

Evolutionary Rate Variation at Multiple Levels of Biological Organization in Plant Mitochondrial DNA

Daniel B. Sloan,* Camille M. Barr,† Matthew S. Olson,‡ Stephen R. Keller,* and Douglas R. Taylor*

*Department of Biology, University of Virginia; †Division of Biological Sciences, University of Montana; and ‡Institute of Arctic Biology and Department of Biology and Wildlife, University of Alaska Fairbanks

We examined patterns of mitochondrial polymorphism and divergence in the angiosperm genus *Silene* and found substantial variation in evolutionary rates among species and among lineages within species. Moreover, we found corresponding differences in the amount of polymorphism within species. We argue that, along with our earlier findings of rate variation among genes, these patterns of rate heterogeneity at multiple phylogenetic scales are most likely explained by differences in underlying mutation rates. In contrast, no rate variation was detected in nuclear or chloroplast loci. We conclude that mutation rate heterogeneity is a characteristic of plant mitochondrial sequence evolution at multiple biological scales and may be a crucial determinant of how much polymorphism is maintained within species. These dramatic patterns of variation raise intriguing questions about the mechanisms driving and maintaining mutation rate heterogeneity in plant mitochondrial genomes. Additionally, they should alter our interpretation of many common phylogenetic and population genetic analyses.

Introduction

Classic molecular evolutionary studies have established a general pattern of low substitution rates in plant mitochondrial DNA (Wolfe et al. 1987; Palmer and Herbon 1988). In recent years, however, exceptions to this pattern of slow plant mitochondrial sequence evolution have been found, highlighted by major rate accelerations in *Plantago* and *Pelargonium* (Cho et al. 2004; Parkinson et al. 2005). Given that the mechanistic basis remains elusive in these cases of elevated substitution rate, the full implications and extent of rate variation in plant mitochondrial genomes are unclear. For example, at what biological levels does rate variation occur? Does it only occur among species or is it found within species as well? Does it affect the levels of genetic variation (polymorphism) that are maintained within species? Here, we address these questions with an empirical analysis of DNA sequence polymorphism and divergence in *Silene*, expanding on earlier single-species studies in this genus (Städler and Delph 2002; Houlston and Olson 2006; Barr et al. 2007).

In comparing patterns of mitochondrial divergence, we found substantial rate variation among closely related species (fig. 1). Most notably, mitochondrial genes in *Silene noctiflora* showed extreme divergence relative to the rest of the genus—a result that was recently discovered in an independent study of rate variation among species (Mower et al. 2007). Even after excluding *S. noctiflora*, rate variation among species was still evident; a likelihood ratio test found that a model of evolution allowing for rate heterogeneity among species provided a significantly better fit to the mitochondrial data than one that enforced a molecular clock ($\chi^2_{df=5} = 54.6$; $P < 0.0001$). Among the remaining species, substitution rates differed by more than 8-fold between the fastest (*Silene paradoxa*) and slowest (*Silene latifolia*) evolving taxa. In contrast, substitution rates of 2 nuclear (*X4* and *ITS*) and 2 chloroplast loci (*trnL* and *rps16* introns)

showed no evidence for variation among species ($\chi^2_{df=5} = 4.6$; $P = 0.47$).

Analysis of multiple individuals within *Silene vulgaris* and *S. latifolia* found that patterns of rate heterogeneity extended across multiple phylogenetic scales. The concatenation of 7 mitochondrial loci revealed extensive rate variation among the different lineages of *S. vulgaris* (fig. 2), and a molecular clock test strongly rejected homogeneous rates in this species ($\chi^2_{df=39} = 111.7$; $P < 0.0001$). The consequences of intraspecific rate variation were startling. For example, slowly evolving lineages in both species have not experienced a single substitution in the highly polymorphic *atp1* locus so that both species retain the same common ancestral haplotype (as identified by haplotype reconstruction in PAML). At the other extreme, rapidly evolving lineages within *S. vulgaris* have accumulated as many as 16 substitutions in this gene. In contrast to *S. vulgaris*, no rate variation was detected within the largely invariant *S. latifolia* sample ($\chi^2_{df=27} = 30.3$; $P = 0.30$). Findings of rate heterogeneity both within and among species were consistently supported with pairwise relative rate tests (data not shown; Tajima 1993), which are not sensitive to recombination or phylogenetic uncertainty (Posada 2001).

