontaneous transition from a sexually reproducing *T. poppensis–T. californicum* hybrid species, or (3), a spontaneous origin from *T. poppensis* followed by secondary introgression mediated by *T. californicum* males mating with facultatively parthenogenetic females (see Fig. 5 for illustration). As expected with gene flow or reticulate speciation, there was less support for the association of *T. shepardi* with *T. californicum* than for the other sexual:asexual species pairs (Fig. 4, Table 2). Taken together these results suggest that *T. shepardi* and *T. douglasi* represent independent transitions to asexuality from the same maternal ancestor (*T. poppensis*) and that gene flow from a second species (*T. californicum*) was involved in the evolution of *T. shepardi*.

To further estimate the relative contribution of the two parents *T. californicum* and *T. poppensis* to the nuclear genome of

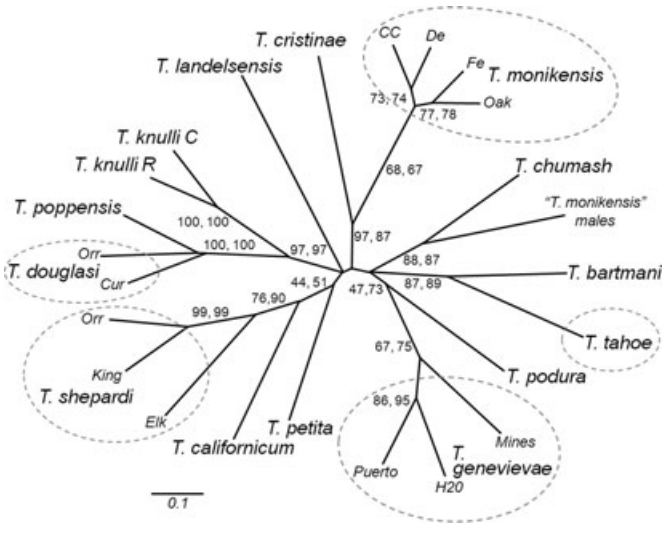

Figure 4. NJ tree of Cavalli-Sforza genetic distance (Cavalli-Sforza and Edwards 1967) based on nine microsatellite markers. Numbers indicate bootstrap values over loci and individuals. Circles group different locations of a given parthenogen. Populations of the sexual species were pooled for the analysis.

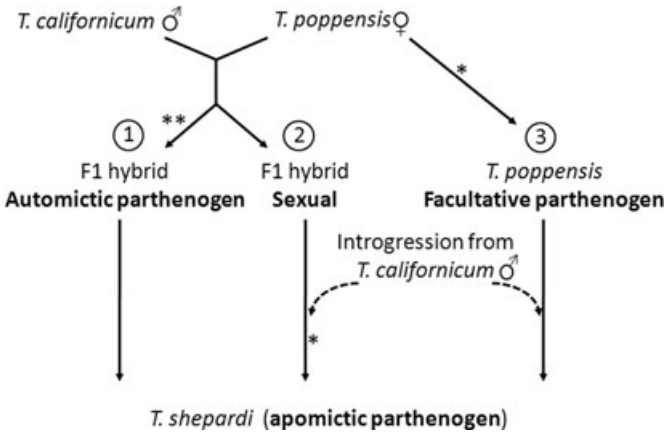

Figure 5. Possible transitions to parthenogenesis in *T. shepardi*. In pathway (1), parthenogenesis is automatic, because a direct transition from sexual reproduction in the parental species to apomixis in F1 hybrids should result in relatively equal contributions of *T. poppensis* and *T. californicum* to the *T. shepardi* nuclear genome. An equal contribution of the two sexuals to *T. shepardi* was not supported by the microsatellite allele frequencies (see text for details). In pathway (2) apomixis appeared spontaneously in a sexually reproducing hybrid species. This is the most likely origin for *T. shepardi* as suggested by the microsatellite analyses. In pathway (3) *T. shepardi* would derive from facultatively parthenogenetic *T. poppensis* females mating with *T. californicum* males (parthenogenesis in *T. poppensis* could be either apo- or automictic). **hybrid origin, *spontaneous origin of asexuality.

T. shepardi, we estimated admixture proportions with the maximum-likelihood methods implemented in LEADMIX2 (Wang 2003). This revealed that *T. shepardi* retained a very small proportion of *T. poppensis* alleles as compared to alleles deriving from *T. californicum* (relative contributions and 95% CI: *T. poppensis*: 0.117; 0.002, 0.259; *T. californicum*: 0.883; 0.741, 0.998) as expected if after an initial hybridization event between *T. poppensis* females and *T. californicum* males, there were multiple generations of backcrossing with *T. californicum*.

A detailed analysis of microsatellite genotypes in the five parthenogens suggests that a considerable proportion of their genotypic diversity stems from the conservation of ancestral alleles inherited from the sexual relative rather than from new mutations. Of 332 heterozygous genotypes in the parthenogens, 58.4% involved alleles also present in the sexual ancestors; the remaining 41.6% comprised one or two alleles specific to the parthenogens. This proportion of alleles specific to the parthenogens varied among the five lineages consistent with the accumulation of new mutations over time. Indeed, the proportion of heterozygous genotypes involving alleles specific to the parthenogen increased significantly with an increasing level of COI sequence divergence from the sexual ancestor (one-tailed Spearman rank correlation test: $S = 2$, $P = 0.042$, rho $= 0.9$; *T. douglasi*: 0.09, *T. shepardi*: 0.32, *T. tahoe*: 0.52, *T. monikensis*: *0.57, T. genevievae*: 0.60). In contrast, there was no significant correlation between heterozygosity levels and sequence divergence if alleles specific to each asexual lineage were excluded ($S = 30$, $P = 0.225$, rho $= -0.5$).

In line with the high rate of spontaneous transitions from sexuality to obligate parthenogenesis in the genus *Timema*, we also found a high rate of spontaneous parthenogenesis in all nine analyzed sexual species. Overall, 30.4% (62 out of 204) of the virgin females produced unfertilized eggs that gave rise to viable offspring. In 24 of 25 genotyped families descended from virgin females, the offspring were genetically identical neither to each other nor to their mother. The differences resulted from offspring being homozygous at some loci where the mother was heterozygous. This is the expected pattern under automictic parthenogenesis with variable proportions of central and terminal fusions. With central fusion, the offspring have the same genotype as their mother for the loci that did not crossover, whereas the offspring are homozygous for one of the two maternal alleles if crossing-over did occur; the contrary is true for terminal fusion (Suomalainen et al. 1987, Fig. 1). Under both central and terminal fusion, the frequency of transition from heterozygosity to homozygosity is expected to vary across loci depending on their distance to the centromere. Consistent with this prediction, the frequency varied significantly across the seven loci we used for offspring genotyping, presumably reflecting differences in the distance between each locus and the centromere ($\chi^2_6 = 22.6$, $P = 0.001$; percentage of homozygous offspring for a given locus when the mother was heterozygous at that locus: Tim1: 100%; Tim3: 100%; Tim4: 83.3%; Tim5: 91.4%; Tim6: 83.3%; Tim7: 66.7%, and Tim8: 95.0%). For the remaining family, descended from a *T. poppensis* female, all 10 genotyped offspring were heterozygous and genetically identical to their mother at all investigated markers, suggesting this female produced her eggs via apomixis.

