

Male-Driven Evolution of Mitochondrial and Chloroplastial DNA Sequences in Plants

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Although there is substantial evidence that, in animals, male-inherited neutral DNA evolves at a higher rate than female-inherited DNA, the relative evolutionary rate of male- versus female-inherited DNA has not been investigated in plants. We compared the substitution rates at neutral sites of maternally and paternally inherited organellar DNA in gymnosperms. The analysis provided substantial support for the presence of a higher evolutionary rate in both the mitochondrial and chloroplastial DNA when the organelle was inherited paternally than when inherited maternally. These results suggest that, compared with eggs, sperm tend to carry a greater number of mutations in mitochondrial and chloroplastial DNA. The existence of a male mutation bias in plants is remarkable because, unlike animals, the germ-lines are not separated from the somatic cells throughout an individual's lifetime. The data therefore suggest that even a brief period of male and female germ-line separation can cause gender-specific mutation rates. These results are the first to show that, at least in some species, germ-lines influence the number of mutations carried in the gametes. Possible causes of male mutation bias in plants are discussed.

Introduction

Higher evolutionary rates at neutral sites in Y-linked than in X-linked genes in mammals and higher rates in Z-linked than in W-linked genes in birds indicate that the mutation rate is higher in the male germ-line than in the female germ-line (Shimmin, Chang, and Li 1993; Chang et al. 1994; Agulnik et al. 1997; Ellegren and Fridolfsson 1997; McVean and Hurst 1997; Kahn and Quinn 1999; Bohoslan, Skaletsky, and Page 2000; Huttley et al. 2000). The presence of a higher mutation rate in males has often been attributed to the greater number of cell divisions in the male germ-line (based on the assumption that most mutations arise from replication errors, Haldane 1947; Vogel and Rathenberg 1975). This opinion, however, has been questioned as increasing evidence indicates that replication-independent factors may be responsible (Shimmin, Chang, and Li 1993; McVean and Hurst 1997; Hurst and Ellegren 1998; Smith and Hurst 1999; Bohoslan, Skaletsky, and Page 2000; Huttley et al. 2000). Even though a number of studies have detected male-driven evolution among mammals and birds, to date there has not been an equivalent examination of the relative evolutionary rates of male- versus female-inherited DNA in plants. Comparisons of male- versus female-inherited DNA may not have been undertaken in plants because it may have been assumed that gender-specific mutation rates cannot exist. This assumption could be reasonable because somatic cells, and not germ-lines (Klekowski 1988, 1997), are believed to be the primary source of mutations in plant gametes and because it has often been stated that plants do not have germ-lines (Klekowski 1988, 1989, 1997). Gender-specific mutation rates could exist in plants, however, given that, first, somatic mutations may not be the only or even the primary factor affecting the

mutation rates in plant gametes and, second, separate germ-lines do develop during reproduction (Slatkin 1984). In particular, the sperm and eggs develop by separate and distinct pathways (Pennell 1988), differing perhaps in the cellular environment and number of cell divisions.

In animals, the germ-lines are maintained separately from the somatic cells; therefore, the mutations in the gametes can arise only from within the germ cells. In plants, the case is quite different. In most plants, the germ-lines develop from apical cells after they have undergone many somatic cell divisions to form the body of a plant. Because of this pattern of growth, it is believed that somatic mutations, arising either from replication errors or from replication-independent causes, can be passed to the gametes; thus, these are the primary source of all new genetic variation (Klekowski 1988, 1997). One factor that is overlooked in this perspective, however, is that germ-lines do develop in plants and that the germ-lines could be an important source or a regulator (or both) of gamete mutations (Slatkin 1984). For plants, the germ-line can be described as the lineage of cells that are committed to give rise to either a sperm or an egg following the transition of a vegetative apex to a reproductive apex (i.e., a separate cell lineage for the sperm and the egg). The cell lineage that ultimately gives rise to the gamete appears well before meiosis and originates at least as early as the formation of the archaespore cell, the precursor to the archaesporeium (Pennell 1988). The germ-line therefore consists of the cells that arise after the formation of the archaesporeium cell, including the pregametic sporogeneous cells, and ends with the formation of the gamete (because the origin of the archaesporeium cell is unknown in most taxa, it is possible that the progenitor cells of the archaesporeium cell are committed to give rise to the gamete, such that the germ-line begins before the archaesporeium cell [Pennell 1988]). Therefore, any differences between the male and the female germ-lines in plants, either replication dependent or replication independent, could lead to gender-specific mutation rates; thus, the relative evo-

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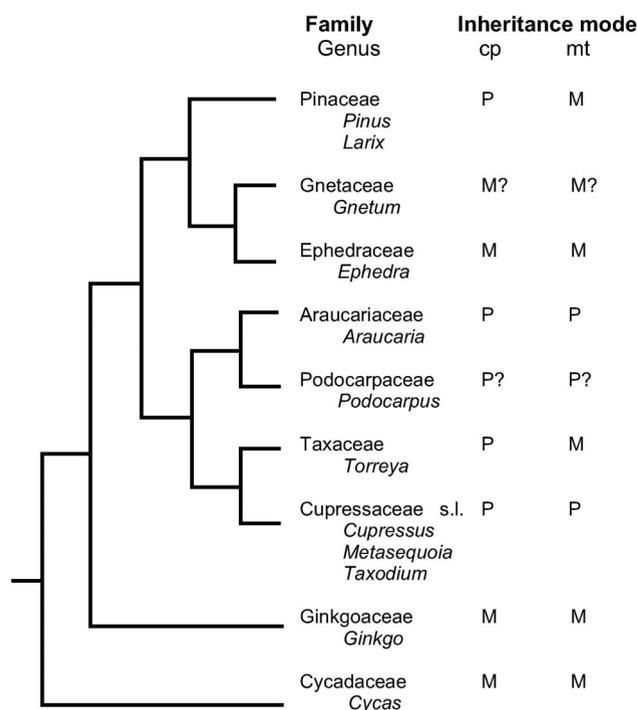


FIG. 1.—Gymnosperm phylogeny of families and genera utilized in this study according to Chaw et al. (2000) and the associated inheritance modes of the chloroplasts and the mitochondria (Mogensen 1996). “P” represents paternal inheritance, “M” maternal inheritance, “cp” chloroplast, and “mt” mitochondrion. The symbol “?” means not known with certainty. Cupressaceae s.l. (*sensu lato*) includes the Taxodiaceae.

lutionary rates of male- versus female-inherited DNA should be investigated.

