INFLUENCE OF GENERATION TIME, GENDER AND DNA DAMAGE ON THE MUTATION RATE IN SEED PLANTS

by

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ABSTRACT

Although mutation is a fundamental process in evolution and disease, little is known about the factors that affect the mutation rate, especially for plants. Five separate studies were conducted, each aimed towards the assessment of factors that affect the mutation rate in seed plants. Firstly, from an examination of molecular evolutionary rates of selectively neutral DNA (which equals the mutation rate) of phylogenetically independent pairs of closely related plant species, it was shown that, contrary to widely accepted opinion, generation time does not generally affect molecular evolutionary rates in plants. Secondly, an analysis of molecular evolutionary rates of selectively neutral maternally and paternally inherited organellar DNA of gymnosperms, lead to the discovery that the sperm contains more selectively neutral mutations than an egg. Thirdly, from the comparison of the fitness effects of UV on maternal and pollen parents, it was demonstrated that more deleterious mutations in the nuclear genome are transmitted by a sperm than by an egg in *Arabidopsis thaliana*. Fourthly, an assessment of the parallels between the p53-related pathway, the most well-characterized process for DNA repair in humans and mice, and the process of germination of aged seeds (which contain DNA damage) indicated that plants may eliminate DNA damage by a p53-related pathway. Fifthly, it is described why, in many cases, the mutation rate does not almost entirely depend on replication errors arising during the synthesis (S) stage of the cell cycle, as contended by many studies, but also depends on factors other than S-stage replication errors. Overall, the results suggest that although generation time does not influence the mutation rate in plants, gender does affect both the neutral mutation rate and the transmission of deleterious mutations. The implications of these results to understanding the cause of most mutations are discussed.

LIST OF ABBREVIATIONS

ANOVA, analysis of variance

cp, chloroplast

CI, confidence interval

CDK, cyclin dependent kinase

CKI, cyclin dependent kinase inhibitor

coxI, cytochrome I oxidase

C, cytosine

DNA, deoxyribonucleic acid

E2F, E2F transcription factor

ETS, external transcribed spacer

F1, first generation of offspring

HDM2, human double minute 2

HYPHY software, Hypothesis Testing using Phylogenetics

ITS1 and *ITS2*, internal transcribed spacer 1 and 2

M, maternal

MGT, minimum generation time

mt, mitochondria

MEGA software, Molecular Evolutionary Genetics Analysis

MDM2, murine double minute 2

-, mutant

dNTP, (one of four) nucleotide triphophates

P, paternal

PAR, photosynthetically active radiation

Pol δ, (DNA) polymerase δ

PCR, Polymerase chain reaction

PCNA, proliferating cell nuclear antigen

PAML, Phylogenetic Analysis using Maximum Liklihood

PHYLIP software, Phylogeny Inference Package

Rb, retinoblastoma

RNA, ribonucleic acid

rbcL, ribulose 1, 5, bisphosphate

F2, second generation of offspring

TAQ, *Thermus aquaticus*

T, thymine

trnL intron, transcribed RNA Leucine intron

UV, ultraviolet radiation

U, uracil

VGR, vertical growth rates

+, wild type

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GENERAL INTRODUCTION

Mutation is the ultimate source of genetic variation and plays a critical role in biological processes including the onset of diseases (Crow 1997) and the evolution of sex (Kondrashov 1994, Keightley and Eyre-Walker 2000), ploidy levels (Kondrashov and Crow 1991, Ramsey and Schemske 1998), mating systems (Byers and Waller 1999), Y chromosomes (Gordo and Charlesworth 2001) and aging (Yampolsky *et al.* 2000) as well as the magnitude of inbreeding depression (Johnston and Schoen 1994, Deng and Lynch 1997), the rate of adaptation (Orr 2000) and the occurrence of species extinctions (Higgins and Lynch 2001). Information about the types of factors that influence the mutation rate is therefore central to evaluating and understanding many evolutionary processes as well as the pattern of disease. To date, however, there is considerable uncertainty about the types of factors influencing the mutation rate, especially for plants.

Most information about the types of factors that influence the mutation rate has been based on evolutionary rate analysis of DNA sequences. Such analyses are based on the assumption that the mutation rate equals the nucleotide substitution rate in selectively neutral DNA (Kimura 1983). By using DNA sequence analysis, it has been shown that generation time is an important factor associated with the substitution rate in animals. Specifically, the generation-time hypothesis predicts that the substitution rate is higher in selectively neutral DNA of animal taxa with shorter generation times because they undergo more DNA replications, and therefore have more replication errors, per unit time (Laird *et al.* 1969, Wu and Li 1985). Recently, many studies have suggested that generation-time effects also exist in plants (Gaut *et al.*1996, Laroche and Bousquet 1997,

Andreason and Baldwin 1999). The relationship between generation time and evolutionary rates in plants, however, has not been firmly established because the studies conducted to date have examined very few species and/or genes and often have lacked phylogenetic independence. A broader range of species is therefore needed to evaluate whether there is a general relationship between generation time and mutation rates in plants.

Another factor in addition to generation time that has been found to be associated with mutation rates in animals is gender. Specifically, analyses of substitution rates of selectively neutral DNA on sex chromosomes indicates that mutation rates are higher in male-inherited DNA, a trend that is commonly believed to be caused by the higher number of DNA replications, and therefore replication errors, in the male germline (Haldane 1947, Miyata *et al.*1987, Shimmin *et al.* 1993). Although higher mutation rates in males have been reported in many animals, there has not been, to date, an assessment of the influence of gender on mutation rates in plants. There are at least two probable explanations. Firstly, it has probably been assumed that gender cannot affect mutation rates because plants do not have separate germlines throughout their lifespan. Secondly, an experimental system has not been available to distinguish between male and female mutations in plants (i.e., most plants lack sex chromosomes). Probably for similar reasons, there has not been any study of the relative transmission of deleterious mutations, those that have substantial evolutionary consequences, by a plant sperm and an egg. In order to develop a better understanding of the influence of gender on the mutation rate and the transmission of mutations to progeny in plants, new types of scientific approaches, which can distinguish between male and female mutations, need to be developed.

An important component to understanding the factors that influence the mutation rate is the development of an appreciation of how most mutations arise. To date, the cause of most mutations has fallen into one of two broad categories. Firstly, and most commonly, it is believed that mutations are caused by replication errors occurring during the synthesis (S) stage of the cell cycle (Laird *et al.* 1996, Wu and Li 1985). Secondly, it has been hypothesized that mutations are caused by other factors including DNA damage or by events occurring during the resting stage (i.e., G0 or G1 stage, Evans *et al.* 1994, Advin *et al.* 2002). The former case is supported by the presence of generation time-effects and male-mutation bias in many animals. The latter case is supported by the higher evolutionary rate in organisms with higher metabolic rates (and thus contain more DNA damaging byproducts, Martin 1999), the lack of generation time- and gender-effects in some organisms, and the occurrence of mutations in non-dividing cells (Evans *et al.* 1994). An assessment of the types of factors associated with evolutionary rates of selectively neutral DNA across a range of organisms is an important step in the development of an understanding of whether replication errors are, as is often contended, almost entirely responsible for all mutations or whether many mutations arise from other factors.

The level of mutations that ultimately arise from DNA damage largely depends on the ability of a cell to remove such damage. For animals, the p53 pathway is known to be essential for this purpose. Following DNA damage, the p53 pathway becomes activated, which causes the cell to arrest in the G1 stage, which in turn, allows DNA repair to occur before DNA replication (Burns and El-Deiry 1999). In contrast to animals, there is relatively little information about how plants respond to DNA damage, or whether a p53 pathway may exist. A potentially useful experimental system from which to examine

whether a p53 pathway exists in plants is aged seeds. Aged seeds may be suitable for this purpose because they tend to contain substantial levels of DNA damage, which needs to be managed before or during germination. Therefore, an assessment of the known parallels between the germination of aged seeds and the animal p53 pathway is an important preliminary step in determining whether plants respond to DNA damage by a p53-related pathway.