In contrast to offspring produced by the vast majority of virgin sexual females, offspring of females in the five asexual species had genotypes identical to the genotype of their mother at all investigated markers, with a complete maintenance of heterozygosity (250 offspring, 910 heterozygous genotypes). The complete conservation of heterozygosity across generations suggests that parthenogenetic females produce their offspring by apomixis (none out of the 910 offspring genotypes was recombined), or that, if offspring are still produced by automictic parthenogenesis, recombination is less than 0.33% (upper 95% CI assuming 1 out of 911 offspring genotypes would be homozygous as a consequence of recombination).

The microsatellite and mitochondrial haplotype analyses also revealed that the presumed *T. monikensis* males actually belong to an isolated *T. chumash* population located outside the currently known distribution range for *T. chumash*. Thus, microsatellite allele frequencies revealed that males collected along with *T. monikensis* females on *Cercocarpus* plants were genetically more similar to *T. chumash* than to *T. monikensis* (Fig. 4) with several markers displaying fixed differences between *T. monikensis* and *T. chumash*. The assignment of "*T. monikensis*" males to *T. chumash* was also supported by mitochondrial haplotypes and karyotype structure. The haplotypes from males collected at *T. monikensis* sites (GenBank accession numbers EU860194-EU860196 and EU860199-EU860203) displayed $3.3 \pm 1.6\%$ divergence from the most similar *T. chumash* sequences (GenBank accession numbers AF410138, AF410141, and EU860189-EU860192) as compared to $12.5 \pm 1.5\%$ divergence from *T. monikensis* (GenBank accession numbers AF410100-AF410104, EU860193, EU860197, and EU860198). The males also had $2n = 21$ chromosomes, which corresponds to one of the three *T. chumash* karyotypes but not to the *T. monikensis* karyotypes ($2n = 26$ in females). Because *T. chumash* was initially only found among males at *T. monikensis* sites, we genotyped at two microsatellite markers 55 presumed "*T. monikensis*" females that were not included in the previous analyses. The additional genotyping revealed the presence of three *T. chumash* females in the *T. monikensis* population (5.4% overall). Altogether, these results show that the parthenogen *T. monikensis* co-occurs with the sexual species *T. chumash* at several locations in the distribution range described for *T. monikensis* and that there is thus no evidence for male production in *T. monikensis*.

Discussion

Our study suggests that at least four of the five *Timema* parthenogens evolved through a spontaneous loss of sex. The congruence of the most likely sexual ancestor inferred from mitochondrial and nuclear markers for *T. douglasi*, *T. monikensis*, *T. tahoe*, and *T. genevievae* revealed that they evolved from four different sexual ancestors (respectively *T. poppensis*, *T. cristinae*, *T. bartmani*, and *T. tahoe*). The fifth asexual, *T. shepardi* likely derives from a hybrid between *T. poppensis* females and *T. californicum* males. Notably, *T. poppensis*, *T. californicum*, and *T. shepardi* all have the same number of chromosomes (Table 1). Hybridization between *T. poppensis* and T. *californicum* may have been a relatively rare event given that the two species currently have no overlap in host plant species (Table 1) and that the use of different host plants is one of the main factors mediating reproductive isolation in *Timema* (Nosil 2007) and other phytophagous insects (Feder et al. 1994; Funk 1998).

The *T. shepardi* microsatellite allele frequencies further suggest that the *T. poppensis* nuclear genome has been almost completely swamped by T. *californicum* alleles. Two additional lines of evidence also support very high levels of gene flow from *T. californicum*. First, *T. shepardi* females are morphologically similar to *T. californicum* females and relatively different from *T. poppensis* females (Vickery and Sandoval 1999, 2001). Second, the only known host plant for *T. shepardi* is manzanita (*Arctostaphylos* spp.), which is one of the host plants used by *T. californicum* but is not used by *T. poppensis* (which occurs on the conifers *Pseudotsuga menziesii* and *S. sempervirens*, Table 1). Host preference has a strong genetic basis in *Timema* (Nosil et al. 2006) so that a high level of gene flow from *T. californicum* is likely to have occurred at potential host preference loci as well as at the neutral genetic markers.

Hybridization is more commonly asymmetric than symmetric (Dowling and Secor 1997) and has probably contributed to asexual origins in a wide range of taxa including other stick insects (Bullini and Nascetti 1990; Mantovani et al. 2001), *Meloidogyne* root knot nematodes (Castagnone-Sereno et al. 1993; Castagnone-Sereno 2006), rock lizards (Murphy et al. 2000), and *Calligrapha* leaf beetles (Gomez-Zurita et al. 2006). However, it is usually not possible to infer whether hybridization per se triggers the transition to asexuality or if the two events occur independently. Because in *Timema*, the evolution of asexuality likely involved a direct transition to apomictic parthenogenesis without passing through stages of automixis (see below), hybridization between *T. poppensis* and *T. californicum* and the transition to asexuality in *T. shepardi* should have been independent events. The nearly complete replacement of the *T. poppensis* nuclear genome by *T. californicum* alleles found in *T. shepardi* could only occur under multiple generations of backcrossing with *T. californicum* males. If hybridization per se induced the switch to parthenogenesis in F1 hybrids, then the allelic contribution of the two parents to the hybrid would be relatively equal under apomictic parthenogenesis. The finding of a sexually reproducing *T. poppensis–T. californicum* hybrid population with genetic signatures similar to *T. shepardi* would further bolster our inference of a spontaneous origin of asexuality in *T. shepardi*. Independently of the detailed evolutionary history of *T. shepardi*, our results strongly support five independent transitions from sexual to asexual reproduction in the genus *Timema* with two of the five transitions involving the same maternal sexual species.

The independent transitions to apomictic reproduction in *Timema* are also consistent with asexuality induced by endosymbionts, such as *Wolbachia* and *Cardinium* (Suomalainen et al. 1987; Stouthamer and Kazmer 1994; Werren 1997). Parthenogenetic production of female-only offspring as a consequence of an endosymbiont infection are only documented in species with haplo–diploid sex determination, not in species with other sex determination mechanisms (e.g., Werren 1997; Normark 2003). It is possible that *Wolbachia* or other maternally inherited elements are involved in other genetic-system transitions as well, but such involvement has yet to be clearly demonstrated (Normark 2003). The potential role of endosymbionts in asexual reproduction in *Timema* remains to be investigated.