The rate of substitution of selectively neutral DNA (introns, intergenic DNA, synonymous sites of coding DNA) should be equal to the mutation rate (Kimura 1983; Li 1997). The comparison of evolutionary rates of male- versus female-inherited neutral DNA in plants could therefore help determine the relative mutation rate inherent to sperm versus eggs. Although most plants do not have sex chromosomes (*Ginkgo* is an exception), differences between the evolutionary rates of male- and female-inherited DNA may be revealed by comparing the genetic distance (i.e., number of nucleotide substitutions per site) at neutral sites of paternally versus maternally inherited organellar DNA, a technique that has been utilized to disclose a male bias in mutation in the mitochondrial DNA of mussels (Rawson and Hilbish 1995; Liu, Mitton, and Wu 1996). The evolutionary rates of male- and female-inherited organellar DNA may be compared in gymnosperms because this group of plants has the unusual characteristic that some families inherit their mitochondria or their chloroplasts (or both) along a paternal line of descent, whereas others inherit them maternally (Mogensen 1996; fig. 1). The most obvious approach to detect differences between male- and female-inherited organellar DNA would be to determine the genetic distances among three taxa, two that have different organellar inheritance modes (one maternal and one paternal) and an outgroup. Subsequently, the branch

length to the taxon with maternal inheritance and the branch length to the taxon with paternal inheritance after their divergence can be compared. This approach is problematic, however, because any gender-specific differences will be confounded with all the other differences between the taxa that could affect the substitution rates (e.g., population size, taxon-specific mutation rates). This problem can be overcome by standardizing the branch length for each taxon (i.e., for the DNA region where inheritance mode differs) relative to the branch length from another part of the genome. For example, for each taxon being compared, the genetic distance for a particular mitochondrial region may be standardized by dividing the branch length for the mitochondrial DNA by the branch length in a chloroplastidial region. When all compared taxa have the same chloroplastidial inheritance mode, the chloroplast will then control for taxon-specific effects for mitochondrial comparisons. Similarly, male and female rates in chloroplastidial DNA can be compared by standardizing the chloroplastidial rates relative to the rates from another DNA region, such as the mitochondrial DNA, where inheritance mode does not differ. Thus, by comparing the standardized distances between two taxa with different inheritance modes, taxon-level effects on substitution rates are removed, and only proportionately higher or lower evolutionary rates associated with maternal or paternal organellar inheritance will be detected.

One challenge with conducting comparisons of mitochondrial and chloroplastidial DNA in plants is the current lack of DNA sequence data available for analysis. Even though chloroplastidial *rbcL* sequences tend to be readily available (Soltis et al. 1990; Bousquet et al. 1992; Gadek and Quinn 1993; Kron and Chase 1993; Morgan and Soltis 1993), other plant DNA sequences remain less so. The chloroplastidial *trnL* intron may be particularly suitable for studying evolutionary rates because it is known to have an exceptionally wide distribution in land plants, having been identified in over 330 species (Besendahl et al. 2000), and universal primers have been described that can successfully amplify DNA segments in a diverse range of species (Taberlet et al. 1991; Fangan et al. 1994; Gielly et al. 1996). In addition, this group-I intron contains a common conserved core containing four domains (P, Q, R, and S-domains) used for self-splicing, ensuring an accurate means to align sequences for even highly divergent species before phylogenetic analysis (Bhattacharya et al. 1994). In contrast to chloroplastidial DNA sequences, use of mitochondrial DNA, such as *coxI*, for studying evolutionary rates may be more complicated because of RNA editing of cytosine (C) to uracil (U) at some first and second base positions (Hiesel, Combettes, and Brennicke 1994; Steinhäuser et al. 1999; Odintsova and Yurina 2000). Evidence indicates, however, that mRNA-edited sequences and genomic DNA sequences result in similar phylogenetic relationships, both in gymnosperms and in angiosperms (Bowe and dePamphilis 1996; Pesole et al. 1996) and that genomic DNA alone serves as an effective tool for revealing evolutionary relationships. Although edited sites may be removed from genomic DNA

before analysis, this would require the collection and comparison of the mRNA and DNA sequences, a process that is unnecessary for the determination of synonymous substitution rates because the edited sites are automatically excluded, for the following reason. The main effect of RNA editing on protein-coding DNA is an increase in the rate of T-C nonsynonymous substitutions (Bowe and dePamphilis 1996; Lu, Szmidi, and Wang 1998; Szmidi, Lu, and Wang 2001), which may result from the fact that T-C mutations are, in effect, selectively neutral T-T mutations because RNA editing converts the C to U. Because these T-C substitutions are scored as nonsynonymous, they are excluded from the calculation of synonymous substitution rates; therefore, there is no benefit to removing the edited sites before analysis. Thus, the combined use of mitochondrial and chloroplastial DNA data provides an effective means to evaluate whether paternally and maternally inherited DNA evolve at different rates. Here, we examine the evolutionary rates of maternally and paternally inherited mitochondrial and chloroplastial DNA at neutral sites using *rbcL*, the *trnL* intron, and *coxI* in gymnosperms.

Materials and Methods

Sequences for *coxI*, *rbcL*, and the *trnL* intron were obtained from a total of 12 genera representing nine gymnosperm families. DNA sequences were obtained either from GenBank or by conducting sequencing using universal primers. In five cases a species from the same genus as that utilized for *rbcL* and *trnL* intron was substituted as a placeholder for *coxI*.

DNA Isolation and Amplification

Total genomic DNA was isolated from approximately 1 g of seed or leaf tissue based on the protocol of Doyle and Doyle (1990). This DNA was used as a template for PCR amplification of both *rbcL* and the *trnL* intron. *rbcL* was amplified using two pairs of overlapping primers in separate PCR reactions: (1) the forward primer 5'-ATGTCACCACAAACAGAGACTAAAGC-3' combined with reverse primer 5'-CTTCTGCTCAAATAAGATCGATCTCTCCA-3', and (2) the forward primer 5'-TGAAAACGTGAATTCCCAA-CCGTTTATGCG-3' and reverse primer 5'-GCAGCAGCTAGTTCCGGGCTCCA-3', as described by Hasebe et al. (1994). Two separate reactions were utilized because the PCR reactions using the two end primers did not yield a PCR product for most taxa. The *trnL* intron was amplified with the forward primer 5'-CGAAATCGGTAGACGCTACG-3' and reverse primer 5'-GGGGATAGAGGGACTTGAAC-3' (Taberlet et al. 1991). In some instances it was necessary to utilize the reverse primer 5'-ATTTGAACTGGTGACACGAG-3' downstream from the *trnL* intron. M13 forward and reverse extensions were added to the 5' ends of each primer to allow efficient direct sequencing of PCR products on a LICOR sequencer (M13 forward: 5'-CAGGACGTTGTAAAACGAC-3', M13 reverse: 5'-GGATAACAATTTACACAGG-3'). Each PCR reaction contained 100 ng genomic DNA, 1 × PCR buffer, 1 × Q

solution (Qiagen, Mississauga, Canada), 200 μM each dNTP, 200 nM of each primer, and 2 U *Taq* DNA polymerase (Qiagen TAQ polymerase #201203). Thermal cycling was conducted with 1 min at 94°C, 1 min at 45°C, and 1 min at 72°C for a total of 40 cycles. The PCR products were then run on 0.7% low-melting temperature agarose gels at 4°C, the desired fragments cut out and purified using Qiagen Qiaquick Gel Extraction Kit (#28704). PCR products were then quantified on a 1% agarose gel relative to a standard, to ensure that at least 500 ng was available for sequencing.

