Genealogical evidence for epidemics of selfish genes

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Some genetic elements spread infectiously in populations by increasing their rate of genetic transmission at the expense of other genes in the genome. These so-called selfish genetic elements comprise a substantial portion of eukaryotic genomes and have long been viewed as a potent evolutionary force. Despite this view, little is known about the evolutionary history of selfish genetic elements in natural populations, or their genetic effects on other portions of the genome. Here we use nuclear and chloroplast gene genealogies in two species of *Silene* to show the historical pattern of selection on a well known selfish genetic element, cytoplasmic male sterility. We provide evidence that evolution of cytoplasmic male sterility has been characterized by frequent turnovers of mutations in natural populations, thus supporting an epidemic model for the evolution of selfish genes, where new mutations repeatedly arise and rapidly sweep through populations.

A wide variety of genetic elements that spread infectiously in populations exist because they have a replication advantage over other genes in the genome. These are often called selfish genetic elements because their transmission advantage permits them to spread even when they are neutral or detrimental to the fitness of individuals that carry them (1). Selfish genetic elements have long been viewed as a potent evolutionary force (2) and are now known to comprise a substantial portion of eukaryotic genomes (3). A remarkable diversity of selfish genetic elements exists in nature. They include repetitive sequences such as transposable elements that autonomously replicate within the genome. Segregation distorters enhance their genetic transmission by altering meiosis or gametogenesis to increase their representation in the gametes. Maternally inherited genes and cytoplasmically transmitted parasites enhance their genetic transmission by increasing reproductive allocation toward the production of females, because only females transmit those elements to the next generation (3, 4). Theoretical studies suggest that these elements may have been important for such diverse phenomena as the evolution of genome architecture, the evolution of sex determination and sex chromosomes, and reproductive isolation between species (3). In this paper we focus on cytoplasmic male sterility (CMS), a maternally inherited selfish gene that is widespread among angiosperms.

CMS has been reported from more than 150 different plant species and has long been a model system for the study of selfish genetic elements occurring in nature (5, 6). Furthermore, CMS is thought to be an important intermediate step in the evolution of separate sexes and it has been put to widespread use in the production of hybrid seed for agriculture (5). CMS is caused by the creation of chimeric genes through rearrangements within the mitochondrial genome (5, 6). These chimeric genes produce peptides that interfere with the development of male reproductive organs (stamens) (5). Because mitochondria and chloroplast genomes are maternally inherited in most angiosperms, mutations in those genomes are favored by natural selection if they reallocate resources to the production of female gametes, even if this reallocation occurs at the expense of gamete production in males. The initial spread of a CMS mutation then creates strong selection for nuclear loci that restore male fertility. Restored populations may then be susceptible to invasion by new CMS mutations that are counteracted by different restorer alleles, and so on. As a result, classical genetic studies of natural systems have often shown a complex genetic basis of male sterility, involving several different CMS mutations and associated nuclear restorer alleles (7–11).

The complex genetics of CMS systems have made it difficult to study even the most general features of the evolutionary dynamics of these systems in natural populations. Instead, the evolutionary dynamics of CMS systems have been explored by using a variety of theoretical models (7, 12–14). These models can be grouped into two general classes that predict qualitatively different evolutionary dynamics. One class of models has shown that even though CMS mutations may arise infrequently, they are maintained indefinitely in a stable polymorphic state, possibly with accompanying polymorphism at restorer loci (12, 13). Another class of models suggests repeated epidemics of CMS mutations occur, either because CMS mutations oscillate in frequency over time (14) or because populations repeatedly invaded by novel CMS alleles (7) that spread and are then restored. A fundamental issue for understanding CMS evolution, therefore, is whether relatively few CMS elements are maintained in populations over long evolutionary time scales or whether a continual replacement of CMS types occurs in a species. The answer to this question in systems with CMS, or in systems with other types of selfish genetic elements, contributes to the broader issue of whether selfish genetic elements are rare curiosities or recurring phenomena with important evolutionary consequences.