This thesis consists of five separate studies, each aimed towards the identification and characterization of factors that influence the mutation rate in seed plants. The first chapter describes an assessment of whether generation-time effects exist in plants using more than 40 pairs of phylogenetically independent comparisons of closely related taxa with different generation times. This chapter has been published in the Journal of Molecular Evolution. The second chapter is a study of whether male-mutation bias, which has been reported in many animal species, also exists in plants. A new method is described for assessing any gender-effect on mutation in plants that involves the comparison of the evolutionary rates of selectively neutral male and female inherited organellar DNA. The chapter has been published in Molecular Biology and Evolution. The third chapter describes an experimental approach to assessing whether pollen and maternal parents tend to transmit different levels of UV-induced deleterious mutations to the progeny in *Arabidopsis thaliana* and has been published in Proceedings of the National Academy of Sciences USA. Chapter Four outlines the circumstantial case for why a p53 related DNA repair pathway may exist in plants, based on the many parallel events between the germination of aged seeds and p53 pathway in humans and mice. This chapter has been published in Trends in Plant Science. The fifth chapter describes an analysis of the currently available scientific information, including that described in

Chapters One to Four, for and against the theory that most mutations are S-stage replication errors. It is suggested that the mutation rate depends, in many cases, on the level of DNA damage and the levels of mutation that occur during the resting stage of the cell cycle. Finally, a conclusion section is provided, describing what the results of Chapters one to five indicate about the types of factors that affect the mutation rate in plants and about how most mutations arise. Permission was granted by each journal to reprint the article for the purposes of this thesis given that the full citation was provided. Each respective journal holds the copyright© and the complete citations are listed below.

Chapter 1
Whittle, C.A. and M.O. Johnston. 2003. Broad-scale analysis contradicts the theory that generation time affects molecular evolutionary rates in plants. Journal of Molecular Evolution **56**: 223-233.

Chapter 2
Whittle, C.A. and M.O. Johnston. 2002. Male-driven evolution of mitochondrial and chloroplastidial DNA sequences in plants. Molecular Biology and Evolution **19**: 937-949.

Chapter 3
Whittle, C.A. and M.O. Johnston. 2003. Male-biased transmission of deleterious mutations to the progeny in *Arabidopsis thaliana*. Proceedings of the National Academy of Sciences USA. **100**: 4055-4059.

Chapter 4
Whittle, C.A., T.L. Beardmore, and M.O. Johnston. 2001. Is G1 arrest in plant seeds induced by a p53 pathway? Trends in Plant Science **6**: 248-251.

CHAPTER 1

BROAD-SCALE ANALYSIS CONTRADICTS THE THEORY THAT GENERATION TIME AFFECTS MOLECULAR EVOLUTIONARY RATES IN PLANTS

INTRODUCTION

The generation-time theory originates from studies in animals and predicts that taxa with shorter generation times have a higher molecular evolutionary rate at selectively neutral DNA because there is an inverse correlation between generation time and the number of germ-line cell divisions, and therefore replication-induced mutations, per unit time (Laird *et al.* 1969, Wu and Li 1985, Li 1997). The most well documented case of a generation-time effect is the faster evolution of rodents than primates (Laird *et al.* 1969, Ohta 1993, Easteal and Collet 1994, Wu and Li 1985, Li *et al.* 1987, Li 1997, Weinreich 2001). The generation-time theory has also been applied to plants. There is now a widely accepted opinion that the selectively neutral DNA of annuals evolves faster than in perennials (Charlesworth *et al.* 2001) and that taxa with shorter minimum generation times (time to first flowering) evolve faster than those with longer minimum generation times. Specifically, minimum generation time has been proposed as an explanation for the higher evolutionary rate of grasses than palms at synonymous sites of the chloroplastidial *rbcL* and *ndhF* genes and the nuclear *Adh* gene (Gaut *et al.* 1996,1997) and the rate variation of monocotyledonous taxa at the *rbcL* gene (Gaut *et al.* 1992). In addition, differences in annual/perennial life history are believed to explain the higher molecular evolutionary rates in annual than perennial angiosperms at synonymous sites of the mitochondrial *coxI* gene and at the *rps3* introns (Laroche *et al.* 1997, Laroche

and Bousquet 1999), and in annual than perennial species of *Lupinus* (Fabaceae) and *Sidalcea* (Malvaceae) at the nuclear internal transcribed spacer sequences (*ITS1* and *ITS2*, Aïnouche and Bayer 1999, Andreasen and Baldwin 2001). The notion that the generation-time pattern applies to plants is precarious (Gaut *et al.* 1992, 1996,1997, Aïnouche and Bayer 1999), however, because the studies conducted to date either compare widely divergent groups, which differ in many aspects other than generation time, or are limited to several closely related species within a genus, where the changes in life history may not be independent. In addition, there is no theoretical basis to support generation-time effects in plants. The notion that generation-time effects exist in plants may be partially attributable to increased tendency to publish studies that show a relationship between evolutionary rates and generation times over those that do not show such effects. Consequently, there is a need for a thorough analysis across a broad range of plant taxa to assess whether generation time, including annual/perennial life history, can explain molecular rate variation in plants.

To effectively evaluate whether minimum generation time or annual/perennial life history can explain rate variation in selectively neutral DNA of plants, it is first necessary to address the limitations of studies conducted to date. Failure to control for phylogenetic bias has been nearly unavoidable in the studies that have utilized a large number of pairwise relative rate tests between taxa with different annual/perennial life histories within a single genus (e.g., Aïnouche and Bayer 1999, Andreasen and Baldwin 2001). The bias, which arises from the multiple use of a single portion of a branch length in multiple tests (Felsenstein 1985), seriously impedes the ability to make generalizations about generation-time effects. This problem may be addressed by ensuring that no more

than one pairwise comparison is conducted within a single genus or family. A benefit to such an approach is that the difference in the phylogenetic branch lengths between the two species following their divergence constitutes an independent data point, which when combined with other such points can reveal statistically sound relationships between generation time and evolutionary rates. Another problem with studies that have shown generation-time or life-history effects is that the compared taxa have often been highly divergent. For example, the proposition that the faster evolution of grasses than palms may be due to minimum generation time and that the faster evolution of primrose (*Oenothera*, Onagraceae) and petunia (*Petunia*, Solanaceae) than birch (*Betula*, Betulaceae) and alder (*Alnus*, Betulaceae) (Gaut *et al.* 1992, 1996,1997, Laroche and Bousquet 1999) may be attributed to annual/perennial life history is, as noted by the respective authors, inconclusive because the compared taxa are so divergent that many other differences could explain the rate variation. These differences include plant size at maturity, the pattern and number of pre-gametic cell divisions per generation, physiological properties, developmental patterns, and/or exposure to environmental agents resulting from light conditions, temperature or microenvironment. In order t o attribute rate variation to minimum generation time or annual/perennial life history, it is imperative that the compared taxa be as closely related as possible to minimize all other differences between them (Thorne *et al.* 1998). The use of phylogenetically independent comparisons combined with the close relatedness of each species per comparison, provides an effective means to assess whether generation time or life history are related to molecular evolutionary rates across a range of plant taxonomic groups (Bromham *et al.* 1996).

A critical factor in determining whether rate variation is related to minimum generation time or annual/perennial life history, within the context of phylogenetically independent comparisons of species pairs, is the statistical test utilized. The statistical analysis must capture the direction of the difference in the genetic distance between each taxon per species pair, without incorporating the magnitude of the difference. There are several important reasons why the magnitude of the difference must be excluded. First, the difference in the phylogenetic branch lengths of the two compared species per pair partly depends on their degree of relatedness, which will vary considerably among species pairs. Therefore, if the magnitude of the difference is included, the pairs that are most highly divergent may be weighted more. To avoid this, each pair must be weighted equally, based on the direction of the difference and not its magnitude. Second, within a species pair, the timing of the switch from perenniality to annuality or from short to long minimum generation times (or visa-versa) could occur near the branch tip, such that most of the branch length would have evolved under the opposite life form. In such cases, the differences in genetic distances are likely to be small, and would be diluted unless all comparisons are weighted equally. The issue of the timing of the switch in life history or in minimum generation time is a major drawback to studies that use relative rate tests, because such tests cannot distinguish whether a statistically insignificant comparison results from the lack of generation-time effects or from a small difference in branch lengths resulting from a recent change in annual/perennial life history or minimum generation time (Aïnouche and Bayer 1999, Muse and Gaut 1994). By equally weighting each pair, even when the rate difference is small, and by including a range of species pairs, one avoids this problem. Two statistical tests that meet all these requirements are

the sign test and the G-test (Sokal and Rohlf 1995). These tests can compare the number of positive to the number of negative differences between the branch lengths of the two taxa per pair, across all species pairs. Although the G-test is slightly more powerful when assessing deviation from a 50:50 ratio, it cannot be used when expected frequencies are less than five, or when all comparisons are in one direction. The sign test is therefore more generally suitable. An important additional advantage of the sign test over both parametric and other nonparametric tests is that it does not require a symmetrical error distribution (Hollander and Wolfe 1999). In this study, a generically extensive analysis was conducted, based on independent comparisons of species pairs, that incorporates sign tests to assess whether minimum generation time or annual/perennial life history can explain molecular rate variation in seed plants.