Challenges to the concept of a molecular clock in which the accumulation of substitutions occurs at a constant rate over time have arisen since its inception (Zuckerkandl and Pauling 1965), and violations of the clock are now well established including examples in plant mitochondrial DNA (Britten 1986; Martin and Palumbi 1993; Eyre-Walker and Gaut 1997; Whittle and Johnston 2002; Mower et al. 2007). The present study and previous work showing rate variation among genes in *S. vulgaris* (Barr et al. 2007) demonstrate not only that rate variation occurs but also that it extends broadly across multiple biological scales.

As seen in other examples of rate acceleration in plant mitochondria (Cho et al. 2004; Parkinson et al. 2005), rate heterogeneity within *Silene* predominates at synonymous sites (data not shown), suggesting that differences in mutation rate are the underlying cause (Kimura 1983). The existence of rate heterogeneity both within and among species raises important and as yet unanswered questions about the relationship of mutation rate variability at different scales. Is the variation in substitution rate among *Silene* species

Key words: molecular clock, mtDNA, mutators, *Silene latifolia*, *Silene noctiflora*, *Silene vulgaris*.

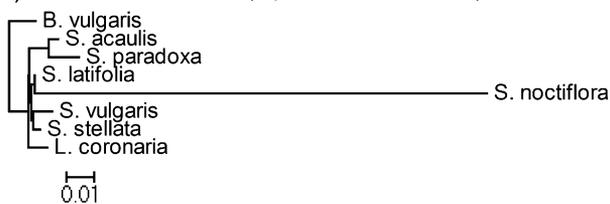
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(a) Mitochondrial Genes (atp1, cob, cox3, nad9)



(b) Nuclear/Chloroplast Genes (X4, ITS, trnL, rps16)

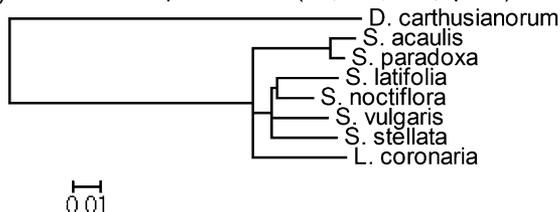


FIG. 1.—(a) Mitochondrial and (b) nuclear/chloroplast trees with branch length estimates (substitutions per site) performed in PAML (BASEML).

caused by the same mechanisms that produce rate variation among genes or among lineages within *S. vulgaris*? Is the extreme divergence of *S. noctiflora* simply a case of the more modest interspecific variation writ large, or is it the result of an entirely different mechanism? How does selection act on mutation rate variation? Identifying the mechanistic basis of mutation rate variation at these different scales is an important goal to resolve these questions and thereby better understand the processes that drive sequence evolution in the plant mitochondrial genome.

Given mutation rate variation among species, neutral theory would predict differences in intraspecific polymorphism. Comparison of 7 mitochondrial loci found substantially greater polymorphism in *S. vulgaris* than *S. latifolia* (table 1). A maximum likelihood model of the neutral coalescent process produced an estimate of the scaled mutation rate (θ) based on synonymous segregating sites that was more than 5-fold higher for *S. vulgaris* than for *S. latifolia*. For each species-specific estimate of θ , the approximate 95% confidence intervals do not overlap the corresponding maximum likelihood estimate of the other species (fig. 3); therefore, we consider these differences in polymorphism significant. In contrast, analysis of a single locus in both the nuclear and chloroplast genomes found that polymorphism in *S. latifolia* was equal to or greater than in *S. vulgaris*, consistent with previous studies of other loci in these genomes (Ingvarsson and Taylor 2002; Taylor and Keller 2007; but see Ingvarsson 2004).