Our approach to this problem is to study gene genealogies of genomic regions linked to CMS elements to analyze the historical patterns of selection on CMS. The two classes of models set up different expectations for the amount of neutral genetic diversity that is maintained in the population, at the CMS "locus" and at neutral sites linked to the CMS "locus". Models involving stable polymorphisms predict that high levels genetic diversity, relative to the neutral expectation, should occur, because different lineages accumulate mutations independently in the absence of recombination (15). By contrast, the rapid turnover of lineages (i.e., through selective sweeps or recurrent "epidemics" of CMS mutations) tends to reduce genetic variation at linked sites compared with the neutral case (16). Both of these effects are expected to be particularly pronounced in genomes that do not undergo recombination, such as the mitochondrial or chloroplast genomes of plants.

To test whether CMS evolution is characterized by stable polymorphisms or recurrent epidemics, we therefore estimated sequence diversity at nuclear and cytoplasmic loci in *Silene vulgaris*, a plant that is known to have a CMS under intense selection in nature. As a control, we used another species that has no recent evolutionary history of CMS. We show that CMS acts to reduce diversity in cytoplasmic genomes, suggesting the historical evolutionary dynamics of CMS in *S. vulgaris* is characterized by recurrent epidemics.

EVOLUTION

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Abbreviations: CMS, cytoplasmic male sterility; HKA, Hudson–Kreitman–Aguade.

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Materials and Methods

S. vulgaris is a weedy perennial herb common throughout Europe. It is gynodioecious, i.e., populations consist of a mixture of hermaphrodite and female individuals, and sex expression is known to be under cytoplasmic control (11, 17). Natural populations often contain high frequencies of females, sometimes reaching 75% or higher (18). We compared cytoplasmic diversity in *S. vulgaris* with another species, *Silene latifolia*, which does not have a recent history of CMS. *S. latifolia* is a closely related species with ecological and life history characteristics similar to *S. vulgaris*. However, *S. latifolia* is dioecious (i.e., has separate male and female individuals) with a chromosomal sex determination system (females are XX, males are XY). These two species provide a uniquely powerful test for the effect of CMS evolution on lineage diversity. Because *S. latifolia* is dioecious, a CMS mutation cannot gain any transmission advantage by turning off male function and reallocating resources to seed production, so no possibility exists for selection favoring CMS. Hence, crossing studies in *S. latifolia* have shown no cytoplasmic genetic effects on sex ratio or sex allocation (19), as there is in *S. vulgaris* (11, 20).

We collected seeds from populations of both species scattered throughout Europe (from the Mediterranean northward into Scotland, and from the Atlantic Ocean eastward as far as Armenia). We isolated DNA from one plant per population. Total genomic DNA was isolated from leaf tissue by using DNeasy plant miniprep kit (Qiagen, Valencia, CA) from 25 *S. vulgaris* and 29 *S. latifolia* plants. We amplified three chloroplast intergenic spacers (*trnL-trnF*, *trnH-psbA*, and *trnG-trnS*) and one intron (*trnL*) by using PCR. For all amplifications of cpDNA we used universal primers (21, 22). We chose to sequence chloroplast DNA rather than mitochondrial DNA for two reasons. First, plant mtDNA has the lowest substitution rate of the three genomes, whereas the chloroplast genome has a substitution rate that is intermediate between nuclear and mitochondrial DNA. Using cpDNA therefore increased our power to detect any changes in cytoplasmic genetic variation between the two species. Second, plant mitochondria are subject to frequent rearrangements, sometimes making direct sequencing difficult. The cpDNA and mtDNA genomes are in complete linkage disequilibria in *Silene*, so the two genomes share a common evolutionary history (23).