METHODS

Evaluation of Life-History and Minimum Generation-Time Effects

Species pairs were chosen based on availability of the complete DNA sequences of18S *ITS1* and *ITS2* regions from Genbank and the availability of information regarding annual/perennial life history and minimum generation time. A total of 24 independent species pairs of an annual and a perennial and a total of nine independent species pairs of long-lived woody taxa containing a taxon with a short and a long minimum generation time were chosen (Table 1). Each pair was chosen from a single genus or from a single family. Because phylogenetic relationships are often uncertain, the following approach was used to ensure independence of pairs. No more than one pair per genus was examined. For those cases where inter-generic taxa were paired, no other taxa from that

Table 1. Species pairs and their taxonomic families.

Family	Species 1	Accession Number	Species 2	Accession Number	Family of Reference Species	Reference Species	Accession Number	Phylogeny Citation
Comparisons of Annual/Perennial Life History								
	Annual		Perennial					
Rosaceae	*Aphanes arvensis*	AF183538 AF183515	*Pyrus calleyana*	U16202	Moraceae	*Ficus albipila*	AF165375	Reference from another family
Brassicaceae	*Arabidopsis thaliana*	AJ232900	*Arabidopsis lyrata*	AJ232889	Brassicaceae	*Arabis turrita*	AJ232906	Koch *et al.* 2001[a]
Fabaceae	*Astragalus epiglottis*	U50506	*Astragalus membran-aceous*	AF121675	Fabaceae	*Glycyrrhiza echinata*	AJ232906	Wojciech-owski *et al.* 1999[b]
Poaceae	*Bromus briziformis*	U83356 U83357	*Bromus racemosus*	U83372 U83373	Poaceae	*Brachypo-dium arbuscula*	U56000 U55999	Aïnouche and Bayer 1997[b]
Portulacaceae	*Claytonia perfoliata*	AF084152 AF084173	*Claytonia megarhiza*	L78027	Cactaceae	*Pereskia aculeata*	L78035	Downie and Palmer 1994[a]

Table 1

Polemoniaceae	*Collomia heterophylla*	AF020703	*Collomia rawsoniana*	AF208201	Polemoniaceae	*Gilia stellata*	AF208212	Johnson *et al.* 1996[a]
Onagraceae	*Epilobium cleistogamum*	L28017	*Epilobium canum*	L28013	Lythraceae	*Cuphea hookeriana*	AF201691	Reference from another family
Asteraceae	*Erigeron annuus*	AF118489	*Erigeron glabellus*	AF118498	Asteraceae	*Bidens alba*	U67107	Noyes and Rieseberg 1999[c]
Asteraceae	*Helianthus annuus*	AF047924	*Helianthus nuttallii*	AF047950	Asteraceae	*Erigeron annuus*	AF118489	Schilling *et al.* 1998[b]
Convolvulaceae	*Ipomoea nil*	AF110948	*Ipomoea batatas*	AF256642 AF256643	Solanaceae	*Capsicum baccatum*	AF244708	Reference from another family
Polemoniaceae	*Linanthus aciculatis*	AF119424 AF119450	*Linanthus floribundus*	AF119429 AF119455	Myrsinaceae	*Cyclamen africanum*	AF163999	Reference from another family
Fabaceae	*Lupinus microanthus*	AF007480	*Lupinus perennis*	Z72163 Z72162	Fabaceae	*Glycyrrhiza echinata*	U55999 U56000	Ainouche *et al.* 1999[b]; Kass and Wink 1997[b]
Asteraceae	*Machaer-anthera*	U97622	*Machaeran-thera*	AF251567	Asteraceae	*Erigeron annuus*	AF118489	Morgan 1997[b]

Table 1

	canescens		*tanacetifolia*					
Malvaceae	*Malva parviflora*	AF303031	*Malva sylvestris*	AF303021	Malvaceae	*Durio acutifolius*	AF287700	Ray 1995[c]
Fabaceae	*Medicago lupulina*	AF028388 AF028448	*Medicago prostrata*	AJ288249 AF288247	Fabaceae	*Trifolium alpinum*	AF154379 AF154603	Watson *et al.* 2000[d]
Rosaceae	*Potentilla norvegica*	U90790	*Potentilla palustris*	U90789	Rosaceae	*Malus prunifolia*	AF186500	Eriksson *et al.* 1998[c]
Ranunculaceae	*Ranunculus sceleratus*	AF323322	*Ranunculus circinatus*	AF323321	Berberidaceae	*Podophyllum hexandrum*	AF328965	Jensen 1995[b]
Saxifragaceae	*Saxifraga cymbalaria*	AF087599 AF087629	*Saxifraga latepetiolata*	AF261183	Saxifragaceae	*Saxifragella albowiana*	AF374825 AF374826	Mort *et al.* 1999[c]
Caryophyllaceae	*Silene gallica*	U30959 U30985	*Silene vulgaris*	U30969 U30996	Amaranthaceae	*Amaranthus albus*	AF210918	Reference from another family
Apiaceae	*Smyrnium olusatrum*	AH003553 U30595	*Myrrhis odorata*	AH00348 1 U30531	Araliaceae	*Stilbocarpa lyalli*	U72387	Reference from another family
Solanaceae	*Solanum nigrum*	AJ300211	*Solanum elaeagnifolium*	AF244730	Solanaceae	*Capsicum baccatum*	AF244708	Borisjuk *et al.* 1994[b]
Fabaceae	*Vicia*	AF335210	*Vicia cracca*	AF335189	Fabaceae	*Arachis*	AF203553	Raina and

Table 1

	tetrasperma			AF335190		*batizocoi*		Ogihara 1994[c]; Gimenes *et al.* 2000[d]
Violoaceae	*Viola arvensis*	AF097242 AF097288	*Viola calcarata*	AF097243 AF097289	Euphobiaceae	*Macaranga angulata*	AF361112	Reference from another family
Asteraceae	*Volutaria lippi*	L35870	*Cheirolo-phus arboreus*	AF021147 AF021164	Asteraceae	*Gazania krebsiana*	U84770	

Comparisons of Minimum Generation Time (*MGT*)

	Short *MGT*		Long *MGT*					
Aceraceae	*Acer macrophy-llum*	AF020367	*Acer saccharum*	AF401152	Aceraceae	*Dipteronia senensis*	AF020386	Suh *et al.* 2000[a]
Betulaceae	*Betula pendula*	AJ006445	*Betula alleghanie-nsis*	X68133	Betulaceae	*Alnus maritima*	X68135	Chen *et al.* 1999[a]
Juglandaceae	*Carya illinoensis*	AF303825	*Carya cordiformis*	AF303820	Juglandaceae	*Juglans nigra*	AF179579	Manos and Stone 2001[a]

Table 1

Myrtaceae	*Eucalyptis grandis*	AF390471	*Eucalyptus globulus*	AF058467	Myrtaceae	*Angophora costata*	AF190356	Steane *et al.* 1999[a]
Oleaceae	*Fraxinus excelsior*	AH004997 AH004996	*Fraxinus ornus*	AH00491 U82893	Oleaceae	*Syringa amurensis*	AF297074	Wallander and Albert 2000[d]
Juglandaceae	*Juglans nigra*	AF179579	*Juglans microcarpa*	AF179577	Juglandaceae	*Alfaroa costaric-ensis*	AF303803	Stanford *et al.* 2000[b]
Pinaceae	*Larix decidua*	AF041343	*Larix laricina*	AF041348	Pinaceae	*Picea abies*	AJ243166A J243167	Govindara ju *et al.* 1992[a]
Rosaceae	*Prunus besseyi*	AF318732	*Prunus cerasifera*	AF318755	Rosaceae	*Exochorda racemosa*	AF318740	Bortiri *et al.* 2001[a]
Fagaceae	*Quercus acutissima*	AF098428	*Quercus robur*	AF098424	Fagaceae	*Fagus sylvatica*	U93099 U93100	Samuel *et al.* 1998[b]
Ulmaceae	*Ulmus americana*	AF174640	*Ulmus rubra*	AF174642	Ulmaceae	*Celtis laevigata*	AF174621	Wiegrefe *et al.* 1994[c]

[a]Phylogeny indicates that reference taxon is from outside the clade containing the two compared taxa, [b]Phylogeny shows that the compared taxa are from a genus that is monophyletic and therefore the reference is from outside the clade containing the two compared taxa, [c]No specific evidence from phylogeny that reference taxon is from a clade separate from the two compared taxa, [d]Reference taxon is from a monophyletic genus and therefore is from outside the clade of the two compared taxa.

family were included. The reference taxon in each comparison was chosen from a closely related genus in the same family for intra-generic comparisons (from the same family as the pair of compared taxa) and from a closely related family for inter-generic comparisons (i.e., from the same order). Monophyly of families was assumed. Even though monophyly of genera may be reasonably assumed for most of the intra-generic comparisons, the appropriate phylogeny was examined in the literature whenever possible to ensure that the reference taxon is outside the clade containing the two compared taxa (Table 1). The reference was not necessarily a basal taxon, which is not necessary, but was outside the genus or family of the pair being compared.