The exact transmission of multilocus heterozygous genotypes from asexual females to their offspring and the apparent complete lack of haploid cells in the asexual females' ovaries strongly suggest that egg production in all five *Timema* asexuals is apomictic, which is the most frequent mode of parthenogenesis in all-female insects (Suomalainen et al. 1987). In cases in which apomictic parthenogenesis arose independently of hybridization and polyploidy, its origin is, so far, obscure in its details. Two main hypotheses have been proposed (White 1973; Bell 1982; Suomalainen et al. 1987). First, given that sexual species with some capacity for facultative asexual reproduction typically produce parthenogenetic eggs via automixis, asexual lineages could initially reproduce by automictic parthenogenesis and then undergo a stepwise transition to apomictic parthenogenesis (White 1973; Loki and Saura 1980; Bell 1982; Dalmasso and Bergé 1983; Suomalainen et al. 1987; Castagnone-Sereno 2006). The change from sexual reproduction and fertilization to automictic parthenogenesis does not require any drastic departure in the cytological mechanism of meiosis (Fig. 1). Thus, the fusion of nuclei of two different individuals is replaced by fusion of the nuclei of a single individual. Next, the efficiency of automixis may improve, through preferential fusion between nonsister nuclei (which maintains heterozygosity) and the suppression of crossing over (Templeton 1982; Suomalainen et al. 1987). The final step in such a transition to apomixis would then require the suppression of the first meiotic division (Bell 1982; Suomalainen

et al. 1987). Second, apomictic lineages could evolve from rare sexual females that produce their eggs mitotically (White 1964, 1973; Bell 1982; Suomalainen et al. 1987). White (1964, 1973) has considered that such drastic alterations from the usual course of meiosis as observed in apomictic parthenogenesis must have originated by sudden large cytological events.

Although cytologically, the transition from sexual reproduction to automixis is associated with smaller changes than a transition to apomixis, an incipient automictic lineage is probably less likely to become established in the face of competition with its sexual ancestor than an apomictic one. Automictic egg production, especially in normally sexually reproducing species, is characterized by very low hatching success (Stalker 1956; Carson 1967; Lamb and Willey 1979; Hong and Ando 1998). Detailed studies of selected asexual laboratory strains derived from the normally sexually reproducing species *Drosophila mercatorum* revealed that this low hatching success results in extreme bottlenecks during the early parthenogenetic generations (Templeton et al. 1976; Annest and Templeton 1978; Templeton 1982). Incipient parthenogenetic populations must successfully go through several intense selective events, which alone constitutes a severe barrier to the utilization of a parthenogenetic capacity in the sexual species (Templeton et al. 1976; Templeton 1982). Given the costs of automictic parthenogenesis at least during the early stages, automictic asexuals should have a very low competitive ability relative to their sexually reproducing ancestors (Carson 1967; Kramer and Templeton 2001) and they are expected to be favored only in environments in which finding a mate is difficult or impossible, such as in marginal habitat with such low densities that stochastic fluctuations in the sex ratio may by chance eliminate males (Stalker 1956; Kramer and Templeton 2001).

In contrast to automixis, apomictic parthenogenesis is often characterized by a relatively high egg hatching success (Bell 1982). Incipient parthenogens that reproduce via apomixis may thus be considerably more viable and demographically competitive than automicts. Our study strongly suggests that apomictic parthenogenesis can arise suddenly, without passing through a stage of automictic parthenogenesis. Although a large (30.3% overall) proportion of virgin females of the normally sexually reproducing species were able to produce small numbers of offspring by automictic parthenogenesis, this cytological mechanism is unlikely to have generated the obligately parthenogenetic lineages in *Timema*. Automictic parthenogenesis leads to a rapid increase in homozygosity over generations (Bell 1982; Suomalainen et al. 1987). In sexual *Timema*, we observed a homozygosity increase ranging from 66.7% to complete homozygosity (depending on loci) within a single generation. Yet all five asexual *Timema* lineages display high heterozygosity levels. Heterozygosity may have appeared secondarily from new mutations and have accumulated over time because of the lack of recombination in the apomicts. However, new mutations should mostly result in novel alleles, whereas the parthenogenetic *Timema* lineages had relatively few alleles that did not occur in their sexual ancestors (11–30%, Table 3). Furthermore, for the majority (58.4%) of heterozygous genotypes found in the parthenogens, both alleles were present in their sexual ancestor. These data suggest that the majority of heterozygous genotypes in the parthenogens derive from the conservation of initial allelic diversity present in the sexual ancestor rather than from new mutations. An additional line of evidence for a sudden transition to apomictic egg production in *Timema* stems from the single virgin *T. poppensis* female that produced large numbers of offspring via apomixis. The degree to which females of *T. poppensis* have the capacity for facultative apomictic parthenogenesis, or if apomitotic parthenogenesis is obligate, remains to be investigated. If future research corroborates the potential for spontaneous apomictic parthenogenesis in *Timema*, then this process might explain the large proportion of obligatorily asexual lineages in this genus.

The karyotype analyses revealed that none of the transitions from sexual to asexual reproduction was associated with polyploidy. *Timema* thus appears to be one of the few taxa where geographical parthenogenesis (Law and Crespi 2002a) is independent of polyploidy. Four of the five asexuals had the same chromosome number and similar chromosome structures as their sexual ancestors. The fifth asexual, *T. genevievae*, was characterized by an uneven number of chromosomes and different chromosome numbers at different localities. The comparison of the *T. podura* ($2n = 28$) and *T. genevievae* karyotypes suggests than one of the smaller chromosomes was lost in *T. genevievae* and resulted in the 27-chromosome karyotype. In a second step, pairwise fusion of 12 acrocentric chromosomes (including the four largest chromosomes) into six metacentric chromosomes may have resulted in the transition from 27 to 21 chromosomes. Fusions of pairs of initially acrocentric chromosomes into metacentric chromosomes could also account for the differences in chromosome numbers between the different species groups overall. The species with fewer chromosomes ($2n = 26$ and $2n = 24$) have correspondingly more metacentrics (one pair in the $2n = 26$ and two pairs in the $2n = 24$) as compared to the species with $2n = 28$ chromosomes.