Alignment of Sequences and the Relative Ratio Test

Amino acid sequences for the translated *rbcL* and *coxI* genes and the DNA sequences for *trnL* intron were each aligned for all 12 taxa using CLUSTALW (Higgins, Thompson, and Gibson 1996), and the gaps were removed. These sequences were utilized for all further analysis to ensure that the same DNA regions were assessed in all comparisons (*coxI* = 441 bp, *rbcL* = 1,293 bp, *trnL* intron = 230 bp). To evaluate whether synonymous substitution rate differences between taxa remain proportional across loci, a maximum likelihood relative ratio test (Muse and Gaut 1997) was conducted on branch lengths for *coxI* and *rbcL* (across synonymous sites only) based on the codon substitution model, as described by Muse and Gaut (1994), and the gymnosperm phylogeny, as described by Chaw et al. (2000, fig. 1). The analysis was conducted using the software package HYPHYv0.7 (Muse and Pond 2000).

Calculation and Statistical Comparison of Standardized Distances

The principal analysis for the detection of differences in the evolutionary rates of male- versus female-inherited DNA consisted of pairwise comparisons between the standardized distance for a taxon with paternal mitochondrial inheritance (Taxon 1 in fig. 2) versus a taxon with maternal mitochondrial inheritance (Taxon 2), and pairwise comparisons between the standardized distance for a taxon with paternal chloroplastial inheritance (Taxon 1) versus a taxon with maternal chloroplastial inheritance (Taxon 2; see fig. 2). For the mitochondrial comparisons, the chloroplasts were always inherited paternally; for the chloroplastial comparisons, the mitochondria were always inherited maternally. To calculate the standardized distance of each of the two taxa to be compared, it was first necessary to determine mitochondrial and chloroplastial branch lengths. The branch length to each of the two taxa after their divergence was determined separately at *coxI*, *rbcL*, and the *trnL* intron as: $d_{01} = (d_{A1} + d_{12} - d_{A2})/2$ and $d_{02} = (d_{A2} + d_{12} - d_{A1})/2$ (Li 1997), where d_{01} = the branch length from the common node to Taxon 1, d_{02} = the branch length from the common node to Taxon 2, d_{A1} = the genetic distance from the outgroup to Taxon 1, d_{A2} = the genetic distance from the outgroup to Taxon 2, and d_{12} = the genetic distance between Taxon 1 and Taxon 2. These branch lengths were then used to calculate the standardized distance of *coxI*

Comparisons of Maternally versus Paternally Inherited Organellar DNA

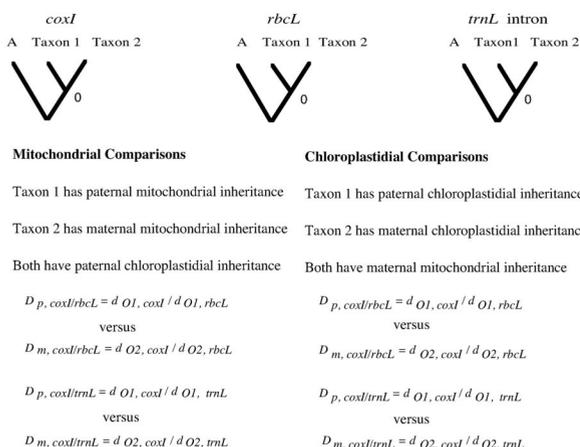


FIG. 2.—Pairwise comparisons of standardized distances. For each pairwise comparison the branch length to Taxon 1 (d_{O1}) and Taxon 2 (d_{O2}) was determined for *coxI*, *rbcL*, and for the *trnL* intron. Standardized distances were calculated as the ratio of the branch length of *coxI* relative to *rbcL* and the ratio of *coxI* relative to the *trnL* intron. Comparisons were conducted for the standardized distance for Taxon 1 versus the standardized distance for Taxon 2. See text for details.

relative to *rbcL*: $D_{p,coxI/rbcL} = d_{O1,coxI}/d_{O1,rbcL}$ (taxon with paternal inheritance) and $D_{m,coxI/rbcL} = d_{O2,coxI}/d_{O2,rbcL}$ (taxon with maternal inheritance) and the standardized distance of *coxI* relative to the *trnL* intron: $D_{p,coxI/trnL} = d_{O1,coxI}/d_{O1,trnL}$ (taxon with paternal inheritance) and $D_{m,coxI/trnL} = d_{O2,coxI}/d_{O2,trnL}$ (taxon with maternal inheritance; fig. 2), for each of the two compared taxa. The standardized distance was then compared between Taxon 1 (paternal inheritance) and Taxon 2 (maternal inheritance), once for *coxI* relative to *rbcL* and separately for *coxI* relative to the *trnL* intron. Thus, the mitochondrial rate was standardized by the chloroplastidial rate in both the mitochondrial and chloroplastidial comparisons. Use of the same standardization method in the mitochondrial and chloroplastidial comparisons not only provides consistency but also stabilizes the ratios by having the larger value (i.e., chloroplastidial) in the denominator. Thus, in contrast to the mitochondrial comparisons, in the chloroplastidial comparisons the taxon with the higher evolutionary rate will have a lower standardized distance.