At least a portion of the elevated mitochondrial polymorphism in *S. vulgaris* appears to result from accelerated mutation in a subset of lineages within the species. A local clock comparison in PAML (Yoder and Yang 2000) found the substitution rate in *S. vulgaris* to be 2-fold higher than in *S. latifolia*, but this difference was not significant. A model allowing for separate rate estimates for each species offered only a marginal improvement over one that enforced a single rate ($\chi^2_{df=1} = 3.1$; $P = 0.08$). This species-wide analysis, however, obscures the rate variation among *S. vulgaris* lineages. Relative rate tests found that the most rapidly

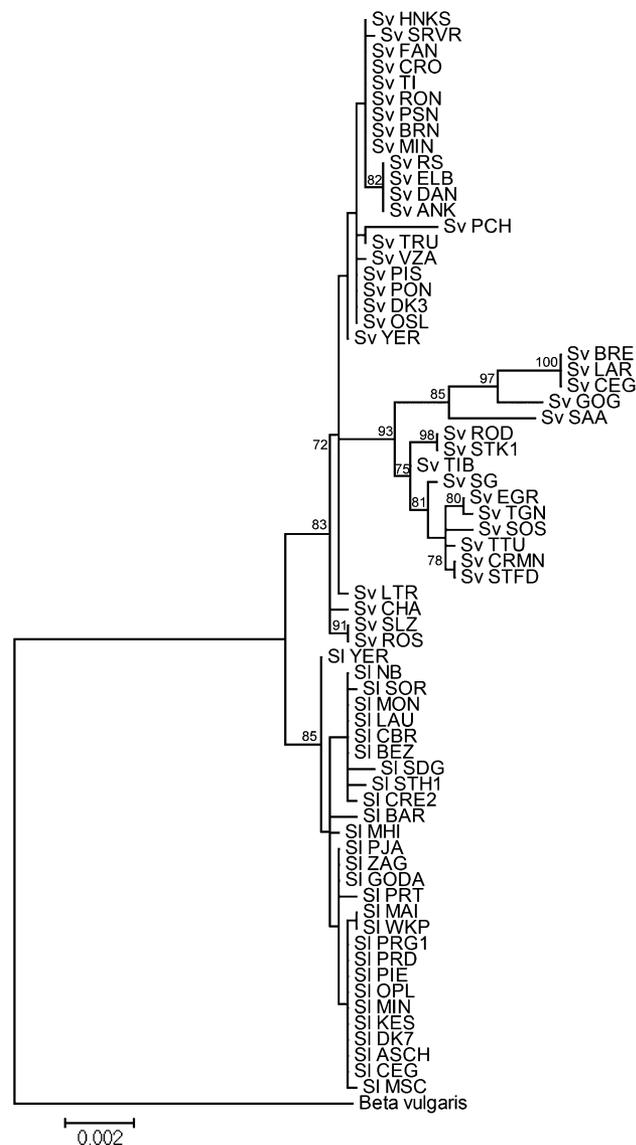


FIG. 2.—Mitochondrial tree for all *Silene vulgaris* and *Silene latifolia* individuals based on 7 concatenated loci with branch length estimates (substitutions per site) performed in PAML (BASEML).

evolving *S. vulgaris* lineage has a significantly elevated rate compared with *S. latifolia* even after considering the multiple comparisons based on 40 *S. vulgaris* samples ($\chi^2_{df=1} = 11.7$; $P = 0.0006$). At the other extreme, the most slowly evolving *S. vulgaris* lineages were indistinguishable in rate from *S. latifolia* ($\chi^2_{df=1} = 0.3$; $P = 0.57$).

Previous studies in *Silene* have interpreted patterns of polymorphism as possible evidence for balancing selection or selective sweeps acting on mitochondrial genomes (Ingvarsson and Taylor 2002; Städler and Delph 2002; Houliston and Olson 2006). Although historical patterns of selection in these species may contribute to the difference in mitochondrial polymorphism, these interpretations are generally made under an assumption of a constant mutation rate. Our findings of extensive mitochondrial rate variation suggest that the role of mutation should be taken into account even when comparing closely related species.