As a control for differences in effective population size between the two species we also sequenced a region covering exons 13 (partial), 14, and 15, two intervening introns, and part of the 3′ noncoding region (24) from *Sl*X1, an X-linked locus in *S. latifolia* that is autosomal in *S. vulgaris*. We sequenced a total of eight *S. vulgaris* and nine *S. latifolia* individuals, selected at random from among the populations sampled for cpDNA variation. *Sl*X1 was amplified by using primer −7 from Filatov *et al.* (24) as well as a newly designed forward primer: 5′-TGGAAACAGAGAGCGGAGGT-3′. All PCR products were cleaned by using QIAquick PCR purification kit (Qiagen, Valencia, CA). The cleaned PCR product was cycle-sequenced directly with BigDye terminator ready reaction mix (Applied Biosystems) and applied to an ABI377 automated sequencer (Applied Biosystems). Sequences were verified manually and contigs were assembled by using the computer program SEQUENCHER (Gene Codes, Ann Arbor, MI). Multiple sequence alignments were made by using the software package GCG (WISCONSIN PACKAGE, V. 10.0, Accelrys, Burlington, MA) and adjusted manually. The combined and aligned data sets were 1,849 bp long for the chloroplast data and 915 bp for *Sl*X1, including gaps caused by insertions and deletions. The computer program DNASP V3.53 (www.bio.ub.es/~julio/DnaSP.html) was used to calculate the number of exclusive and shared segregating sites (S_x and S_s), fixed differences (S_f), nucleotide diversity (π), sequence divergence (d_{xy}), and Tajima's D (25). The neighbor-joining trees were constructed by using the software program MEGA 2.1 (www.megasoftware.net/), based on pairwise divergence values by using a Jukes–Cantor nucleotide substitution model. Coalescent simulations were performed by using standard coalescent algorithms (26) with a program written in C++ (available from P.K.I. upon request). Coalescent simulations were performed assuming no recombination and constant population size, by using estimates of the scaled mutation rate ($\theta = 4N_e\mu$) derived from the number of segregating sites in the two species. In each sample, a random genealogy was generated with the same sample size as the original samples and mutations were distributed along the branches of the genealogy according to an exponential distribution with mean $\theta/2$ (26). The number of segregating sites in the sample was then scored. Each simulation was replicated 10^6 times to generate the distribution of S conditional on θ. A 95% confidence of S interval was calculated from this distribution, and we then determined whether the observed number of segregating sites in the two species were compatible with the value θ used to generate the distribution.

Table 1. The number of exclusive (S_x) and shared (S_s) segregating sites and the number of fixed differences (S_f) between *S. vulgaris* and *S. latifolia* for the cpDNA data and the nuclear gene (*Sl*X1)

	S_x (*S. vulgaris*)	S_x (*S. latifolia*)	S_s	S_f
k_{cpDNA}	14	28	2	26
$k_{nuclear}$	26	30*	1	80
π_{cpDNA}	0.0015 ± 0.0012	0.0022 ± 0.002		
$\pi_{nuclear}$	0.011 ± 0.0056	0.012* ± 0.006		
$d_{xy(cpDNA)}$				0.024 ± 0.001
$d_{xy(nuclear)}$				0.125 ± 0.015

Also shown is nucleotide diversity ($\pi \pm$ standard deviation) within species and the nucleotide divergence between the two species ($d_{xy} \pm$ standard deviation) calculated by using an Jukes–Cantor nucleotide substitution model. *Number of segregating sites and expected sequence diversity for the *Sl*X1 gene in *S. latifolia* after correcting for X-linkage.