To statistically test whether the standardized distances differed between the taxon with paternal versus maternal inheritance, the standardized distance was calculated for 100 bootstrap replicates for each taxon per comparison. Codon sequences of *coxI* and *rbcL* were bootstrapped 100 times using the program Codonbootstrap 2.22 (Bollback 2001), and the *trnL* intron was bootstrapped 100 times using the software program PHYLIP 3.5c (Felsenstein 1993). The genetic distance was determined at synonymous sites of *coxI* and *rbcL* using the codon substitution model described by Yang and Nielsen (2000) and as implemented in the PAML software package (Yang 2000). For the *trnL* intron, genetic distance was determined at all sites using the substitution model described by Tamura and Nei (1993) in the software package MEGA (Kumar, Tamura, and Nei

1994) using the default value of gamma = 1. For each paired comparison, this procedure resulted in 100 bootstrapped genetic-distance values among the two compared taxa and the outgroup at *coxI*, *rbcL*, and the *trnL* intron. These values were used to calculate the mean bootstrapped standardized distances. Two-tailed *P*-values for each comparison were obtained by doubling the proportion of bootstrap replicates where the standardized distance of Taxon 1 with paternal inheritance (D_p) exceeded or was less than (whichever was smaller) the standardized distance for Taxon 2 with maternal inheritance (D_m).

To quantify the size of any gender effect on mitochondrial evolutionary rates, the ratio of the mean standardized distances was determined as \bar{D}_p/\bar{D}_m for each comparison. For comparisons of maternally and paternally inherited chloroplastidial DNA, in contrast, the evolutionary rate of paternally inherited DNA relative to maternally inherited DNA was quantified as \bar{D}_m/\bar{D}_p because the chloroplastidial DNA is in the denominator of the standardized distance. Thus, for both mitochondrial and chloroplastidial DNA, a ratio of mean standardized distances exceeding unity signifies a higher paternal rate.

Relative Rate Tests

To discern any absolute differences in evolutionary rates among male- and female-inherited DNA, maximum likelihood relative rate tests were conducted for: (1) each pairwise comparison between a taxon with maternal versus a taxon with paternal mitochondrial inheritance at *coxI* based on the codon substitution model described by Muse and Gaut (1994), (2) each pairwise comparison of a taxon with maternal chloroplastidial inheritance versus a taxon with paternal chloroplastidial inheritance at *rbcL* using the model of Muse and Gaut (1994) and at the *trnL* intron based on the model described by Tamura and Nei (1993), using the software package HYPHYv0.7. These relative rate tests were conducted to determine whether the standardized distances allowed the detection of rate differences between male- versus female-inherited DNA that would not have been apparent from the comparison of absolute differences in rates between taxa.

Reference Taxa

For relative rate tests and the calculation of branch lengths for standardized distances, *Ginkgo biloba* was used as the reference taxon for all comparisons not involving itself (i.e., for any comparisons among the conifers: *Araucaria* spp., *Cupressus corneyana*, *Metasequoia glyptostroboides*, *Podocarpus* spp., *Taxodium distichum*, *Ephedra* spp., *Gnetum ula*, *Larix* spp., *Pinus strobus*, or *Torreya nucifera*) because it is a member of the most closely related family that can be used as an outgroup (fig. 1). For those cases where evolutionary rate of *G. biloba* was compared to the conifers, *Cycas* spp. was used as the outgroup because it is a member of the most closely related family that is an outgroup to both *G. biloba* and the conifers (fig. 1).

Results

In the mitochondrial analyses, the five taxa representing paternal inheritance were *C. corneyana*, *M. glyptostrobooides*, *Podocarpus* spp., *Araucaria* spp., and *T. distichum*. The three taxa representing maternal mitochondrial inheritance were *Larix* spp., *P. strobus*, and *T. nucifera*. In each pairwise comparison, mitochondrial (*coxI*) branch lengths following the divergence of the two taxa were standardized by branch lengths in chloroplastidial regions, either *rbcL* (15 comparisons) or the *trnL* intron (9 comparisons). Eleven of the 15 mitochondrial comparisons in which branch lengths in *coxI* were standardized by *rbcL* were statistically significant, all with the paternal standardized distance, $D_{p,coxI/rbcL}$, exceeding the maternal standardized distance, $D_{m,coxI/rbcL}$ (table 1). The four cases of nonsignificance involved *T. nucifera* as the taxon with maternal mitochondrial inheritance. All nine of the comparisons in which *coxI* branch lengths were standardized by those in the *trnL* intron showed a statistically significantly greater standardized distance in the taxon with paternal mitochondrial inheritance ($D_{p,coxI/trnL} > D_{m,coxI/trnL}$; table 1).

In contrast to mitochondrial comparisons, a higher evolutionary rate in male-inherited chloroplastidial DNA would give lower values for the paternal standardized distance than the maternal standardized distance because the chloroplastidial DNA is in the denominator of the standardized distance. In the chloroplastidial analyses, the three taxa representing paternal chloroplastidial inheritance were *Larix* spp., *P. strobus*, and *T. nucifera*. The two taxa with maternal chloroplastidial inheritance were *Ephedra* spp. and *G. biloba*. The standardized paternal distance was smaller in all seven comparisons; it was statistically significantly smaller in two of the three *rbcL* comparisons and all four of the *trnL* comparisons. The smaller paternal values indicate that the male-inherited chloroplastidial DNA evolved faster in all seven comparisons.

Overall (both mitochondrial and chloroplastidial DNA), 29 of 31 comparisons of standardized distances showed a higher neutral substitution rate in paternally than in maternally inherited DNA. A total of 26 of the 31 comparisons were individually statistically significant, all showing a higher paternal rate.

The relative ratio test between *coxI* and *rbcL* across all 12 genera was statistically significant at $P < 1.0 \times 10^{-6}$, indicating that synonymous substitution rates for the two DNA regions are not correlated. Relative rate tests at *coxI* indicated that taxa with paternal mitochondrial inheritance, namely *A. heterophylla*, *C. corneyana*, *M. glyptostrobooides*, and *P. macrophyllus*, had significantly higher evolutionary rates in this gene than *Larix* sp., *P. strobus*, and *G. biloba*, with maternal mitochondrial inheritance (table 2). In addition, *Ephedra* spp., with maternal mitochondrial and chloroplastidial inheritance, evolved significantly faster at the mitochondrial gene *coxI* than each taxon with paternal mitochondrial inheritance and also evolved faster at the *trnL* intron than each taxon with paternal chloroplastidial inheritance (table 2).