Despite the uneven chromosome number in *T. genevievae*, the size of the chromosomes (and their shape in the 21 chromosome karyotype) suggests that the chromosomes may still form pairs. This is surprising as under mitotic egg production, there should be no selective pressure to maintain chromosomal structures in pairs, so in relatively old asexuals such as *T. genevievae*, chromosomal rearrangements might result in the loss of karyotypic diploidy (Judson and Normark 1996; Normark et al. 2003). Possibly, as suggested by the somewhat minor changes in karyotype structure throughout the genus, major chromosomal rearrangements other than simple fusions are rare overall in *Timema*. As a consequence,

karyotypic diploidy may be maintained over evolutionary long time periods even under exclusively mitotic reproduction.

In conclusion, our study suggests that apomixis can evolve suddenly from a sexually reproducing species without passing through stages of automictic parthenogenesis. A direct transition to apomixis results in a relatively high level of initial allelic diversity in a new asexual lineage and probably in a high efficiency for parthenogenesis that should exceed the efficiency found among automicts by orders of magnitude. Both of these traits should positively affect the demographic success of asexual lineages relative to their sexual relatives (Bell 1982) and thus contribute to the origin and maintenance of asexuality.

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Appendix 1. Sampling sites per species.

Species	Population	Host plant	County	Coordinates	Sample size
T. bartmani	JL	W	San Bernadino	34°10′200″N/117°00′121″W	13
	YmCA	W	San Bernadino	34°09′813″N/116°54′377″W	13
	RS	W	San Bernadino	34°12′600″N/117°05′900″W	5
T. californicum	LiObs	O	Santa Clara	37°20′590″N/121°38′188″W	20
	Shiv	M, C	Humboldt	40°26′759″N/123°58′581″W	5
	Loma	O	Santa Clara	37°13′343″N/122°05′271″W	5
	Cuesta	M, C	San Luis Obispo	35°21′430″N/120°39′351″W	5
	Summit	M, O	Santa Cruz	37°02′720″N/121°45′193″W	5
T. chumash	JL	O	San Bernadino	34°16′182″N/118°10′105″W	9
	PF	C	Riverside	33°51′160″N/116°49′081″W	6
	pop30	O	San Bernadino	34°17′643″N/117°59′957″W	20
	Baldy	C, O	Los Angeles	34°11′433″N/117°40′737″W	12
T. cristinae	CA	A	Santa Barbara	34°29′269″N/120°13′569″W	10
	Mat	A	Santa Barbara	34°30′897″N/120°04′278″W	10
	SM	A	Santa Barbara	34°31′500″N/119°51′000″W	10
	WTA	A	Santa Barbara	34°30′950″N/120°04′389″W	10
	OJ	C	Santa Barbara	34°29′488″N/119°18′367″W	10
T. douglasi	Orr	D	Mendocino	39°12′047″N/123°17′636″W	20
	Curry	D	Curry (Oregon)	42°16′583″N/124°06′328″W	19
T. knulli	HW1	R	Monterey	36°17′202″N/121°50′504″W	7
	BC	R	Monterey	36°04′306″N/121°36′019″W	20
	HW1	C	Monterey	36°10′115″N/121°40′944″W	6
	BC	C	Monterey	36°04′261″N/121°35′734″W	17
T. landelsensis	LH	M	Monterey	36 1°1′365″N/121°33′100″W	20
T. monikensis	CC	CC	Los Angeles	34°07′129″N/118°51′058″W	20
	De	CC	Los Angeles	34°07′172″N/118°50′441″W	17
	Fe	CC	Los Angeles	34°15′129″N/118°58′667″W	14
	Oak	CC	Los Angeles	34°07′255″N/118°51′178″W	10
T. petita	SS	C	San Luis Obispo	35°43′724″N/121°18′963″W	15
	Mo	C	Monterey	36°28′528″N/121°56′155″W	8
T. podura	DZR	A	Riverside	33°51′160″N/116°49′810″W	10
	243–2	A	Riverside	33°51′096″N/116°49′568″W	7
	Hwy 166E	A	Santa Barbara	35°05′687″N/120°07′477″W	5
	Tecate	A	San Diego	32°40′152″N/116°19′004″W	9
T. poppensis	King Mtn	R, D	Humboldt	40°08′223″N/124°04′358″W	12
	Sum	R	Santa Clara	37°01′692″N/121°44′415″W	20
	Fish	R, D	Sonoma	38°53′117″N/123°31′029″W	9
T. shepardi	King	M	Sonoma	38°35′734″N/123°09′589″W	14
	Orr	M	Mendocino	39°11′559″N/123°15′707″W	17
	Elk	M	Mendocino	39°16′729″N/122°55′546″W	11
T. tahoe	Sn	W	El Dorado	38°45′580″N/120°11′954″W	10
T. genevievae	H20	A	Lake	38°59′747″N/122°55′546″W	15
	Puerto	A	Santa Clara	37°23′392″N/121°23′389″W	18
	Mines	A	Santa Clara	37°25′431″N/121°30′336″W	5

Appendix 2. Microsatellite allele frequencies for the 14 *Timema* species analyzed.