Results

The total aligned chloroplast data set was 1,849 bp, including insertions and deletions, and 1,351 bp when sites with indels are excluded. Ignoring variation caused by insertions/deletions, 14 and 28 exclusive polymorphisms occurred in *S. vulgaris* and *S. latifolia*, respectively, and also two shared polymorphisms. The average number of nucleotide differences between the two species was 31.8. The *Sl*X1 data set was 915 bp, including insertion and deletions, and 730 bp, excluding sites with indels. Of these sites, 258 were sites in coding regions. For *Sl*X1, also ignoring insertions and deletions, we found 25 exclusive polymorphisms within *S. vulgaris,* 23 exclusive polymorphisms within *S. latifolia,* 1 shared polymorphism, and on average 91.1 nucleotide differences between the two species. After correcting for the smaller effective population size of *Sl*X1 in *S. latifolia* (because of X-linkage) the number of segregating sites in *S. latifolia* was 30 (Table 1).

The distribution of chloroplast variation within both species deviated from a neutral model of evolution. Tajima's D (25) was significantly negative for both *S. vulgaris* ($D = -2.28$, $P < 0.001$) and *S. latifolia* ($D = -2.09$, $P < 0.001$). We did not detect any deviation from neutrality for the nuclear locus in either species (*S. vulgaris*: $D = -0.75$, $P > 0.4$ and *S. latifolia*: $D = -0.14$, $P > 0.25$).

To test whether the patterns of polymorphism and divergence in the sequence data fit a neutral model of evolution, we performed an HKA (Hudson–Kreitman–Aguade) test (27). The HKA test estimates whether the amount of within-species poly-

morphism is correlated with the degree of divergence between the two species. The HKA test showed a significant departure from the neutral expectation ($\chi^2 = 15.5$, $P < 0.01$). After inspecting the data, however, it is clear that the significance of the HKA test is either because of high levels of intraspecific polymorphism in both species or a low divergence between the two species for the chloroplast data. We are therefore not able to use the significance of the HKA test to make statements about the differences in the level of intraspecific cpDNA polymorphism in the two species.

We used direct simulations of the coalescent process (26–28) to test specifically whether the difference in levels of polymorphism in the chloroplast genomes of *S. vulgaris* and *S. latifolia* was statistically significant. We used estimates of the scaled mutation rate ($\theta = 4N_e\mu$) derived from the number of segregating sites in *S. latifolia* ($\theta = 7.24$) and *S. vulgaris* ($\theta = 3.28$), respectively. Specifically, we used coalescent simulations conditional on θ to generate a probability distribution for the number of segregating sites (28). The lower number of segregating sites within *S. vulgaris* ($S = 14$) was unlikely to be observed by chance ($P = 0.027$) given the expected distribution of the number of segregating sites generated by the value of θ for *S. latifolia*. Similarly, the higher number of segregating sites within *S. latifolia* ($S = 28$) was unlikely to be observed by chance ($P = 0.02$) given the expected distribution of the number of segregating sites generated by the value of θ for *S. vulgaris*. The number of segregating sites in the chloroplast sequences was therefore significantly different for the two species (28). By contrast, the number of segregating sites in the nuclear locus was not significantly different in the two species. Specifically, the number of segregating sites observed for the nuclear locus in *S. vulgaris* was well within the distribution of values generated by the value of θ for *S. latifolia* ($P = 0.52$), and *vice versa* ($P = 0.30$). The general pattern of reduced cytoplasmic variation within *S. vulgaris*, but no significant difference in nuclear diversity, is straightforward to see by inspection of the genealogies (Fig. 1).

Discussion

The results show that sequence variation at cytoplasmic loci within *S. vulgaris* has been reduced relative to *S. latifolia*, a closely related species with no recent evolutionary history of CMS. The fact that the two species do not differ in diversity at the nuclear gene suggests the two species have not had dramatically different effective population sizes during their recent evolutionary history. Taken together, the data indicate that purifying selection has occurred on the cytoplasmic genome of *S. vulgaris*. These results counter the predictions from several theoretical models that selection acts to maintain CMS mutations in a stable polymorphic state. The fact that selection has acted to purge variation from the cytoplasmic genome of *S. vulgaris* supports the notion that the evolution of CMS in this species has been characterized by the frequent turnover of lineages through selective sweeps or recurrent "epidemics" of CMS mutations.