Discussion

Standardized Distances

That the standardized distance of taxa with paternal mitochondrial inheritance, namely *C. corneyana*, *M. glyptostrobooides*, *T. distichum*, *Araucaria* spp., and *Podocarpus* spp., was consistently greater than the standardized distance of taxa with maternal mitochondrial inheritance, namely *Larix* spp. and *P. strobus*, for *coxI* relative to *rbcL* suggests that the synonymous sites of the mitochondrial gene *coxI* evolve faster in taxa with paternal mitochondrial inheritance, relative to the chloroplastidial DNA (table 1). A relatively higher evolutionary rate in paternally inherited DNA is also supported by the significantly higher values of the standardized distance for *Podocarpus* spp. (with paternal mitochondrial inheritance) than the standardized distance for *T. nucifera* (with maternal mitochondrial inheritance) of *coxI* relative to *rbcL* and by the consistently higher values of the standardized distance in the taxa that have paternal mitochondrial inheritance for *coxI* relative to the *trnL* intron (table 1). The consistency of results across both standardized distance measures, *coxI* relative to *rbcL* and *coxI* relative to the *trnL* intron, is remarkable, given that *rbcL* and the *trnL* intron have very different absolute evolutionary rates, suggesting that the "paternal effect" may be of substantial magnitude. Altogether, 20 of the 24 comparisons of the standardized distances indicated that the mitochondrial DNA evolved faster in taxa with paternal mitochondrial inheritance (four were inconclusive for *T. nucifera*), thereby suggesting that the sperm tends to carry a higher number of mutations in mitochondrial DNA than the eggs.

In contrast to the mitochondrial DNA, a higher evolutionary rate in paternally inherited chloroplastidial DNA leads to lower values for the standardized paternal distance than for the standardized maternal distance, resulting from a relatively higher evolutionary rate in the *trnL* intron and the *rbcL* gene. The higher standardized distance for *Larix* spp. and *P. strobus*, with paternal chloroplastidial inheritance, than the standardized distance for *Ephedra* spp. and *G. biloba*, with maternal chloroplastidial inheritance, both for *coxI* relative to *rbcL* and for *coxI* relative to the *trnL* intron, suggests that paternally inherited chloroplastidial DNA does evolve faster than maternally inherited DNA (table 1). A lowered evolutionary rate in the maternally inherited chloroplastidial DNA of *Ephedra* spp. is remarkable, given that this taxon has a significantly higher absolute substitution rate than *Larix* spp. and *P. strobus* for both *coxI* and the *trnL* intron (table 2). Furthermore, *G. biloba* showed no significant differences in the relative rate tests when compared with *Larix* spp. or *P. strobus* at the *trnL* intron (table 2), suggesting that the use of standardized distances was again critical to the detection of differences in maternally and paternally inherited DNA and that relative rate tests alone would have impeded the detection of such differences. Nevertheless, the relatively higher evolutionary rate in paternally inherited chloroplastidial DNA suggests that sperm tends

Table 1
Pairwise Comparisons of the Standardized Distances Between Taxa with Paternal (D_p) and Taxa with Maternal (D_m) Mitochondrial Inheritance and Between the Standardized Distances for Taxa with Paternal (D_p) and Taxa with Maternal Chloroplastidial Inheritance (D_m), d_{01} and d_{02} Represent the Branch Lengths for the Taxon with Paternal Inheritance and the Taxon with Maternal Inheritance, Respectively. Statistical Differences were Detected as the Proportion of Bootstrap Replicates Where D_p was Greater than or Less than D_m . The Use of 100 Bootstrap Iterations Causes the Minimum Detectable P -Value to be “ <0.01 .”

TAXON WITH PATERNAL INHERITANCE	MEAN COXI		MEAN RBCL		MEAN TRNL INTRON		COXI RELATIVE TO RBCL				COXI RELATIVE TO TRNL INTRON			
	d_{01}	d_{02}	d_{01}	d_{02}	d_{01}	d_{02}	Mean D_p coxI/rbcl	Mean D_m coxI/rbcl	\bar{D}_p/\bar{D}_m^a	P	Mean D_p coxI/trnL	Mean D_m coxI/trnL	\bar{D}_p/\bar{D}_m^a	P
Mitochondrial comparisons														
<i>Cupressus corneyana</i> ...	0.121	-0.004	0.348	0.118	0.115	0.081	0.368	-0.038	> ^b	0.02	0.116	-0.055	>1	<0.01
<i>Larix</i> spp.	0.119	0.005	0.354	0.142	0.115	-0.071	0.352	0.051	6.9	0.02	0.352	NC ^c	—	—
<i>Pinus strobus</i>	0.016	0.012	0.223	0.027	—	—	0.076	-0.126	>1	0.52	—	—	—	—
<i>Torreya nucifera</i>														
<i>Metasequoia glyptostroboides</i> ...	0.053	-0.004	0.295	0.154	0.236	0.046	0.203	-0.030	>1	<0.01	0.243	0.000	>1	0.02
<i>Larix</i> spp.	0.111	0.008	0.309	0.186	0.242	0.024	0.374	0.039	9.6	<0.01	0.483	0.065	7.4	0.01
<i>Pinus strobus</i>	0.019	0.012	0.155	0.044	—	—	0.082	0.044	1.9	0.48	—	—	—	—
<i>Torreya nucifera</i>	0.191	-0.007	0.325	0.157	0.105	0.069	0.644	-0.036	>1	<0.01	2.020	-0.206	>1	<0.01
<i>Podocarpus</i> spp.	0.194	0.007	0.341	0.191	0.118	0.053	0.589	0.033	17.8	<0.01	1.880	0.031	60.6	0.02
<i>Pinus strobus</i>	0.115	0.042	0.289	0.154	—	—	0.411	0.293	1.4	0.04	—	—	—	—
<i>Torreya nucifera</i>	0.105	0.003	0.201	0.153	0.048	0.074	0.536	0.032	16.8	<0.01	2.760	-0.077	>1	<0.01
<i>Larix</i> spp.	0.103	0.013	0.189	0.159	0.052	0.052	0.582	0.081	7.2	<0.01	2.300	0.281	8.2	<0.01
<i>Pinus strobus</i>	0.055	0.076	0.152	0.141	—	—	0.371	0.571	0.4	<0.46	—	—	—	—
<i>Torreya nucifera</i>	0.119	-0.007	0.299	0.152	0.112	0.048	0.429	-0.064	>1	<0.01	1.160	-1.150	>1	<0.01
<i>Taxodium distichum</i> ...	0.121	0.005	0.303	0.174	0.120	0.029	0.415	0.021	19.8	0.02	1.100	0.180	6.11	0.04
<i>Pinus strobus</i>	0.020	0.006	0.461	0.017	—	—	0.152	0.283	0.5	0.98	—	—	—	—
<i>Torreya nucifera</i>														
Chloroplastidial comparisons														
<i>Larix</i> spp.	0.006	0.316	—	—	0.050	0.414	—	—	—	—	0.206	0.798	3.9	<0.01
<i>Ephedra</i> spp. ^d	0.017	0.314	—	—	0.026	0.417	—	—	—	—	0.343	0.786	2.3	0.02
<i>Pinus strobus</i> ...	-0.001	0.027	0.208	0.086	0.088	0.060	-0.017	0.292	>1	<0.01	-0.017	0.581	>1	<0.01
<i>Ephedra</i> spp.	0.009	0.024	0.215	0.097	0.056	0.062	0.044	0.261	5.9	<0.01	0.172	0.435	2.5	0.02
<i>Ginkgo biloba</i>	0.118	0.039	0.267	0.224	—	—	0.477	0.817	1.7	<0.10	—	—	—	—
<i>Pinus strobus</i> ...														
<i>Torreya nucifera</i> ...														