The *ITS1* and *ITS2* sequences each contain approximately 200-270 sites. For each set of three taxa, the two compared taxa and the reference taxon, DNA sequences of *ITS1* and *ITS2* were separately aligned using Clustal W (Higgins *et al.*1996), and gaps were removed. Most comparisons had very few or no gaps. The edited sequences had between182 and 266 sites for *ITS1* and between 151 and 225 sites for *ITS2*. The branch length to each taxon per comparison following their divergence (i.e., the number of nucleotide substitutions per nucleotide site) was determined based on the maximum likelihood model described by Tamura and Nei (1993) with gamma variation and three rate classes in the software HYPHY (Muse and Pond 2000). For the comparisons of annual/perennial life history, a sign test (Wilkinson *et al.* 1992) was conducted on the branch length of the annual versus the perennial species across all 24 species pairs at *ITS1* and across all 22 species pairs at *ITS2*. For the comparisons of minimum generation time, a sign test was conducted between the branch length for the species with the shorter

versus the species with the longer minimum generation time across the nine species pairs with different minimum generation times at *ITS1* and *ITS2*.

Supplemental Analyses

To effectively interpret results obtained from the sign tests, the following supplemental analyses were conducted. First, maximum likelihood relative rate tests were conducted for each species pair for annual/perennial life history comparisons and minimum generation time comparisons according to the substitution models described above in the software package HYPHY. Second, Pearson correlation coefficients were determined between the branch lengths between the *ITS1* and *ITS2* regions for annuals, for perennials, for taxa with short minimum generation times and for taxa having longer minimum generation times to assess whether these two DNA regions evolve proportionately across the wide range of taxa examined. Third, it was assessed whether vertical growth rates, and therefore the approximate number of pre-gametic apical cell divisions per unit time, were related to phylogenetic branch lengths in the long-lived taxa. The vertical growth rates for taxa with short (*S*) and long (*L*) minimum generation times were determined as H_S/MGT_S and H_L/MGT_L, respectively, where H is the mean height at maturity, and MGT is minimum generation time. A sign test was conducted between the branch lengths for the species with the higher vertical growth rate relative to the species with the lower vertical growth rate, across all pairs. Because the generation-time theory is based on the notion that the number of pre-gametic cell divisions per unit time causes higher mutation rates, this comparison was used to assess whether pre-gametic apical cell divisions can explain rate variation in plants.

Results

For 14 of the 24 comparisons of annual/perennial life history at *ITS1* ($P = 0.541$) and 15 of 22 comparisons at *ITS2* ($P = 0.133$) the branch length was longer for the perennial taxon than for the annual (Table 2). Statistical significance ($P<0.05$) required that the annual or the perennial taxon had a longer branch length for at least18 of the 24 comparisons at *ITS1* and at least 17 of the 22 comparisons at *ITS2*. Comparisons of minimum generation time in long-lived taxa indicated that the taxon with a relatively longer minimum generation time evolved faster than the taxon with a shorter minimum generation time for five of the nine contrasts at *ITS1* ($P = 1.0$) and for three of the nine contrasts at *ITS2* ($P = 0.508$, Table 3). Statistical significance in this case required that eight of nine comparisons were in the same direction. There was no evidence for an effect of vertical growth rate on evolutionary rate at *ITS1* ($P =1.0$) or at *ITS2* ($P = 0.508$, Table 3).

For the comparisons of annual/perennial life history, none of the individual relative rate tests was statistically significant at *ITS1* and only two cases were statistically significant at *ITS2*, in opposite directions (Table 2). For the comparisons of minimum generation time, none of the relative rate tests was statistically significant at *ITS2* and only three were statistically significant at *ITS1*. Two of these tests were in the opposite direction of the third. The Pearson correlation coefficients between *ITS1* and *ITS2* branch lengths were statistically significant for both annuals ($r = 0.57$, $P = 0.007$) and for

Table 2. Comparisons of annual/perennial life history. Sign represents the direction of the difference in branch lengths. Bold entries indicate individually statistically significant comparisons using relative rate tests (two-tailed *P*-value).

Annual	Perennial	*ITS1*			*ITS2*		
		Branch Length			Branch Length		
		Annual	Perennial	Sign	Annual	Perennial	Sign
Aphanes arvensis	*Pyrus callayana*	0.105	0.233 (0.266)	-	0.209	0.335 (0.247)	-
Arabidopsis thaliana	*Arabidopsis lyrata*	0.043	0.030 (0.556)	+	0.088	0.098 (0.918)	-
Astragalus epiglottis	*Astragalus membranaceus*	0.006	0.113 (0.362)	-	0.060	0.119 (0.828)	-
Bromus briziformis	*Bromus racemosus*	0	>0 (0.997)	-	0.004	0 (0.240)	+
Claytonia perfoliata	*Claytonia megarhiza*	0.077	0.028 (0.923)	+	**0.122**	**0 (0.007)**	**+**
Collomia heterophylla	*Collomia rawsoniana*	0.020	0.029 (0.716)	-	0.077	0.028 (0.713)	+
Epilobium cleistogamum	*Epilobium canum*	0	0.029 (0.640)	-	0.004	0.016 (0.495)	-
Erigeron annuus	*Erigeron glabellus*	0.036	0.021 (0.773)	+	0.031	0.062 (0.577)	-
Helianthus annuus	*Helianthus nuttallii*	0.012	0 (0.069)	+	0	0.021 (0.573)	-

Table 2

Ipomoea nil	*Ipomoea batatas*	0.181	0	(0.163)	+	0.171	0 (0.078)	+
Linanthus acicularis	*Linanthus floribundes*	0.058	0.021	(0.108)	+	0.097	0 (0.067)	+
Lupinus microanthus	*Lupinus perennis*	0.038	0.004	(0.588)	+	0	0.020 (0.337)	-
Machaeranthera canescens	*Machaeranthera tanacetifolia*	0.028	0.014	(0.501)	+	0	0.026 (0.065)	-
Malva parviflora	*Malva sylvestris*	0.010	0.020	(0.836)	-	0	0.009 (0.132)	-
Medicago lupulina	*Medicago prostrata*	0.039	0.008	(0.252)	+	0.014	0.030 (0.303)	-
Potentilla norvegica	*Potentilla palustris*	0.048	0.060	(0.892)	-	0.083	0.346 (0.086)	-
Ranunculus sceleratus[a]	*Ranunculus circinatus*	0.005	0.037	(0.229)	-	–	–	
Saxifraga cymbalaria	*Saxifraga latepetiolata*	0.175	0.267	(0.380)	-	0.117	0.126 (0.897)	-
Silene gallica	*Silene vulgaris*	0.046	0.052	(0.863)	-	0.008	0.095 (0.266)	-
Smyrnium olusatrum	*Myrrhis odorata*	0.150	0.191	(0.689)	-	0.216	0.101 (0.451)	+
Solanum nigrum	*Solanum elaeagnifolium*	0.045	0.084	(0.255)	-	**0.049**	**0.189 (0.006)**	-
Vicia tetrasperma	*Vicia cracca*	0.019	0.047	(0.722)	-	0.020	0.047 (0.239)	-
Viola arvensis[a]	*Viola calcarata*	0	0.018	(0.074)	-	–	–	

Table 2

Volutaria lippi	*Cheirolophus arboreus*	0.091	0.047 (0.296)	+	0.051	0.043 (0.855)	+

Number of positive differences between annual and perennial branch lengths	10	7
Number of negative differences between annual and perennial branch lengths	14	15
P-value of sign-test	0.541	0.133

Annual/perennial life history provided by (Tutin *et al.* 1964, Hickman 1993). [a] The nucleotide substitution values are saturated relative to reference taxon at *ITS2*

Table 3. Comparisons of minimum generation time. *S* and *L* represents short and long minimum generation time, respectively. Sign represents the direction of the difference in branch lengths. Bold entries indicate individually statistically significant comparisons using relative rate tests (two-tailed *P*-value).