Although our data reveal a history of purifying selection on the cytoplasmic genomes in *S. vulgaris*, abundant mitochondrial and chloroplast DNA diversity still exists in natural populations (ref. 29; Fig. 1). This diversity suggests local, rather than species-wide, selective sweeps of CMS elements have occurred. Local CMS epidemics are to be expected because CMS factors may only spread on specific genetic backgrounds (because of the presence of variation at restorer loci) or in certain geographic locations (because of population structure). In fact, recent studies of molecular markers of the mitochondrial genome (29) and of the CMS loci themselves (17) show local population structure exists at the CMS loci in *S. vulgaris*. Although no theoretical models predict the effects of local selective sweeps on species-wide levels of genetic diversity, we expect the net effect of local selective sweeps will be qualitatively similar to species-wide sweeps. Local selective sweeps should reduce diversity relative to the neutral expectation and/or relative to species without CMS, although the effects might be less pronounced than with a species-wide sweep.

It is important to point out that, although our results highlight an important feature of the evolutionary dynamics of CMS in this species, they neither support nor refute a specific model of CMS evolution. For example, some models of CMS evolution are truly nonequilibrium, with male sterility systems going to fixation, or being restored, followed by the spread of a new CMS mutation (7). Other models predict stable limit cycles (14) or a damped cyclic approach to a stable equilibrium (12). The latter models show that selection can maintain polymorphisms in some deterministic systems, but they also predict that selection drives large fluctuations in gene frequency that in a finite population could lead to the loss of one allele or another. That situation is essentially a selective sweep. Therefore, although CMS evolution in *S. vulgaris* seems to involve selective sweeps of CMS factors, the specific model could involve the continual replacement of old CMS systems by new ones (cf. ref. 7), or one that could in principle have limit cycles or a balanced polymorphism but where the evolutionary dynamics and the chance loss of rare alleles combine to make the deterministic equilibrium largely irrelevant.

Our estimate of the reduction in sequence diversity of the chloroplast genome of *S. vulgaris* is likely to be conservative. *S. latifolia*, being dioecious, is expected to have an effective population size for the cytoplasmic genomes that is one fourth of the nuclear genome (31). Because *S. vulgaris* has a mixture of females and hermaphrodites, we expected the effective population size for the cytoplasmic genome to fall between one half of the nuclear genome (if there were 0% females) and one fourth of the nuclear genome (if there were 50% females) (30). Because the average frequency of females in natural populations is roughly 25% (18), we would have expected *S. vulgaris* to have a greater cytoplasmic diversity under a strictly neutral model.

Another interesting pattern to the data was a far lower among-species divergence for the chloroplast sequences than expected relative to the observed levels of intraspecific polymorphism. A potential explanation for the low divergence/high polymorphism is that both *S. latifolia* and *S. vulgaris* have pronounced population structure, with the chloroplast genome being much more structured that the nuclear genome (31, 32). Wakeley (33) has shown that the coalescent process remains unaffected by population subdivision if time is measured in units of the global effective population size $N_s = N_eD(1 + 1/2M)$, where N_e is the local population size, D is the number of demes, and M is the scaled migration rate ($M = Nm$ for haploid and $2Nm$ for diploid genomes). The species-wide diversity is thus proportional to $\theta_s = 4N_s\mu = 4N_eD\mu(1 + 1/2M)$. It is easy to see from this expression that strong population subdivision (i.e., low M) will result in an increase in levels of intraspecific polymorphism maintained. Theoretical analyses have also shown that population structure will result in reduced divergence between species and this effect gets more pronounced for low migration rates (34), because for a mutation to be classified as a fixed difference between species, the mutation has to spread throughout a species range. In a subdivided population this divergence is obviously harder to achieve with low migration rates, such as for the chloroplast genome in plants.