Locus	Allele[1]	*T. bartmani*	*T. californicum*	*T. chumash*	*T. chumash*[2]	*T. cristinae*	*T. knulli*[3]	*T. knulli*[4]	*T. landelsensis*	*T. petita*	*T. podura*	*T. poppensis*	*T. douglasi*	*T. shepardi*	*T. tahoe*	*T. monikensis*	*T. genevievae*
Tim-1	213	–	0.01	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	216	0.05	–	–	–	–	–	–	–	–	–	–	0.03	–	–	–	–
	219	0.02	–	–	–	–	–	–	0.44	–	–	–	–	–	–	–	–
	222	0.24	–	–	–	–	–	–	–	–	–	–	–	–	0.08	–	–
	228	–	–	0.01	–	–	–	–	–	–	–	–	–	–	–	–	–
	231	0.03	–	0.06	–	–	0.23	0.05	–	–	0.07	0.39	0.63	0.33	–	–	0.12
	234	0.29	0.19	0.07	–	–	0.23	0.45	–	1.00	0.26	0.21	–	0.06	0.03	–	0.54
	237	–	0.01	0.02	–	–	–	–	–	–	0.05	0.01	–	–	0.05	–	–
	240	–	–	–	–	–	–	–	–	–	–	0.01	–	–	–	–	0.08
	243	–	–	0.06	–	–	0.02	–	0.09	–	0.36	0.10	0.05	0.22	–	–	–
	246	0.28	0.73	0.49	1.00	–	0.52	0.50	–	–	0.26	0.27	0.30	–	0.55	–	0.23
	249	0.09	0.03	0.27	–	–	–	–	–	–	0.02	–	–	–	0.29	–	0.04
	252	–	0.03	0.01	–	–	–	–	0.47	–	–	–	–	0.39	–	–	–
	255	–	–	–	–	0.06	–	–	–	–	–	–	–	–	–	–	–
	258	–	–	–	–	0.06	–	–	–	–	–	–	–	–	–	–	–
	261	–	–	–	–	0.07	–	–	–	–	–	–	–	–	–	–	–
	264	–	–	–	–	0.07	–	–	–	–	–	0.01	–	–	–	–	–
	267	–	–	–	–	0.10	–	–	–	–	–	–	–	–	–	0.97	–
	270	–	–	–	–	0.10	–	–	–	–	–	–	–	–	–	–	–
	273	–	–	–	–	0.06	–	–	–	–	–	–	–	–	–	0.03	–
	276	–	–	0.01	–	0.09	–	–	–	–	–	–	–	–	–	–	–
	279	–	–	–	–	0.13	–	–	–	–	–	–	–	–	–	–	–
	282	–	–	0.01	–	0.14	–	–	–	–	–	–	–	–	–	–	–
	285	–	–	–	–	0.03	–	–	–	–	–	–	–	–	–	–	–
	288	–	–	–	–	0.09	–	–	–	–	–	–	–	–	–	–	–
	297	–	–	–	–	0.01	–	–	–	–	–	–	–	–	–	–	–
Tim-2	91	–	–	–	–	–	–	–	0.10	–	–	–	–	–	–	–	–
	94	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.04	–
	115	–	–	–	–	0.03	–	–	–	–	–	–	–	–	–	–	–
	130	–	–	–	0.88	–	–	–	–	–	–	–	–	–	–	–	–
	133	–	–	–	–	–	–	–	–	–	0.02	–	–	–	–	–	0.08
	136	–	0.03	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	139	0.08	–	–	–	0.01	–	–	–	–	–	–	–	–	–	–	–
	142	–	–	–	–	–	–	–	–	–	0.27	–	–	–	–	–	0.04
	145	–	–	–	–	–	–	–	–	–	0.34	–	–	–	–	–	0.33
	148	–	–	0.06	0.13	–	–	–	–	–	0.03	–	–	–	–	–	0.04
	151	0.45	–	0.12	–	0.07	–	–	0.25	–	0.03	–	–	–	1.00	–	–
	154	0.38	–	–	–	0.01	–	–	–	–	–	–	–	–	–	–	–
	157	0.05	0.03	0.06	–	0.07	–	–	–	–	–	–	–	0.25	–	–	–
	160	–	0.09	0.55	–	0.01	–	–	0.05	–	–	–	–	0.07	–	–	0.04
	163	0.02	0.01	0.17	–	0.10	–	–	–	–	0.20	–	–	–	–	–	0.13
	166	–	–	0.02	–	0.04	–	–	–	–	0.05	–	–	–	–	0.02	0.08
	169	–	–	0.01	–	0.03	–	–	–	–	0.03	–	–	–	–	0.05	–
	172	–	0.09	–	–	0.04	–	–	–	–	0.03	–	–	–	–	0.18	–
	175	–	–	–	–	0.08	–	–	–	–	–	–	–	–	–	0.64	–
	178	–	–	–	–	0.07	–	–	–	–	–	–	–	–	–	0.07	0.04

Continued.

Appendix 2. Continued.

Locus	Allele[1]	*T. bartmani*	*T. californicum*	*T. chumash*	*T. chumash*[2]	*T. cristinae*	*T. knulli*[3]	*T. knulli*[4]	*T. landelsensis*	*T. petita*	*T. podura*	*T. poppensis*	*T. douglasi*	*T. shepardi*	*T. tahoe*	*T. monikensis*	*T. genevievae*
	181	0.02	0.01	0.01	–	0.12	–	–	–	–	–	–	–	–	–	–	0.04
	184	–	0.03	–	–	0.05	–	–	–	–	–	–	–	–	–	–	0.08
	187	–	–	–	–	0.05	–	–	–	–	–	–	–	–	–	–	–
	190	–	0.01	–	–	0.07	0.16	0.43	–	–	–	–	–	0.18	–	–	–
	193	–	0.53	–	–	–	0.20	0.23	0.25	1.00	–	–	–	0.21	–	–	–
	196	–	0.01	–	–	0.08	0.06	0.18	0.15	–	–	–	–	0.09	–	–	0.08
	199	–	0.14	–	–	–	0.36	0.18	0.10	–	–	–	–	0.15	–	–	–
	202	–	–	–	–	0.03	–	–	0.10	–	–	0.43	–	–	–	–	–
	205	–	–	–	–	–	0.06	–	–	–	–	0.33	–	–	–	–	–
	208	–	0.01	–	–	0.01	0.14	–	–	–	–	0.02	–	–	–	–	–
	211	–	–	–	–	0.01	–	–	–	–	–	0.09	–	–	–	–	–
	214	–	–	–	–	–	0.02	–	–	–	–	0.02	–	–	–	–	–
	223	–	–	–	–	0.01	–	–	–	–	–	–	–	–	–	–	–
	229	–	–	–	–	–	–	–	–	–	–	–	–	0.06	–	–	–
	316	–	–	–	–	–	–	–	–	–	–	0.11	0.95	–	–	–	–
	319	–	–	–	–	–	–	–	–	–	–	–	0.05	–	–	–	–
Tim-3	82	–	–	–	–	0.01	–	–	1.00	–	–	–	–	–	–	–	–
	88	–	–	0.01	–	0.01	–	–	–	–	–	–	–	0.75	–	–	–
	91	–	–	–	–	0.14	–	–	–	–	–	–	–	–	–	–	0.20
	94	–	–	–	–	0.22	–	–	–	–	–	–	–	–	–	–	–
	97	–	–	–	–	0.29	–	–	–	–	–	–	–	–	–	–	–
	100	–	–	–	–	0.10	–	–	–	–	–	–	–	–	–	1.00	–
	103	–	–	–	–	0.18	–	–	–	–	–	–	–	–	–	–	–
	106	–	–	–	–	0.03	–	–	–	–	–	–	–	–	–	–	–
	109	–	–	–	–	0.01	–	–	–	–	0.02	–	–	–	–	–	–
	112	–	–	–	–	–	–	–	–	–	–	–	–	0.25	–	–	–
	118	–	0.01	–	–	–	–	–	–	0.38	0.02	–	–	–	–	–	0.30
	121	0.07	–	–	–	–	–	–	–	0.06	0.09	–	–	–	–	–	0.50
	124	0.87	0.69	0.99	1.00	–	–	–	–	0.56	0.84	–	–	–	1.00	–	–
	139	–	0.07	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	142	–	0.04	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	154	0.06	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	157	–	–	–	–	–	–	–	–	–	0.02	–	–	–	–	–	–
	166	–	–	–	–	–	0.12	0.07	–	–	–	–	–	–	–	–	–
	169	–	0.03	–	–	–	–	–	–	–	–	0.68	1.00	–	–	–	–
	172	–	0.03	–	–	–	0.88	0.88	–	–	–	0.02	–	–	–	–	–
	175	–	0.11	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	178	–	0.01	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	181	–	–	–	–	–	–	0.02	–	–	–	–	–	–	–	–	–
	199	–	–	–	–	–	–	0.02	–	–	–	–	–	–	–	–	–
	217	–	–	–	–	–	–	–	–	–	–	0.23	–	–	–	–	–
	238	–	–	–	–	–	–	–	–	–	–	0.06	–	–	–	–	–
Tim-4	65	–	0.02	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	77	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.54
	80	–	–	–	–	–	–	–	–	–	0.13	–	–	–	–	–	–

Continued.