^a The relative evolutionary rate of paternally inherited DNA equals D_p/D_m for mitochondrial comparisons and equals D_p/D_p for chloroplastidial comparisons.
^b >1 , the ratio of evolutionary rates for paternally inherited DNA relative to maternally inherited DNA cannot be calculated because either the numerator or denominator is equal to or less than zero. Nevertheless, it is evident that D_p and D_m are significantly different in the direction indicated by their means.
^c NC, not calculable because the denominator is zero in some of the bootstrap replicates.
^d The branch lengths for *Ephedra* spp. could not be calculated for *rbcl* because it is saturated at synonymous sites relative to the outgroup.

Table 2
Relative Rate Tests for *coxI*, *trnL* Intron and *rbcL* (entries in bold are statistically significant, probabilities are in parenthesis).

Relative rate tests for <i>coxI</i>	TAXON WITH MATERNAL MITOCHONDRIAL INHERITANCE			
	<i>Ephedra viridis</i>	<i>Ginkgo biloba</i>	<i>Larix</i> spp.	<i>Pinus strobus</i>
<i>Araucaria heterophylla</i>	Mat > Pat (1.8 × 10 ⁻⁴)	Pat > Mat (0.015)	Pat > Mat (3.7 × 10 ⁻⁵)	Pat > Mat (7.2 × 10 ⁻⁴)
<i>Cupressus corneyana</i>	Mat > Pat (3.2 × 10 ⁻³)	Pat > Mat (0.011)	Pat > Mat (3.0 × 10 ⁻⁶)	Pat > Mat (6.6 × 10 ⁻⁵)
<i>Metasequoia glyptostroboides</i>	Mat > Pat (1.6 × 10 ⁻³)	Pat > Mat (0.019)	Pat > Mat (5.0 × 10 ⁻⁶)	Pat > Mat (1.3 × 10 ⁻⁴)
<i>Podocarpus macrophyllus</i>	Mat > Pat (0.065)	Pat > Mat (2.9 × 10 ⁻⁴)	Pat > Mat (1.0 × 10 ⁻⁶)	Pat > Mat (1.0 × 10 ⁻⁶)
<i>Taxodium distichum</i>	Mat > Pat (1.9 × 10 ⁻³)	Pat > Mat (0.011)	Pat > Mat (1.0 × 10 ⁻⁶)	Pat > Mat (6.6 × 10 ⁻⁵)

Relative rate tests for <i>rbcL</i> and <i>trnL</i> intron	TAXON WITH MATERNAL CHLOROPLASTIDIAL INHERITANCE	
	<i>Ephedra viridis</i>	<i>Ginkgo biloba</i>
<i>Araucaria angustifolia</i>	Mat > Pat (1.0 × 10 ⁻⁶)	Pat > Mat (3.2 × 10 ⁻⁴)
<i>Cupressus corneyana</i>	Mat > Pat (3.5 × 10 ⁻⁴)	Pat > Mat (0.017)
<i>Larix</i> spp.	Mat > Pat (1.0 × 10 ⁻⁶)	Pat > Mat (3.5 × 10 ⁻³)
<i>Metasequoia glyptostroboides</i>	Mat > Pat (0.0234)	Pat > Mat (7.0 × 10 ⁻⁶)
<i>Pinus strobus</i>	Mat > Pat (1.0 × 10 ⁻⁶)	Pat > Mat (7.3 × 10 ⁻³)
<i>Podocarpus reichei</i>	Mat > Pat (7.0 × 10 ⁻⁶)	Pat > Mat (1.0 × 10 ⁻⁶)
<i>Taxodium distichum</i>	Mat > Pat (1.4 × 10 ⁻⁴)	Pat > Mat (1.5 × 10 ⁻⁵)
<i>Torreya nucifera</i>	—	Pat > Mat (1.5 × 10 ⁻⁵)

Note.—Relative rate tests for *Ephedra* spp. could not be calculated for *rbcL* because it is saturated at synonymous sites relative to the outgroup.

to have a relatively greater number of mutations in chloroplastidial DNA than eggs.

Magnitude of the Male Bias

The ratio of the standardized distance for taxa with paternal mitochondrial inheritance relative to the standardized distance of taxa with maternal mitochondrial inheritance (\bar{D}_p/\bar{D}_m) indicates that the substitution rate of male-inherited DNA may be 6 to 10 times higher than the female rate in these gymnosperms and possibly even higher. This range is derived from the ratio of standardized distances for the comparisons between Pinaceae and the members of the clade containing Podocarpaceae, Cupressaceae, and Araucariaceae. These particular comparisons are the most apt to give accurate estimates of the number of mutations in sperm relative to eggs because these taxonomic groups have probably had contrasting inheritance modes for similar time periods (i.e., because the ancestral form is maternal mitochondrial inheritance, the paternal inheritance mode was probably derived in the lineage that gave rise to Podocarpaceae, Cupressaceae, Araucariaceae, and Taxaceae and occurred before these families diverged from one another, see fig. 1). Nevertheless, these values represent the lowermost estimates of male-bias because the difference in inheritance modes did not occur immediately following the phylogenetic divergence.

For the comparisons of maternally versus paternally inherited chloroplastidial DNA, the ratio of standardized distances (\bar{D}_m/\bar{D}_p) for the significant tests indicates that the mutation rate for the sperm is at least two- to sixfold higher than that for the eggs (table 1), close to the lowermost estimates for mitochondrial DNA. This suggests that the male effect could potentially be lower in chloroplastidial DNA than mitochondrial DNA, a phenomenon that could arise because of physiological differences between the male and female germ-lines. For example, a higher metabolic rate or a higher number of mitochondrial replications in the male germ-cells (i.e., the product of the number of cell divisions and number of mitochondrial replications per cell division, Birky and Skavril 1984) could magnify male effects on the mutation rate in the mitochondrial DNA. Similar to mitochondrial comparisons, these ratios underestimate the relative number of mutations in sperm versus the eggs because the difference in inheritance modes did not occur immediately following the phylogenetic divergence of the compared taxa.