One consequence of population subdivision is that it is not meaningful to use the amount of divergence between species to define a neutral expectation for the level of polymorphism within species, as is done with the HKA test, especially when genes with different migration rates are compared (i.e., cytoplasmic vs. nuclear genes). This means that the difference in cytoplasmic polymorphism we observed could be caused by either a defi-

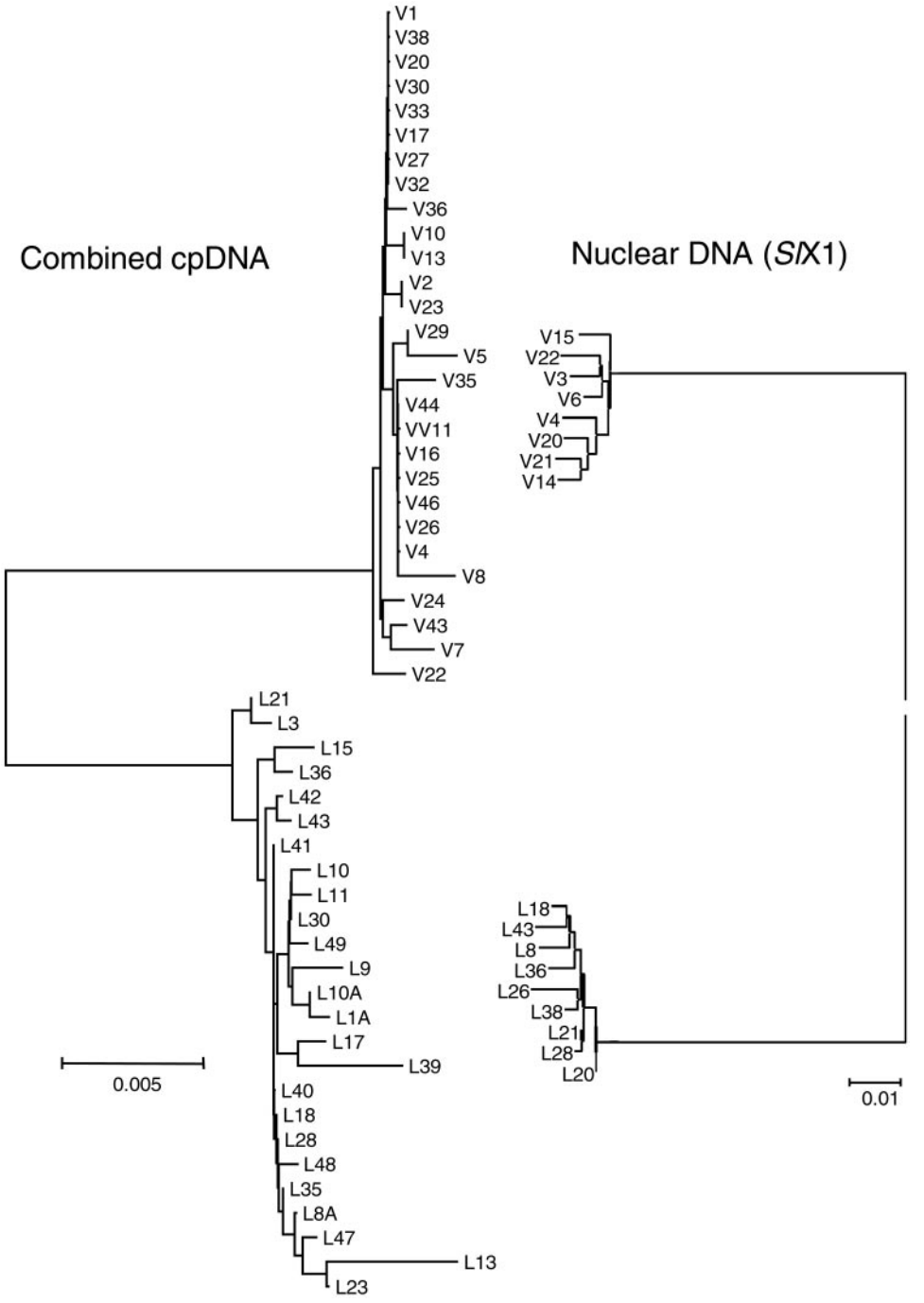

Fig. 1. Neighbor-joining trees for the chloroplast data and the nuclear gene, *Sl*X1, based on Jukes–Cantor distances. Chloroplast sequence diversity in *S. vulgaris* is reduced below neutral expectations as evidenced by the short branch lengths within the *S. vulgaris* clade, compared with *S. latifolia*. No difference in sequence diversity is seen between the two species at the nuclear gene, after correcting for X-linkage of *Sl*X1 in *S. latifolia*. Note that the scale differs between the two neighbor-joining trees. VLG = samples of *S. vulgaris*; LAT = samples of *S. latifolia*.

ciency of segregating sites within *S. vulgaris* or an excess of segregating sites within *S. latifolia*. However, an excess of segregating sites within *S. latifolia* is not supported by the data. Assuming the two species have similar population structures (31, 32), an excess of polymorphism would only be observed if some form of balancing selection existed within *S. latifolia*, unrelated to the phenomenon of CMS. Balancing selection, however, would act to preserve older alleles within populations, which is inconsistent with the significant excess of younger alleles, i.e., the negative Tajima's *D*, we observed in both species. We therefore conclude that selection has acted to reduce genetic diversity in the *S. vulgaris* chloroplast genome below neutral expectations.

Although we have emphasized the difference in cytoplasmic diversity in the two species, it is important to point out that the distribution of chloroplast variation within both species deviated from a neutral model of evolution (Tajima's $D < 0$). By contrast, we detected no such deviations from neutrality for the nuclear locus in either species. This pattern could be caused by some form of purifying selection that has acted on the cytoplasmic genomes of both species, unrelated to the effects of CMS in *S.*