Appendix 2. Continued.

Locus	Allele[1]	*T. bartmani*	*T. californicum*	*T. chumash*	*T. chumash*[2]	*T. cristinae*	*T. knulli*[3]	*T. knulli*[4]	*T. landelsensis*	*T. petita*	*T. podura*	*T. poppensis*	*T. douglasi*	*T. shepardi*	*T. tahoe*	*T. monikensis*	*T. genevievae*
	83	0.50	0.03	–	–	0.01	–	–	0.05	–	0.18	–	–	–	0.50	–	–
	86	0.02	–	–	–	0.03	–	–	–	–	0.04	0.01	–	–	–	–	–
	89	–	–	–	–	0.17	–	–	–	0.31	0.02	–	–	0.13	–	–	–
	92	–	0.02	0.01	–	0.16	0.02	–	–	0.13	0.06	–	–	–	–	–	–
	95	0.03	0.02	–	–	0.11	–	–	–	–	–	–	–	–	–	0.75	–
	98	0.23	–	–	–	0.04	0.02	–	–	–	0.13	–	–	–	0.47	0.11	–
	101	0.21	0.90	0.92	0.88	0.11	0.73	0.83	0.25	0.19	0.19	0.74	1.00	–	–	–	0.46
	104	0.02	0.02	0.07	0.13	0.14	0.06	0.05	0.28	0.31	0.18	0.22	–	0.23	0.03	0.15	–
	107	–	–	–	–	0.06	0.17	0.12	0.10	0.06	0.04	0.02	–	0.63	–	–	–
	110	–	–	–	–	0.06	–	–	0.33	–	–	–	–	–	–	–	–
	113	–	0.02	–	–	0.04	–	–	–	–	–	–	–	–	–	–	–
	119	–	–	–	–	0.03	–	–	–	–	–	–	–	–	–	–	–
	122	–	–	–	–	–	–	–	–	–	0.03	–	–	–	–	–	–
	125	–	–	–	–	0.03	–	–	–	–	–	–	–	–	–	–	–
Tim-5	94	–	–	–	–	–	–	–	–	–	–	–	–	–	1.00	–	–
	109	0.02	–	–	–	–	–	–	–	–	0.03	–	–	–	–	–	–
	118	–	0.03	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	121	–	–	–	–	–	–	–	–	–	0.02	–	–	–	–	–	–
	124	–	0.37	0.15	0.31	0.02	–	–	–	–	0.08	–	–	–	–	0.09	–
	127	–	0.09	0.08	–	–	–	–	–	0.06	0.20	–	0.03	0.55	–	0.01	–
	130	–	0.06	0.14	–	–	–	–	–	0.06	0.06	0.04	–	0.04	–	–	0.50
	133	–	0.02	0.05	0.19	–	–	–	–	–	0.09	–	–	–	–	0.02	–
	136	0.02	0.22	0.29	–	–	–	–	–	–	0.06	–	–	0.10	–	0.01	0.06
	139	–	0.06	0.10	–	–	–	–	–	0.75	–	0.01	–	0.28	–	–	–
	142	0.94	0.04	–	0.13	–	0.05	–	–	0.13	0.30	0.04	–	–	–	–	–
	145	–	–	0.01	–	–	0.11	0.03	1.00	–	0.06	–	–	0.04	–	–	–
	148	–	–	0.09	–	–	0.07	0.20	–	–	0.02	–	–	–	–	–	–
	151	–	–	0.04	–	–	–	–	–	–	–	–	–	–	–	–	–
	154	–	–	0.03	–	–	0.05	0.05	–	–	0.05	–	–	–	–	–	0.44
	157	–	0.06	–	–	–	0.36	0.48	–	–	0.05	0.70	0.73	–	–	–	–
	160	–	–	–	–	0.02	–	–	–	–	–	0.11	0.13	–	–	–	–
	163	–	–	–	–	–	0.09	0.15	–	–	–	0.04	0.13	–	–	–	–
	166	–	–	–	–	–	0.27	–	–	–	–	–	–	–	–	–	–
	172	–	–	–	–	–	–	0.05	–	–	–	–	–	–	–	–	–
	181	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.01	–
	184	–	–	–	–	–	–	–	–	–	–	0.02	–	–	–	–	–
	190	–	0.06	–	–	0.05	–	–	–	–	–	–	–	–	–	–	–
	193	–	–	–	0.38	0.35	–	–	–	–	–	–	–	–	–	0.84	–
	196	–	–	0.01	–	0.26	–	–	–	–	–	–	–	–	–	0.02	–
	199	–	–	–	–	0.11	–	–	–	–	–	–	–	–	–	–	–
	202	–	–	–	–	0.05	–	–	–	–	–	–	–	–	–	–	–
	205	–	–	–	–	0.03	–	–	–	–	–	–	–	–	–	–	–
	208	–	–	–	–	0.03	–	–	–	–	–	–	–	–	–	–	–
	211	–	–	–	–	0.05	–	–	–	–	–	–	–	–	–	–	–
	214	–	–	–	–	0.03	–	–	–	–	–	–	–	–	–	–	–
	220	0.02	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Continued.

Appendix 2. Continued.