Table 1
Polymorphism Statistics for *Silene vulgaris* and *Silene latifolia* by Gene

	<i>S. vulgaris</i> (n = 40)		<i>S. latifolia</i> (n = 28)	
	S	π (%)	S	π (%)
Mitochondrial (in bp)				
<i>atp1</i> (970)	24 (19)	0.65 (2.32)	4 (2)	0.08 (.27)
<i>atp4</i> (291)	0 (0)	0 (0)	1 (0)	0.18 (0)
<i>atp6</i> (696)	14 (6)	0.21 (0.46)	1 (1)	0.02 (0.08)
<i>cob</i> (919)	8 (4)	0.20 (0.55)	1 (0)	0.01 (0)
<i>cox3</i> (602)	6 (0)	0.22 (0)	4 (1)	0.05 (0.05)
<i>nad9</i> (393)	1 (0)	0.09 (0)	4 (1)	0.20 (0.59)
<i>nad4L-atp4</i> (134)	4	0.32	1	0.05
Total (4005)	57 (33)	0.29 (0.75)	16 (6)	0.07 (0.15)
Chloroplast (in bp)				
<i>trnL</i> (504)	6	0.06	7	0.12
Nuclear (in bp)				
<i>X4</i> (578)	57 (36)	1.16 (3.71)	51 (37)	2.65 (8.09)

NOTE.—Values in parentheses reflect polymorphism at synonymous sites. S, segregating sites; π , nucleotide diversity.

The existence of such striking variation at multiple biological scales has far-reaching impacts for phylogenetic and population genetic analyses which commonly disregard mutation rate variation at least at some levels. Meanwhile, these patterns of rate variation suggest that the plant mitochondrial genome and the genus *Silene*, in particular, may be fertile ground for examining the evolutionary causes and consequences of mutation rates.

Materials and Methods

We extracted DNA from a single individual from each of 40 and 28 geographically dispersed populations of *S. vulgaris* and *S. latifolia*, respectively, as well as from a single individual from 6 related species (Supplementary Material online). As described previously (Barr et al. 2007), we polymerase chain reaction (PCR) amplified and sequenced 9 loci representing all 3 genomes (table 1; Supplementary Material online). For the nuclear *X4* gene, heterozygosities were scored manually in Sequencher v4.5, and multiple clones from each PCR product were sequenced to exclude paralogous Y-linked copies in *S. latifolia* males (TOPO TA Cloning Kit, Invitrogen, Carlsbad, CA).

We calculated the number of segregating sites (*S*) and nucleotide diversity (π) for each gene in *S. latifolia* and *S. vulgaris* with DnaSP v4.0 (Rozas et al. 2003). To assess the statistical significance of the difference in mitochondrial polymorphism between the 2 species, we used neutral coalescent estimates of the scaled mutation rate (θ) as described previously (Hudson 1991; Barr et al. 2007).

We performed molecular clock tests and branch length analysis with BASEML within PAML v3.15 (Yang 1997). Intraspecific tree topologies were determined by Neighbor-Joining in MEGA v3.1 (Kumar et al. 2004), whereas interspecific topologies were constrained by a supertree analysis (Supplementary Material online). Models of substitution were chosen with Modeltest v3.7 (Posada and Crandall 1998).

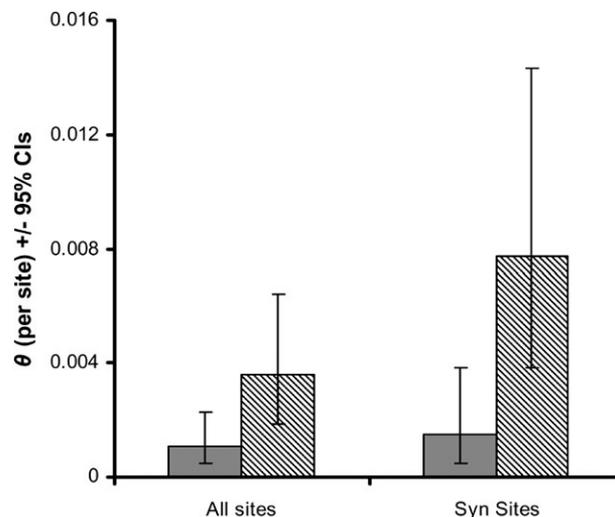


FIG. 3.—Estimates of the scaled mutation rate (θ) based on the number of segregating sites in the mitochondrial genes of *Silene latifolia* (filled) and *Silene vulgaris* (striped).

Supplementary Material

Supplementary files containing geographic information, PCR primer sequences, GenBank accession numbers, sequence alignments, and phylogenetic trees are available at *Molecular Biology and Evolution* online (<http://www.mbe.oxfordjournals.org/>).

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