			Minimum Generation Time (*MGT*)								Vertical Growth Rates (*VGR*)			
Family	Taxon with shorter generation time	Taxon with longer generation time	MGT_S[a]	MGT_L[a]	*ITS1*			*ITS2*			VGR_S (m/ year)[b]	VGR_L (m/ year)[b]	Sign *ITS1*[c]	Sign *ITS2*[c]
					Branch Length		Sign	Branch Length		Sign				
					S	*L*		*S*	*L*					
Aceraceae	*Acer macrophyllum*	*Acer saccharum*	10	30	**0.111**	**0.045 (0.048)**	+	0.036	0.054 (0.481)	-	0.92	0.30	+	-
Betulaceae	*Betula pendula*	*Betula alleghaniensis*	15	40	0.017	0.030 (0.731)	-	0.029	0 (0.672)	+	0.40	0.23	-	+
Juglandaceae	*Carya illinoinensis*	*Carya cordiformis*	10	30	0.016	0 (0.265)	+	0.009	0.004 (0.540)	+	1.3	0.31	+	+
Myrtaceae	*Eucalyptis grandis*	*Eucalyptus globulis*	2	4	0.039	0 (0.222)	+	0.010	0.028	-	21.5	5.75	+	-

Table 3

Oleac-eae	*Fraxinus excelsior*	*Fraxinus ornus*	15	20	0.067	0.102 (0.406)	-	0.070	0.057 (0.575)	+	0.78	0.30	-	+
Jugla-ndace-ae	*Juglans nigra*	*Juglans microca-rpa*	12	20	**0.016**	**0 (0.048)**	+	0.009	0 (0.125)	+	1.15	0.09	+	+
Pinac-eae	*Larix decidua*	*Larix laricina*	10	20	0.001	0.029 (0.571)	-	0.009	0 (0.135)	+	1.20	0.6	-	+
Rosac-eae	*Prunus besseyi*	*Prunus cerasifera*	2	6	0.005	0.012 (0.446)	-	0.008	0.023 (0.243)	-	NA[e]	NA[e]	NA[e]	NA[e]
Fagac-eae	*Quercus acutissima*	*Quercus robur*	5	20	**0**	**0.098 (0.015)**	-	0.089	0 (0.412)	+	0.90	0.51	-	+
Ulma-ceae	*Ulmus americana*	*Ulmus rubra*	15	15	0.023	0.011 (0.715)	N A[d]	0	0.043 (0.482)	N A[d]	0.74	0.44	+	-
Number of positive differences in branch lengths							4			6			5	6
Number of negative differences in branch lengths							5			3			4	3
P- value of sign test							1.0			0.5			1.0	0.5

[a]*MGT* provided by Young and Young (1992), [b] The average heights, provided by Young and Young (1992) and Burns and Honkala (1990), used to calculate *VGR* are: *A. macrophyllum* 9.2m, *A. saccharum*, 9.2m, *B. pendula* 6m, *B. alleghaniensis* 9.3m, *C. illinoensis* 13m, *C. cordiformis* 9.2m, *E. grandis* 43m, *E. globulus* 23m, *F. excelsior*11.7m, *F. ornus* 6m, *J. nigra* 13.8m, *J. microcarpa* 1.8m, *L. decidua* 12m, *L. laricina* 12m, *Q. acumtissima* 4.5m, *Q. robur* 10.2m, *U. americana* 11.1m, *U. rubra* 6.6m, [c] The sign was determined as the branch length difference between VGR_S versus VGR_L, [d] Not applicable, there is no difference in *MGT*, [e] Not applicable, *P. besseyi* is not a vertical plant.

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perennials ($r = 0.60$, $P = 0.004$), but not for taxa with short ($r = 0.209$, $P = 0.563$) and with long (r=0.534, P =0.112) minimum generation times.

DISCUSSION

Annual/Perennial Life History

The absence of statistically significant differences between annuals and perennials from the sign test indicates that life history cannot explain evolutionary rate variation in these plants. That perennials evolved faster than annuals in more than 50% of the cases contradicts previous reports from more taxonomically narrow studies. Given that relative rate tests alone have been the main tool used to show annual/perennial life history effects in other studies, it is worthwhile to examine how these tests compare to the sign test. As shown in Table 2, there were two individually statistically significant differences among the 46 comparisons of annuals versus perennials, precisely what would be expected if generation time was completely unrelated to evolutionary rates (i.e., the number of significant tests = probability of a type I error X number of comparisons = 0.05 X 24 + 0.05 X 22 = 2.3). Furthermore, these two significant cases were in opposite directions. Therefore, the results of the relative rate tests taken alone would have suggested that annual/perennial life history does not affect evolutionary rates.

The lack of life-history effects reported here does not preclude the possibility that life history influenced evolutionary rates within some taxonomic groups. It is possible, for example, that life-history effects do explain the faster evolution in annual than perennial species of *Lupinus* and *Sidalcea* at the 18S *ITS1* and *ITS2* regions and at the 18S *ITS* and *ETS* regions, respectively, and the faster evolution of primrose (*Oenothera*,

Onagraceae) and petunia (*Petunia*, Solanaceae) than birch (*Betula*, Betulaceae) and alder (*Alnus*, Betulaceae) at the *rps3* intron (Aïnouche and Bayer 1999, Laroche and Bousquet 1999, Andreasen and Baldwin 2001). Nevertheless, as noted by the respective authors, the molecular rate heterogeneity in these studies was not universally correlated with differences in habit, and therefore, other factors may account for the variation. It is possible that the annuals coincidentally evolved faster than perennials within these taxonomic groups. Other studies have shown that annual/perennial life history does not explain molecular rate variation. For example, Jansen *et al.* (1991) and Wallace and Jansen (1990) demonstrated that life history could not explain rate variation in *Micorseris* (Asteraceae) and Microseridinae (Asteraceae), respectively, and Bousquet *et al.* (1992) indicated that annuals and perennials evolve at similar rates at the *rbcL* gene among certain seed plants. The results of the present analysis suggest that there is no general effect on evolutionary rate associated with the transition from perenniality to annuality, or visa-versa.

In addition to the analysis of all species pairs, it is worthwhile to consider whether including only the more divergent pairs supports the absence of an effect of annual/perennial life history on evolutionary rates. A more divergent species pair may be considered one where the combined branch lengths are greater than 0.1 substitutions/site. Using only the species pairs that meet this criterion, one sees from Table 2 that the perennial species evolved faster for six of the nine comparisons at *ITS1* and seven of the eleven comparisons at *ITS2*, a result that is consistent with the analysis across all species pairs. Although it could be argued that the results from more divergent pairs could be considered a more effective indicator of substitution-rate differences, because there is

greater time for rate differences to accumulate, they also have a greater potential for multiple changes in annual/perennial life history. Consequently, it could also be argued that the more closely related species pairs, with less opportunity for changes in annual/perennial life history, may more accurately reflect life-history effects. Nevertheless, the fact that the results from the more divergent species pairs are consistent with the results across all species pairs provides further support that annual/perennial life history does not affect evolutionary rates at *ITS1* and *ITS2*.

Minimum Generation Time

Similar to annual/perennial life history, lack of significance of the sign test in the long-lived taxa indicates that minimum generation time cannot explain substitution rate variation at the *ITS1* and *ITS2* regions. Although the analysis consists of a relatively small sample size of nine comparisons, only four of nine comparisons at *ITS1* and six of nine comparisons at *ITS2* showed that taxa with shorter generation times evolved faster. The relative rate tests are consistent with this as they show no tendency for taxa with different minimum generation times to evolve at different rates, with only three of 16 tests showing statistical significance, two of which were in the opposite direction of the third. The absence of minimum generation-time effects in plants reported here contrasts with the results of studies based on relatively narrow taxonomic groups. A major difference between this and other studies is that, here, only very closely related taxonomic groups were examined. Although it has been suggested that the faster evolution of grasses than palms is attributable to minimum generation time, the molecular rate variation may also result from any of the other differences between these two highly

divergent taxonomic groups (Gaut *et al.* 1992, 1996, 1997). The relative rate tests conducted here demonstrate that the pattern does not hold over a wider range of closely related species pairs. It is notable that evolutionary rates at *ITS1* and *ITS2* were correlated within annuals and within perennials, a characteristic that could suggest that the mutation rates of these two regions could be maintained by taxon-specific effects (Eyre Walker and Gaut 1997). Our results indicate that the correlation results from taxon-specific effects other than generation time. It has also been postulated that a correlation between the *ITS1* and *ITS2* regions results from selection arising from interdependency between the two regions because they are part of the same transcriptional unit (Baldwin *et al.* 1995).

Number of Mutations per Unit Time

The proposition that generation time affects evolutionary rates in plants has not been supported by a strong rationale for why such effects might exist. For higher animals, the generation-time theory predicts that taxa with shorter generation times evolve at a higher rate at selectively neutral DNA, because they have a greater number of germ-line cell divisions, and therefore replication-induced mutations, per unit time (Laird *et al.* 1969, Ohta 1993, Easteal and Collet 1994, Wu and Li 1985, Li 1997, Weinreich 2001). This explanation assumes that the higher number of cell divisions per unit time in shorter-generation taxa results from a larger number of gonadal generations per unit time that is not canceled by a possibly greater number of gonadal cell divisions per generation in longer-generation taxa. For plants, in contrast, both somatic and germ-line mutations can be passed to the gametes. Because somatic cell division is a continual process in both

short and long-lived taxa, the lower number of mutations per unit time associated with longer generation times reported in animals should not occur in plants (Gaut *et al.* 1996). In other words, to the degree that somatic mutations contribute to the total number of gametic (or zygotic) mutations, there will be a weakening of any negative correlation between generation time and number of mutations per unit time.