Locus	Allele[1]	*T. bartmani*	*T. californicum*	*T. chumash*	*T. chumash*[2]	*T. cristinae*	*T. knulli*[3]	*T. knulli*[4]	*T. landelsensis*	*T. petita*	*T. podura*	*T. poppensis*	*T. douglasi*	*T. shepardi*	*T. tahoe*	*T. monikensis*	*T. genevievae*
	229	–	–	–	–	0.02	–	–	–	–	–	–	–	–	–	–	–
	232	–	–	–	–	–	–	0.05	–	–	–	–	–	–	–	–	–
	238	–	–	–	–	0.02	–	–	–	–	–	–	–	–	–	–	–
	259	–	–	–	–	–	–	–	–	–	–	0.01	–	–	–	–	–
	265	–	–	–	–	–	–	–	–	–	–	0.04	–	–	–	–	–
Tim-6	199	–	–	–	–	–	–	–	–	–	–	–	–	–	0.38	–	–
	214	–	–	–	0.07	–	–	–	–	–	–	–	–	–	–	–	–
	229	–	–	–	–	–	–	–	–	–	0.02	–	–	0.01	–	–	0.25
	235	–	0.02	–	–	–	–	–	–	–	–	–	–	0.03	–	–	–
	238	0.02	0.16	–	–	–	–	–	0.13	–	–	–	–	–	–	–	–
	241	–	0.05	–	–	–	–	–	–	–	–	–	–	0.84	–	–	–
	244	0.13	0.02	–	–	–	–	–	–	–	0.06	–	–	0.03	–	–	–
	247	0.08	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	250	–	0.03	0.01	–	–	–	–	0.63	–	0.02	0.02	–	0.03	–	–	–
	253	0.08	0.08	–	–	0.01	–	0.08	–	1.00	0.03	0.05	0.05	–	0.35	–	–
	256	0.25	0.19	0.05	–	0.03	0.87	0.65	–	–	0.07	0.27	0.28	0.03	–	1.00	0.13
	259	0.42	0.27	0.03	–	0.21	0.08	0.20	–	–	0.63	0.52	0.60	0.01	0.03	–	–
	262	–	0.08	0.01	–	0.69	0.06	0.08	–	–	–	0.05	0.05	0.01	0.24	–	0.63
	265	–	–	–	–	0.01	–	–	0.21	–	0.09	0.09	0.03	0.03	–	–	–
	268	–	0.03	–	–	0.01	–	–	0.03	–	–	–	–	–	–	–	–
	274	–	–	–	–	0.03	–	–	–	–	–	–	–	–	–	–	–
	277	–	–	–	–	–	–	–	–	–	0.02	–	–	–	–	–	–
	280	–	–	0.74	0.93	–	–	–	–	–	–	–	–	–	–	–	–
	283	–	–	0.15	–	0.01	–	–	–	–	0.03	–	–	–	–	–	–
	286	0.02	–	0.03	–	–	–	–	–	–	–	–	–	–	–	–	–
	292	–	0.06	–	–	–	–	–	–	–	0.04	–	–	–	–	–	–
	295	–	0.03	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Tim-7	84	–	–	–	1.00	–	–	–	–	–	–	–	–	–	–	–	–
	99	–	–	–	–	–	–	–	–	–	–	–	–	–	0.50	–	–
	108	–	–	–	–	–	–	–	–	–	0.03	–	–	–	–	–	–
	114	0.02	–	–	–	–	–	–	–	–	–	–	–	–	0.50	–	–
	120	–	–	–	–	0.01	–	–	–	–	–	–	–	–	–	–	–
	126	–	–	–	–	0.01	–	–	–	0.63	–	–	–	–	–	–	–
	129	0.11	0.30	–	–	0.03	–	–	–	–	0.02	0.01	–	0.20	–	–	–
	132	0.54	0.01	0.13	–	0.04	–	–	–	–	0.83	0.17	–	0.04	–	–	1.00
	135	0.25	0.26	0.60	–	0.11	–	–	–	–	0.03	–	–	0.21	–	–	–
	138	0.04	0.01	0.17	–	0.08	–	–	–	0.38	–	–	–	–	–	–	–
	141	0.04	–	–	–	0.08	–	–	–	–	–	–	–	–	–	–	–
	144	0.02	0.41	0.11	–	0.17	–	–	–	–	–	0.04	–	0.02	–	–	–
	147	–	–	–	–	0.13	–	–	–	–	–	–	–	0.44	–	–	–
	150	–	–	–	–	0.08	–	–	–	–	–	–	–	–	–	0.15	–
	153	–	–	–	–	0.01	–	–	–	–	–	–	–	–	–	–	–
	156	–	–	–	–	0.01	–	–	–	–	–	–	–	–	–	–	–
	159	–	–	–	–	0.21	–	–	–	–	–	0.37	–	–	–	–	–
	162	–	–	–	–	–	0.02	–	0.25	–	–	0.39	1.00	0.10	–	–	–

Continued.

Appendix 2. Continued.

Locus	Allele[1]	*T. bartmani*	*T. californicum*	*T. chumash*	*T. chumash*[2]	*T. cristinae*	*T. knulli*[3]	*T. knulli*[4]	*T. landelsensis*	*T. petita*	*T. podura*	*T. poppensis*	*T. douglasi*	*T. shepardi*	*T. tahoe*	*T. monikensis*	*T. genevievae*
	165	–	–	–	–	–	–	0.02	0.75	–	–	–	–	–	–	–	–
	168	–	–	–	–	–	0.02	0.17	–	–	–	0.02	–	–	–	–	–
	171	–	–	–	–	–	0.50	0.55	–	–	0.02	–	–	–	–	–	–
	174	–	–	–	–	–	0.42	0.24	–	–	–	–	–	–	–	–	–
	177	–	–	–	–	–	0.04	0.02	–	–	–	–	–	–	–	–	–
	180	–	–	–	–	–	–	–	–	–	0.08	–	–	–	–	–	–
	186	–	–	–	–	0.01	–	–	–	–	–	–	–	–	–	0.85	–
	195	–	–	–	–	0.01	–	–	–	–	–	–	–	–	–	–	–
Tim-8	112	–	–	–	–	–	0.15	–	–	–	–	–	–	–	–	–	–
	133	–	–	–	–	0.07	–	–	–	–	–	–	–	–	–	–	–
	136	–	–	–	–	0.49	–	–	–	–	–	–	–	–	–	–	–
	139	–	–	–	–	0.07	–	–	–	–	–	–	–	–	–	–	–
	142	–	–	–	–	0.21	–	–	–	–	–	–	–	–	–	–	–
	145	–	–	–	–	0.01	–	–	–	–	–	–	–	–	–	–	–
	148	–	–	–	–	0.15	–	–	–	–	–	–	–	–	–	–	–
	199	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.00	–
	214	–	–	–	–	–	0.04	–	–	–	–	–	–	–	–	–	–
	226	–	–	–	–	–	–	0.08	–	–	–	–	–	–	–	–	–
	229	–	–	–	–	–	0.06	0.08	–	–	–	–	–	–	–	–	–
	232	–	–	–	–	–	0.02	0.06	–	–	0.86	–	–	–	–	–	0.11
	235	0.27	–	–	–	–	0.04	0.03	–	–	–	–	–	–	–	–	–
	238	–	–	–	–	–	0.02	–	–	–	–	–	–	–	–	–	–
	241	–	–	–	–	–	0.02	0.06	–	–	–	–	–	–	–	–	–
	244	–	–	0.01	–	–	–	–	–	–	–	–	–	–	–	–	–
	247	0.14	–	–	–	–	0.02	–	–	–	–	–	–	–	–	–	–
	250	–	–	–	–	–	0.04	0.25	–	–	–	–	–	–	–	–	–
	253	–	–	–	–	–	0.04	0.03	–	–	–	–	–	–	–	–	–
	256	0.10	0.06	–	–	–	0.13	0.03	–	–	–	0.02	–	–	–	–	–
	259	–	–	–	–	–	0.11	0.22	–	–	–	–	–	–	–	–	–
	262	–	–	–	–	–	0.20	0.03	–	–	–	0.95	1.00	–	–	–	–
	268	–	–	–	–	–	–	0.03	–	–	–	0.02	–	–	–	–	–
	271	–	–	–	–	–	0.02	0.03	–	–	–	–	–	–	–	–	–
	274	–	0.86	–	–	–	0.07	0.03	–	0.44	–	–	–	1.00	–	–	–
	277	0.06	0.09	–	–	–	0.04	0.06	0.90	0.56	–	–	–	–	–	–	–
	286	0.08	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	292	–	–	–	–	–	–	–	–	–	0.12	–	–	–	–	–	–
	295	0.37	–	0.01	–	–	–	–	–	–	–	–	–	–	1.00	–	–
	298	–	–	0.08	–	–	–	–	–	–	0.02	–	–	–	–	–	0.89
	301	–	–	0.50	1.00	–	–	–	0.10	–	–	–	–	–	–	–	–
	304	–	–	0.02	–	–	–	–	–	–	–	–	–	–	–	–	–
	307	–	–	0.02	–	–	–	–	–	–	–	–	–	–	–	–	–
	325	–	–	0.04	–	–	–	–	–	–	–	–	–	–	–	–	–
	331	–	–	0.02	–	–	–	–	–	–	–	–	–	–	–	–	–
	334	–	–	0.08	–	–	–	–	–	–	–	–	–	–	–	–	–
	337	–	–	0.17	–	–	–	–	–	–	–	–	–	–	–	–	–
	340	–	–	0.04	–	–	–	–	–	–	–	–	–	–	–	–	–