The ratio of standardized distances reflects the overall male bias in mature sperm versus mature eggs. These ratios underestimate the relative number of mutations in the male versus the female germ-line. This is because the number of mutations in the gametes could result from both germ-line mutations and somatic mutations. Therefore, the relative number of mutations in the sperm versus the eggs may differ from the relative number of mutations in the male versus the female germ-line, to the extent that somatic mutations are passed to the gametes. Furthermore, it should be noted that the ratio of standardized distances might be an im-

precise measure of the magnitude of the male bias, given the high variance associated with ratios and the fact that these ratios were determined using the means of the standardized distances that themselves have variances. In this regard, the magnitude of the male bias may range considerably beyond that suggested by the ratios of standardized distances.

Possible Causes of the Lack of Significance in Some Comparisons

Even though the totality of the data suggests the presence of a higher substitution rate in paternally inherited DNA, it is noteworthy that the standardized distance for *T. nucifera* (with maternal mitochondrial inheritance) was not statistically significantly different from the standardized distance for *C. corneyana*, *M. glyptostroboides*, *Araucaria* spp., or *T. distichum* (with paternal inheritance) in the comparisons of maternally and paternally inherited mitochondrial DNA. It appears that maternal mitochondrial inheritance is very recently derived in *T. nucifera* of the Taxaceae, which occurs at a branch tip in a clade otherwise exhibiting paternal mitochondrial inheritance (Mogensen 1996; fig. 1) The branch length for this taxon in the comparisons to gymnosperm families with paternal mitochondrial inheritance therefore represents both a period of paternal inheritance and only a short subsequent period of maternal inheritance, a situation that may impede the detection of "maternal effects" in this particular taxon. In contrast, the lack of a significantly higher value for the standardized distance for *G. biloba* (with maternal chloroplastidial inheritance) than for *T. nucifera* (with paternal inheritance) in the chloroplastidial comparisons is inconsistent with a slow maternal rate, given the long period of divergence of maternal and paternal chloroplastidial inheritance for these two taxa. An important factor that may impede the detection of differences in this case may be "leakiness" of chloroplastidial inheritance (whereby some component of the chloroplasts are transmitted paternally, Yamada, Miyamura, and Hori 1993), that could potentially dilute the male or female effect on substitution rates. We do not know whether this phenomenon occurs more often in certain taxa. Nevertheless, the fact that the standardized distance for *T. nucifera* was significantly lower than the standardized distance for *Podocarpus* spp. in the mitochondrial comparisons and that the standardized distance for *G. biloba* was considerably higher than the paternal distance for *T. nucifera* in the chloroplastidial comparisons suggests that the maternally inherited mitochondrial DNA evolves relatively slower and that the paternally inherited chloroplastidial DNA evolves relatively faster in *T. nucifera*, both consistent with male-driven evolution of DNA sequences.

Germ-Lines are Involved in Determining the Number of Mutations in Their Gametes

The evidence suggesting a gender-specific effect on mutation in these gymnosperms is consistent with the results in mussels, humans, primates, and birds, where the silent substitution rates in male- and female-inherited

DNA indicate an excess of male-induced mutations (Shimmin, Chang, and Li 1993; Chang et al. 1994; Rawson and Hilbish 1995; Liu, Mitton, and Wu 1996; Agulnik et al. 1997; Ellegren and Fridolfsson 1997; McVean and Hurst 1997; Kahn and Quinn 1999; Bohossian, Skaletsky, and Page 2000; Huttley et al. 2000). In animals, the higher mutation rate in males has been attributed to a higher number of cell divisions, and therefore an increased number of replication errors, in the male germ-line (Li 1997). In humans, the female germ cells stop dividing before birth and complete with egg maturity, whereas male germ cells continuously divide, resulting in more than 32 male-divisions for each female cell division by age 45 (Vogel and Rathenberg 1975; Crow 1997). There is also strong evidence, however, that the higher mutation rate in males results from replication-independent differences between the germ-lines (Shimmin, Chang, and Li 1993; McVean and Hurst 1997; Hurst and Ellegren 1998; Smith and Hurst 1999; Bohossian, Skaletsky, and Page 2000; Huttley et al. 2000) that may include differences in the expression of DNA repair genes, different levels of cellular mutagens, or a higher level of DNA methylation in the male germ-line (or all) (Bestor 1998; Blackshear et al. 1998; Smith and Hurst 1999; Huttley et al. 2000). In plants, it is possible that any of these factors, either replication dependent or replication independent, could account for the presence of a higher mutation rate in the sperm than in the eggs; thus, these results cannot contribute to the ongoing debate in animals. The trend toward male-driven evolution in these plants does imply, however, that the germ-lines are somehow involved in determining the number of mutations that exist in their gametes.

Analysis of X-, Y-, and autosomal-linked genes in rodents indicates that the relatively higher evolutionary rate in males is not the result of a higher number of cell divisions in the male germ-line but rather is the result of an adaptive link between the hemizygous expression of X-linked genes and a reduction in the mutation rate. For this reason, Smith and Hurst (1999) suggested that the higher mutation rate reported in males is really the result of a lowered mutation rate in X-linked DNA than Y-linked DNA and therefore is not caused by a greater number of mutations arising in the male germ-line. For plants, there is evidence from *Silene* (Caryophyllaceae) indicating that the X-linked DNA has greater neutral genetic variation than Y-linked DNA. In contrast to mammals, however, it was found that this result cannot be explained by different mutation rates in X-linked and Y-linked genes (the purpose of that study was not to compare male and female rates, but it could suggest that the DNA of different chromosomes evolves at different rates, Filatov et al. 2000, 2001). These studies using sex chromosomes suggest that the comparison of evolutionary rates in X- and Y-linked DNA may not be the best means to evaluate whether paternally and maternally inherited DNA evolve at different rates because other explanations for rate variation are possible. The trend of a higher mutation rate in the sperm reported in this study, however, cannot be explained by a different mutation rate in different parts of the genome. This is because we

compared the evolutionary rate of a single DNA region (i.e., *coxI*, *rbcL*, or the *trnL* intron) when it is carried by the male germ-line with the evolutionary rate when it is carried by the female germ-line. Therefore, it may be inferred that the trend of a higher rate of neutral evolution for paternally inherited DNA is the result of being carried in the male germ-line rather than in the female germ-line. The results presented here therefore suggest, at least for plants, that a fundamental difference between the male and female germ-lines may be responsible for male-driven evolution.