The relative importance of soma and germ-line as sources of gametic mutations remains unknown. There are two main schools of thought, one which proposes that the somatic cell divisions are the overwhelming source of gametic mutations (Klekowski 1988, Klekowski and Godfrey 1989, Klekowski 1998), and the other which suggests that replication-independent (Shimmin *et al.* 1993, McVean and Hurst 1997, Hurst and Ellegren 1998, Smith and Hurst 1999, Bohosslan *et al.* 2000, Kumar and Subrananian 2002, Whittle and Johnston 2002) and germ-line mutations (Whittle and Johnston 2002) are also important. That the sign test for the vertical growth rates of taxa with short versus long minimum generation times conducted here (Table 3) was not significant is not consistent with a major role for somatic mutations. Nevertheless, even without any information about how or where gametic mutations arise in plants, there is other evidence that the mutation rate per unit time overlaps considerably among taxa with different generation times. For example, the per-generation mutation rate for achlorophylly is 10 to 25 times higher in taxa with long minimum generation times, such as mangroves (*Rhizophora mangle*) and Scots pine (*Pinus sylvestris*), than in short-lived taxa, such as barley (*Hordeum vulgare*) and buckwheat (*Fagopyrum esculentum*, Klekowski and Godfrey 1989). Therefore, the number of achlorophyllous mutations per unit time is unlikely to be negatively correlated with minimum generation time or associated with

annual/perennial life history in plants, and may instead, be species-specific. This conclusion is consistent with the results of this study, which found no evidence for a general relationship between molecular evolutionary rates and either minimum generation time or annual/perennial life history.

Other Possible Explanations

In addition to species-specific mutation rates, there are several other factors that could contribute to the absence of a relationship between generation time and evolutionary rates at selectively neutral DNA. Given that many plants maintain seed banks for long periods (Parker *et al.*1989, Baldwin *et al.* 1995, Whittle *et al.* 1997, Baskin and Baskin 1998, Andreasen and Baldwin 2001), it is possible that for some annual/perennial comparisons, the annual underwent far fewer generations than would be predicted based on a yearly generation time. Another potential factor that could dilute an effect of annual/perennial life history is that some perennials may reproduce in the first year of growth and could therefore have the same minimum generation time as an annual. Further, some perennials may have continuous vegetative reproduction (Whittle *et al.* 1997) such that the number of generations per unit time could, on average, be higher than for an annual. Therefore, even if minimum generation time did affect evolutionary rates, this may not translate into an effect of annual/perennial life history. It has also been hypothesized that certain regions of *ITS1* and *ITS2* could be under selective constraint (Baldwin *et al.* 1995), possibly affecting evolutionary rates observed in some species pairs. It is noteworthy that although our analysis can effectively detect faster or slower rates across a range of taxa, regardless of when the life history or generation time switch

occurred (e.g., an annual life form develops near the branch tip), it is possible that multiple changes in life history may have occurred along a branch length in some comparisons (Aïnouche and Bayer 1999). If this were a frequent phenomenon, then it could partially explain why there is no general tendency for taxa with shorter minimum generation times or with annual life histories to evolve faster in plants. Although it is worthwhile to be aware of these possibilities as they may hold in particular cases, the results presented here are based on a wide range of taxonomic pairs, where the species in each pair are closely related, making it unlikely that these factors played a significant role. Rather, the lack of annual/perennial life-history and minimum generation-time effects on molecular evolutionary rates are best explained by the absence of an relationship between either of these factors and the number of mutations per unit time.

Conclusions

One of the most important goals of molecular evolutionary biology is the determination of the factors influencing the rate of evolution. This study provides evidence against one factor widely believed to be important in plants. The absence of generation-time effects also has important implications for molecular, population, plant, and evolutionary biologists interested in the factors underlying mutation rates and plant physiology and development. Further investigation into the number of mutations per generation among a range of plant taxa, and the factors that influence the mutational process (e.g., environmental conditions, metabolic rate of pregametic cells, the relative frequency of germ-line and somatic mutations in gametes), will be essential for developing a better understanding of molecular evolutionary rate variation in plants.

CHAPTER 2

MALE-DRIVEN EVOLUTION OF MITOCHONDRIAL AND CHLOROPLASTIDIAL DNA SEQUENCES IN PLANTS

INTRODUCTION

Higher evolutionary rates at neutral sites of 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 *et al.* 1993, Chang *et al.* 1994, Agulnik *et al.* 1997, McVean and Hurst 1997, Ellegren and Fridolfsson 1997, Kahn and Quinn 1999, Bohosslan *et al.* 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 *et al.* 1993, McVean and Hurst 1997, Hurst and Ellegren 1998, Smith and Hurst 1999, Bohosslan *et al.* 2000, Huttley *et al.* 2000). Even though a number of studies have detected male-driven evolution among mammals and birds, there has not been to date 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 reasonably obtained because somatic cells, and not germ-lines (Klekowski 1988,

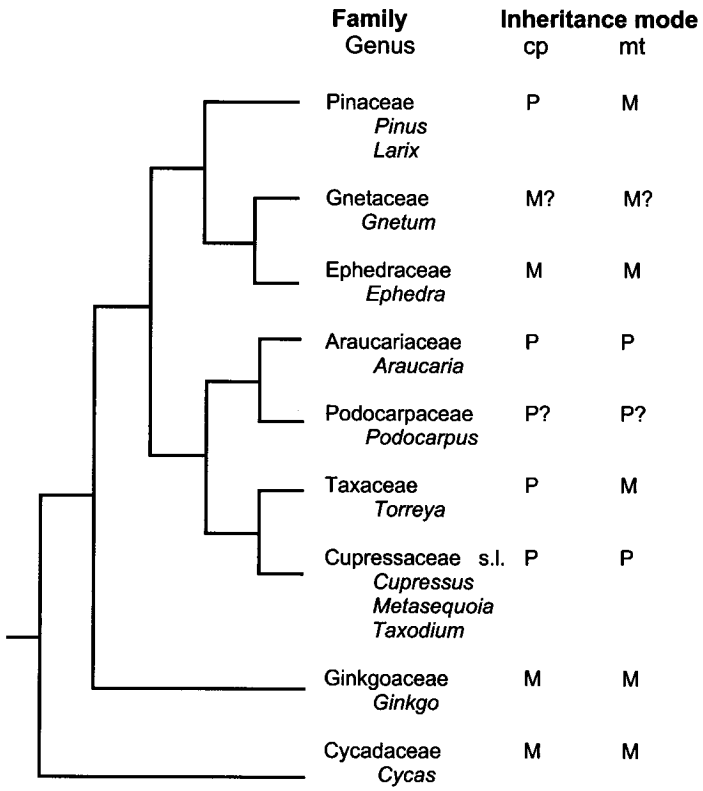
1997), are believed to be the primary source of mutations in plant gametes and because it has often been stated that plants do not have germ-lines (Klekowski 1988, 1989,1997). Gender-specific mutation rates could exist in plants, however, given that, first, somatic mutations may not be the only or even the primary factor affecting the mutation rates in plant gametes and, second, that 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 cellular environment and number of cell divisions.

In animals, the germ-lines are maintained separately from the somatic cells and 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 and 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 of and/or regulator 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 to 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 archaesporial cell, the precursor to the archaesporium (Pennell 1988). The germ-line

therefore consists of the cells that arise following the formation of the archaesporium cell, including the pre-gametic sporogeneous cells, and ends with the formation of the gamete (because the origin of the archaesporium cell is unknown in most taxa, it is possible that the progenitor cells of the archaesporium cell are committed to give rise to the gamete, such that the germ-line begins before the archaesporium 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, and thus the relative evolutionary 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 *et al.* 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 and/or their chloroplasts along a paternal line of descent while others inherit them maternally (Mogensen 1996, Figure 1). The most

Figure 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 and "M" represents maternal inheritance. The symbol "?" means not known with certainty. Cupressaceae s.l. (sensu lato) includes the Taxodiaceae. "cp" represents chloroplasts and "mt" represents mitochondria.



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 were 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 Quin 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 prior to 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 due to RNA editing of cytocine (C) to uracil (U) at some first and second base positions (Hiesel *et al.* 1994, Steinhauser *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 *et al.* 1998, Szmidt *et al.* 2001), which may result from the fact that T-C mutations are in effect selectively neutral T-T mutations as 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, and, therefore, there is no benefit of removing the edited sites before analysis. Thus, the combined use of mitochondrial and chloroplastidial DNA data provides an effective means to evaluate whether paternally and maternally inherited DNA evolve at different rates. Here, the evolutionary rates of maternally and paternally inherited mitochondrial and chloroplastidial DNA at neutral sites using *rbcL*, the *trnL* intron and *coxI* in gymnosperms are examined.