vulgaris. However, the alternative interpretation of a negative Tajima's *D*, that both species have recently undergone a recent bottleneck or a population expansion, should not be completely ruled out. The nonsignificant Tajima's *D* for the nuclear gene would typically be cited as evidence against population expansion because demographic changes should influence the genealogies of all loci in a similar way. However, the low mutation rate of cytoplasmic genomes increases the time it takes for genetic diversity to return to the neutral expectation after a bottleneck, thus making signatures of population expansion potentially easier to detect in cytoplasmic genomes. Both species are thought to have undergone relatively recent range expansions as they spread across Europe during the Neolithic as weeds of cultivation, and like many other European species (35), populations in Northern and Central Europe were probably colonized from southern refugia after the most recent glaciation. Taken together our data show that CMS evolution in *S. vulgaris* has occurred on a background of purifying selection or population expansion that is common to both species.

Selfish genes are notoriously difficult to study because they are often impossible to detect without detailed genetic studies and because they generally involve complex systems of genetic conflict. Studying the genealogies of selfish genetic elements is a powerful tool for studying historical epidemics of genomic parasites, their evolutionary importance, and for evaluating which general theoretical models are most relevant for describing their evolution in natural populations. Earlier studies have established that CMS mutants are currently under strong selection in natural populations of this species (18). Here we show that the pattern of selection that CMS factors exert on the cytoplasmic genome is likely to have taken the form of purifying selection, resulting in reduced levels of genetic diversity in the cytoplasmic genomes. Our data also counter the notion that the diversity of CMS factors in *S. vulgaris* has historically been maintained by balancing selection, which would have enriched the diversity of cytoplasmic lineages. Rather, the data suggest a model of rapid turnover of lineages, such that the CMS system in *S. vulgaris* is characterized by either selective sweeps or recurrent "epidemics" of CMS mutations on a local scale.

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