Possible Scenarios that Could Lead to Male-Driven Evolution in Plants

There are many possible scenarios that could explain why male-driven evolution exists in plants, each of which suggests a different role for somatic mutations. One possible scenario is that somatic cell divisions are the primary cause of mutation in plants and that these somatic mutations are passed on more often to the male germ-line than to the female germ-line, thereby causing the higher number of mutations rate in the sperm. This scenario is possible, given the complexity of the pattern of apical cell divisions, such that the male germ-line may develop from a more mutation-prone somatic cell lineage (e.g., more actively dividing) than the female germ-line. A second possible explanation for male-driven evolution in plants is that the somatic mutations are common but that these mutations are rarely transferred to the gametes. Many somatic mutations occur during the formation of internodes after the apical cells have already progressed; therefore, they are not passed to the gametes (Antolin and Strobeck 1985). In this case, the higher number of mutations in the sperm would necessarily result from differences between the male and female germ-lines, either replication dependent or replication independent. A third possibility is that somatic mutations in plants are rare relative to germ-line mutations; therefore, they are not an important source of mutations in the gametes. According to this scenario, the male bias would have to be caused by differences, either replication dependent or replication independent, in male and female germ-lines. One way that somatic mutations could be rare events compared with germ-line mutations is if the germ-lines arise from a portion of the apical meristem that is largely quiescent during somatic growth, such that the number of somatic mutations arising from replication errors is minimal. Even though quiescent apical cells have been identified in many plant species, whether these regions give rise to the reproductive structures remains uncertain (Klekowski 1988; Lyndon 1998). Nevertheless, it is apparent, under each of these possible scenarios, that the germ-lines are somehow involved in determining the number of mutations that are passed to the gametes.

Given that replication-independent factors could at least partially account for male-driven evolution, it is worthwhile to consider the replication-independent differences between the male and the female germ-lines. Upregulation of the putative nucleotide excision DNA

repair homologue ERCC1 in the male germ-line cells of *Lilium longiflorum* (Xu et al. 1998) indicates that cells of the male germ-line may have increased DNA repair compared with the female germ-line, suggesting that there could be a greater likelihood of DNA repair errors in the sperm because of the higher levels of DNA damage requiring repair than in the eggs. Another possible replication-independent explanation for a higher mutation rate in males is that the sperm is more susceptible to damage-induced mutations because it is released from the parent plant upon maturity and is therefore exposed to solar and UV radiation, chemical mutagens, and fungal and bacterial toxins, and to dehydration (Friedberg 1985) that could lead to increased DNA-damage-induced mutations in the male germ-line. Nevertheless, because little is currently known regarding the physiology of the male and female germ-lines in plants, it is likely that many other, yet unidentified, factors could account for a higher mutation rate in the male gametes.

Lack of Correlation Among Genes

That the relative ratio test between *coxI* and *rbcL* was statistically significant indicates that the synonymous substitution rates for these two DNA regions are not correlated among these gymnosperm taxa. This is in contrast to synonymous substitution rates observed in some studies of angiosperms, where the longer phylogenetic branch length of grasses relative to palms is proportional for three separate DNA regions, the nuclear *Adh* gene, the chloroplastidial *rbcL* gene, and the mitochondrial *atpA* gene (Gaut et al. 1996; Eyre-Walker and Gaut 1997). The correlation among different DNA regions in angiosperms suggests that organism-level effects, such as population size or taxon-specific mutation rates, play an important role in determining the evolutionary rates of selectively neutral DNA. The absence of proportionality between *coxI* and *rbcL* reported in this study, however, does not necessarily mean that organism-level effects do not exist in gymnosperms. Rather, the lack of correlation among these two genes may indicate that organism-level effects are overridden (or hidden) by gender-specific mutation rates. Organism-level effects on neutral substitution rates may be more readily detected in angiosperms because, unlike gymnosperms, the inheritance modes of organellar DNA do not extensively vary among taxa.

Significant Issues Pertaining to the Study of Gender-Specific Mutation Rates

Even though the results here suggest the presence of male-driven evolution of DNA sequences in plants, it is important to consider some of the limitations inherent to the study. In particular, it should be noted that even though the synonymous sites of coding DNA and of introns are generally believed to be selectively neutral, there is evidence that some level of selection can act on these DNA regions. For example, introns may be conserved at their splicing sites for RNA (Long and Deutsch 1999), and synonymous sites of coding DNA can undergo some level of selection because of the bio-

chemistry of translation and the patterns of codon usage within a gene (Akashi, Klimin, and Eyre-Walker 1998). In this regard, it is possible that some of the substitution rates for these taxa were influenced by selective forces that could potentially dilute or magnify the results seen in some of the comparisons. An additional factor to consider is that the mitochondrial replication rate could vary among taxa. Although the mitochondria must replicate before cell division (Brown 1983), mitochondrial replacement may occur during the lifetime of cells that have a high metabolic rate because of damage to the membrane from metabolic byproducts (Rand 1994). This would only be an important factor, however, if the number of mitochondrial replications per unit time is associated with the number of mutations and if taxa with paternal inheritance each coincidentally have a higher metabolic rate in the male germ-line. It would be much more likely that such an association, if it existed, would not be coincidental and would suggest that a higher metabolic rate in the male germ-line causes male mutation bias. Another important consideration is that for the comparisons involving Pinaceae and for the comparisons involving Cupressaceae, more than one genus was utilized. Because Pinaceae is such a large taxonomic group and because Cupressaceae s.l. may represent more than one family (i.e., the Taxodiaceae), we concluded that it was worthwhile to include more than one genus in these comparisons. Nevertheless, it should be made clear that the comparisons involving the different genera within the Pinaceae and the different genera within the Cupressaceae are not completely independent of one another because the branch lengths up to the point of the phylogenetic divergence of the genera would have been shared. It should also be realized that although the phylogeny used is not known with certainty, a revised phylogeny would not alter our conclusions as long as *Cycas* and *Ginkgo* remained as outgroups. In addition to these three considerations, it is important to recognize that we examined mitochondrial and chloroplastidial DNA in this study because they allow the comparison of the relative evolutionary rates of paternally and maternally inherited DNA by assessing the effect of being carried in the male versus the female germ-line. Equivalent comparisons could not be conducted on nuclear DNA because it is inherited biparentally and therefore we cannot make any inference about the nuclear DNA. Further approaches that may help resolve the relative effect of the male and female germ-lines on mutation rates in plants include the examination of shorter-lived taxa that have differential organellar inheritance modes, such as *Cucumis*, where the ratio of somatic mutations to gender-specific mutations is apt to be lowered (Havey 1997; Havey et al. 1998), and the further examination of gymnosperm taxa across a greater range of mitochondrial and chloroplastidial genes. In addition, increased morphological information regarding the pattern of cell division leading to sperm and egg formation in a range of plant taxa as well as studies about the differential expression of DNA repair genes in sperm and egg will help to further resolve the factors that could cause male-driven evolution of DNA sequences in plants.

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