METHODS

Sequences for *coxI*, *rbcL*, and the *trnL* intron were obtained from a total of twelve genera representing nine gymnosperm families. DNA sequences were obtained either from Genbank or from conducting sequencing using universal primers (Table 4). 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 1g 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 i) the forward primer

Table 4. Location and accession number of plant material and the Genbank accession numbers for each taxon used in this study.

Species	Location/Accession Number of Plant Material	Genbank Accession Number		
		rbcL	*trnL* intron	*coxI*
Araucaria angustifolia	Ecology and Evolutionary Biology Greenhouse, University of Connecticut	U96470	AF479866 [a]	*Araucaria heterophylla* AF020558
Cupressus corneyana	University of California Botanical Garden/ 74.1004	AF479876 [a]	AF479868 [a]	AF020561
Cycas circinalis	Cycads and Palms/8318	L12674	AF479867 [a]	*Cycas revoluta* AF020562
Ephedra sinica	Ecology and Evolutionary Biology Greenhouse	D10732	*Ephedra viridis* AF479869 [a]	*Ephedra viridis* AF020564
Ginkgo biloba	National Arboretum USA/ NA56509	D10733	AF479871 [a]	AF020565
Gnetum ula	University of California Botanical Garden/63.0192	AF479877 [a]	AF479870 [a]	*Gnetum leyboldii* AF020566
Larix laricina	National Tree Seed Center Canada	AF479878 [a]	AF479872 [a]	*Larix* sp. X94590
Metasequoia glyptostroboides	National Arboretum USA/ PI161688	AJ235805	AB030050	AF020569

Table 4

Pinus strobus	National Tree Seed Center Canada/ 9310182	AF479880[a]	AF479875[a]	AF020574
Podocarpus reichei	Ecology and Evolutionary Biology Greenhouse, University of Connecticut	AF479879[a]	AF479873[a]	*Podocarpus macrophyllus* AF197620
Taxodium distichum	National Arboretum USA	AF119185	AF479874[a]	AF020578
Torreya nucifera	-	AB027317	-	AF020580

[a], were sequenced in this study.

5'ATGTCACCACAAACAGAGACTAAAGC3' combined with reverse primer 5'CTTCTGCTACAAATAAGAATCGATCTCTCCA3' and ii) the forward primer 5'TGAAAACGTGAATTCCCAACCGTTTATGCG3' and reverse primer 5'GCAGCAGCTAGTTCCGGGCTCCA3'; 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'CGAAATCGGTAGACGCTACG3' and reverse primer 5'GGGGATAGAGGGACTTGAAC3' (Taberlet *et al.*1991). In some instances it was necessary to utilize the reverse primer 5'ATTTGAACTGGTGACACGAG3' downstream from the *trnL* intron. M13 forward and reverse extensions were added the 5' ends of each primer to allow efficient direct sequencing of PCR products on a LICOR sequencer (M13 forward- 5'CAGGACGTTGTAAAACGAC3', M13 reverse-5'GGATAACAATTTCACACAGG3'). Each PCR reaction contained 100ng genomic DNA, 1X PCR buffer, 1X Q solution (Qiagen), 200uM each dNTP, 200nM of each primer, and 2U TAQ DNA polymerase (Qiagen TAQ polymerase # 201203). Thermal cycling was conducted with 1 minute at 94°C, 1 minute at 45°C, and 1 minute 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 Qiagens Qiaquick Gel Extraction Kit (#28704). PCR products were then quantified on a 1% agarose gel relative to a standard, to ensure at least 500ng was available for sequencing.

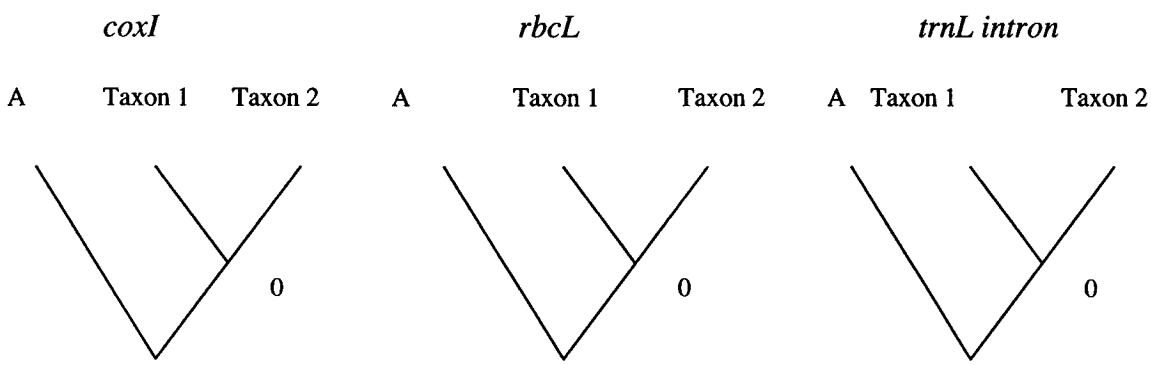
Alignment of Sequences and the Relative Ratio Test

Amino acid sequences for the translated *rbcL* and *coxI* genes and the DNA sequences for *trnL* intron were each aligned for all twelve taxa using CLUSTALW (Higgins *et al.* 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*=441bp, *rbcL*=1293bp, *trnL* intron=230bp). 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 described by Chaw *et al.* 2000 (Figure 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 Figure 2) versus a taxon with maternal mitochondrial inheritance (Taxon 2), and pairwise comparisons between the standardized distance for a taxon with paternal chloroplastidial inheritance (Taxon 1) versus a taxon with maternal chloroplastidial inheritance (Taxon 2; see Figure 2). For the mitochondrial comparisons, the chloroplasts were always paternally inherited, and, for the chloroplastidial comparisons, the mitochondria were always inherited maternally. To calculate the standardized distance of each of the two

Figure 2. Pairwise comparisons of standardized distances. For each pairwise comparison the branch length to Taxon 1 (d_{01}) and Taxon 2 (d_{02}) from their common ancestor 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.



Mitochondrial Comparisons

Taxon 1 has paternal mitochondrial inheritance

Taxon 2 has maternal mitochondrial inheritance

Both have paternal chloroplastidial inheritance

$D_{p,\ coxI/rbcL} = d_{01,\ coxI} / d_{01,\ rbcL}$

versus

$D_{m,\ coxI/rbcL} = d_{02,\ coxI} / d_{02,\ rbcL}$

$D_{p,\ coxI/rbcL} = d_{01,\ coxI} / d_{01,\ trnL}$

versus

$D_{m,\ coxI/rbcL} = d_{02,\ coxI} / d_{02,\ trnL}$

Chloroplastidial Comparisons

Taxon 1 has paternal chloroplastidial inheritance

Taxon 2 has maternal chloroplastidial inheritance

Both have maternal mitochondrial inheritance

$D_{p,\ coxI/rbcL} = d_{01,\ coxI} / d_{01,\ rbcL}$

versus

$D_{m,\ coxI/rbcL} = d_{02,\ coxI} / d_{02,\ rbcL}$

$D_{p,\ coxI/trn\ L} = d_{01,\ coxI} / d_{01,\ trnL}$

versus

$D_{m,\ coxI/rbcL} = d_{02,\ coxI} / d_{02,\ trnL}$

taxa to be compared, it was first necessary to determine mitochondrial and chloroplastidial 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: $d_{O1} = (d_{A1} + d_{12} - d_{A2})/2$ and $d_{O2} = (d_{A2} + d_{12} - d_{A1})/2$ (Li 1997), where d_{O1} = the branch length from the common node to Taxon 1, d_{O2} = 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* relative to *rbcL*: $D_{p,\ coxI/rbcL} = d_{01,\ cox\,I}/d_{01,\ rbcL}$ (taxon with paternal inheritance) and $D_{m,\ coxI/rbcL} = d_{02,\ cox\,I}/d_{02,\ rbcL}$ (taxon with maternal inheritance), and the standardized distance of *coxI* relative to the *trnL* intron: $D_{p,\ coxI/trnL} = d_{01,\ coxI}/d_{01,\ trnL}$ (taxon with paternal inheritance) and $D_{m,\ coxI/trnL} = d_{02,\ coxI}/d_{02,\ trnL}$ (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 both provides consistency and 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 Neilson (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 *et al.* 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 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 $\overline{D_m}/\overline{D_p}$, 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: i) 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), ii) 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 (Figure 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 (Figure 1).

RESULTS

In the mitochondrial analyses, the five taxa representing paternal inheritance were *C. corneyana*, *M. glyptostroboides*, *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 5). 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 5).