Continued.

Appendix 2. Continued.

Locus	Allele[1]	*T. bartmani*	*T. californicum*	*T. chumash*	*T. chumash*[2]	*T. cristinae*	*T. knulli*[3]	*T. knulli*[4]	*T. landelsensis*	*T. petita*	*T. podura*	*T. poppensis*	*T. douglasi*	*T. shepardi*	*T. tahoe*	*T. monikensis*	*T. genevievae*
Tim-9	77	–	–	–	–	–	–	–	–	–	0.04	–	–	–	–	–	–
	98	0.02	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	101	–	0.02	0.22	–	–	–	–	–	–	0.13	–	–	–	–	–	–
	119	–	0.06	–	–	–	–	–	–	–	–	0.01	–	–	–	–	–
	125	–	–	0.03	–	–	0.04	–	–	–	0.02	–	–	–	–	–	–
	128	–	–	0.01	–	–	–	–	–	–	–	–	–	–	–	–	–
	131	0.02	–	0.01	–	–	–	–	–	–	0.04	–	–	–	–	–	–
	134	0.95	0.09	–	–	–	0.59	0.73	–	–	0.04	0.80	0.50	0.13	1.00	–	–
	137	0.02	–	–	–	–	0.02	–	–	–	–	–	–	–	–	–	–
	140	–	–	–	–	–	0.02	–	–	–	0.11	–	–	–	–	–	–
	143	–	0.02	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	146	–	0.02	–	–	–	0.02	–	–	–	–	0.01	–	–	–	–	–
	152	–	0.02	–	–	–	–	–	–	–	0.04	0.01	–	–	–	–	–
	155	–	–	0.01	–	–	0.02	–	–	–	–	0.01	–	–	–	–	–
	164	–	–	0.01	–	–	–	–	–	–	–	–	–	–	–	–	–
	167	–	0.02	0.03	–	–	–	–	–	–	–	–	–	–	–	–	–
	170	–	0.02	–	–	–	–	–	0.03	–	–	–	–	–	–	–	–
	173	–	0.69	–	–	–	0.22	0.28	–	1.00	–	0.13	0.50	0.87	–	–	–
	176	–	0.02	–	–	–	0.06	–	–	–	–	–	–	–	–	–	–
	179	–	–	–	–	–	0.02	–	–	–	–	–	–	–	–	–	–
	182	–	–	0.04	–	–	–	–	–	–	0.02	0.03	–	–	–	–	–
	185	–	–	0.01	–	–	–	–	0.03	–	–	–	–	–	–	0.03	–
	188	–	–	0.11	–	–	–	–	–	–	0.02	–	–	–	–	–	–
	191	–	–	0.14	–	–	–	–	–	–	0.09	–	–	–	–	–	–
	194	–	–	0.27	0.88	–	–	–	0.59	–	0.20	–	–	–	–	0.02	–
	197	–	0.05	0.09	–	–	–	–	–	–	0.07	–	–	–	–	–	–
	203	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.06	–
	206	–	–	–	–	–	–	–	–	–	0.02	–	–	–	–	0.01	–
	209	–	–	–	0.13	0.61	–	–	–	–	–	–	–	–	–	0.37	–
	212	–	–	–	–	0.22	–	–	–	–	–	–	–	–	–	–	–
	215	–	–	–	–	–	–	–	–	–	0.02	–	–	–	–	0.08	–
	218	–	–	0.03	–	–	–	–	0.03	–	–	–	–	–	–	0.01	–
	221	–	–	–	–	–	–	–	0.21	–	–	–	–	–	–	–	–
	227	–	–	–	–	0.02	–	–	0.06	–	–	–	–	–	–	–	–
	230	–	–	–	–	–	–	–	0.03	–	–	–	–	–	–	–	–
	236	–	–	–	–	–	–	–	–	–	0.02	–	–	–	–	–	0.75
	242	–	–	–	–	0.02	–	–	–	–	–	–	–	–	–	–	–
	245	–	–	–	–	–	–	–	–	–	0.13	–	–	–	–	0.02	0.25
	248	–	–	–	–	0.09	–	–	–	–	–	–	–	–	–	–	–
	251	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.24	–
	254	–	–	–	–	0.05	–	–	–	–	–	–	–	–	–	–	–
	266	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.07	–
	275	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.09	–
	287	–	–	–	–	–	–	–	0.03	–	–	–	–	–	–	–	–

[1]Alles are given as PCR-product sizes.

[2]*T. chumash* collected at *T. monikensis* sites.

[3]Redwood hostplant.

[4]*Ceanothus* hostplant.