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.,

Table 5. 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_{O1} and d_{O2} 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".

								coxI relative to *rbcL*				*coxI* relative to *trnL* intron			
Taxon with paternal inheritance	Taxon with maternal inheritance	Mean *coxI* d_{O1}	Mean *coxI* d_{O2}	Mean *rbcL* d_{O1}	Mean *rbcL* d_{O2}	Mean *trnL* intron d_{O1}	Mean *trnL* intron d_{O2}	Mean D_p *coxI*/*rbcL*	Mean D_m *coxI*/*rbcL*	D_p/D_m [a]	*P*	Mean D_p *coxI*/*trnL*	Mean D_m *coxI*/*trnL*	D_p/D_m [a]	*P*
Mitochondrial Comparisons															
Cupressus corneyana	*Larix spp.*	0.121	-0.004	0.348	0.118	0.115	0.081	0.368	-0.038	>1[b]	0.02	0.116	-0.055	>1	0.01
	Pinus strobus	0.119	0.005	0.354	0.142	0.115	-0.071	0.352	0.051	6.9	0.02	0.352	NC[c]	-	-

Table 5

	Torreya nucifera	0.016	0.012	0.223	0.027	-	-	0.076	-0.126	>1	0.52	-	-	-	-
Metase-quoia glyptos-troboides	*Larix spp.*	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
	Pinus strobus	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
	Torreya nucifera	0.019	0.012	0.155	0.044	-	-	0.082	0.044	1.9	0.48	-	-	-	-
Podocar-pus spp.	*Larix spp.*	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
	Pinus strobus	0.194	0.007	0.341	0.191	0.118	0.053	0.589	0.033	18	0.01	1.880	0.031	61	0.02
	Torreya nucifera	0.115	0.042	0.289	0.154	-	-	0.411	0.293	1.4	0.04	-	-	-	-
Arauca-ria spp.	*Larix spp.*	0.105	0.003	0.201	0.153	0.048	0.074	0.536	0.032	17	0.01	2.760	-0.077	>1	0.01

Table 5

	Pinus strobus	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
	Torreya nucifera	0.055	0.076	0.152	0.141	-	-	0.371	0.571	0.4	0.46	-	-	-	-
Taxodium distichum	*Larix spp.*	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
	Pinus strobus	0.121	0.005	0.303	0.174	0.120	0.029	0.415	0.021	20	0.02	1.100	0.180	6.1	0.04
	Torreya nucifera	0.020	0.006	0.461	0.017	-	-	0.152	0.283	0.5	0.98	-	-	-	-
<u>*Chloroplastidial comparisons*</u>															
Larix spp.	*Ephedra* spp.[d]	0.006	0.316	-	-	0.050	0.414	-	-	-	-	0.206	0.798	3.9	0.01
Pinus strobus	*Ephedra* spp.	0.017	0.314	-	-	0.026	0.417	-	-	-	-	0.343	0.786	2.3	0.02
Larix spp.	*Ginkgo biloba*	-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

Table 5

Pinus strobus	*Ginkgo biloba*	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
Torreya nucifera	*Ginkgo biloba*	0.118	0.039	0.267	0.224	-	-	0.477	0.817	1.7	0.10	-	-	-	-

[a], The relative evolutionary rate of paternally to maternally inherited DNA equals D_p / D_m for mitochondrial comparisons and equals D_m / 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.

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 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 twelve genera was statistically significant at $P < 1.0X10^{-6}$, 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. glyptostroboides,* and *P. macrophyllus,* had significantly higher evolutionary rates in this gene than *Larix* sp., *P. strobus* and *G. biloba*, with maternal mitochondrial inheritance (Table 6). 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 6).

Table 6. a. Relative rate tests for *coxI*. b. Relative rate tests for *trnL* intron and *rbcL*.

Entries in bold are statistically significant. Probabilities are in parenthesis.

a.

	Taxon with Maternal Mitochondrial Inheritance				
Taxon with Paternal Mitochondrial Inheritance	*Ephedra viridis*	*Ginkgo biloba*	*Larix sp.*	*Pinus strobus*	*Torreya nucifera*
Araucaria heterophylla	**Mat>Pat** ($1.8X10^{-4}$)	**Pat>Mat** (0.015)	**Pat>Mat** ($3.7X10^{-5}$)	**Pat>Mat** ($7.2X10^{-4}$)	Mat>Pat (0.627)
Cupressus corneyana	**Mat>Pat** ($3.2X10^{-3}$)	**Pat>Mat** (0.011)	**Pat>Mat** ($3.0X10^{-6}$)	**Pat>Mat** ($6.6X10^{-5}$)	**Pat>Mat** ($6.6X10^{-5}$)
Metasequoia glyptostroboides	**Mat>Pat** ($1.6X10^{-3}$)	**Pat>Mat** (0.019)	**Pat>Mat** ($5X10^{-6}$)	**Pat>Mat** ($1.3X10^{-4}$)	Pat>Mat (0.514)
Podocarpus macrophyllus	Mat>Pat (0.065)	**Pat>Mat** ($2.9X10^{-4}$)	**Pat>Mat** ($1.0X\ 10^{-6}$)	**Pat>Mat** ($1.0X10^{-6}$)	**Pat>Mat** (0.0465)
Taxodium distichum	**Mat>Pat** ($1.9X10^{-3}$)	**Pat>Mat** (0.011)	**Pat>Mat** ($1.0.0X10^{-6}$)	**Pat>Mat** ($6.6X10^{-5}$)	Pat>Mat (0.511)

b.

	Taxon with Maternal Chloroplastidial Inheritance		
Taxon with Paternal Chloroplastidial Inheritance	*trnL* intron		*rbcL*
	Ephedra viridis	*Ginkgo biloba*	*Ginkgo biloba*
Araucaria angustifolia	**Mat>Pat** ($1.0X10^{-6}$)	Pat>Mat (0.693)	**Pat>Mat** ($3.2X10^{-4}$)
Cupressus corneyana	**Mat>Pat** ($3.5X10^{-4}$)	Pat>Mat (0.131)	**Pat>Mat** (0.017)
Larix sp.	**Mat>Pat** ($1.0X10^{-6}$)	Pat>Mat (0.654)	**Pat>Mat** ($3.5X10^{-3}$)
Metasequoia glyptostroboides	**Mat>Pat** (0.0234)	**Pat>Mat** ($3.21X10^{-4}$)	**Pat>Mat** ($7.0X10^{-6}$)
Pinus strobus	**Mat>Pat** ($1.0X10^{-6}$)	Pat>Mat (0.787)	**Pat>Mat** ($7.3X10^{-3}$)
Podocarpus reichei	**Mat>Pat** ($7.0X10^{-6}$)	Pat>Mat (0.070)	**Pat>Mat** ($1.0X10^{-6}$)
Taxodium distichum	**Mat>Pat** ($1.4X10^{-4}$)	**Pat>Mat** ($1.4X10^{-4}$)	**Pat>Mat** ($1.5X10^{-5}$)
Torreya nucifera	-	-	**Pat>Mat** ($1.5X10^{-5}$)

[a], relative rate tests for *Ephedra* spp. could not be calculated for *rbcL* because it is saturated at synonymous sites relative to the outgroup.

DISCUSSION

Standardized Distances

That the standardized distance of taxa with paternal mitochondrial inheritance, namely *C. corneyana*, *M. glyptostroboides*, *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 5). A relatively higher evolutionary rate in paternally inherited DNA is also supported by the significantly higher values of the standardized distance for *Podocarpus* spp. (withpaternal 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 5). 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 lower 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 5). 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 6). Furthermore, *G. biloba* showed no significant differences in the relative rate tests when compared to *Larix* spp. or *P. strobus* at the *trnL* intron (Table 6), 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 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 (D_p/D_m) indicates that the substitution rate of male-inherited DNA may be six to ten 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 Figure 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 (D_m/D_p) for the significant tests indicate that the mutation rate for the sperm is at least two- to six-fold higher than for the eggs (Table 5), 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 due to 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 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 distance 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 distance may be an imprecise 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 distance.

Possible Causes of the Lack of Significance in Some Comparisons

Even though the totality of the data suggest 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; Figure 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 *et al.* 1993), that could potentially dilute the male or female effect on substitution rates. It is not known 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 *et al.* 1993, Chang *et al.* 1994, Rawson and Hilbish 1995, Liu *et al.* 1996, Agulnik *et al.* 1997, McVean and Hurst 1997, Ellegren and Fridolfsson 1997, Kahn and Quinn 1999, Huttley *et al.* 2000, Bohosslan *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 are complete with egg maturity while 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 *et al.* 1993, McVean and Hurst 1997, Hurst and Ellegren 1998, Smith and Hurst 1999, Bohosslan *et al.* 2000, Huttley *et al.* 2000) that may include differences in the expression of DNA repair genes, different levels of cellular mutagens, and/or a higher level of DNA methylation in the male germ-line (Bester 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, and thus these results cannot contribute to the ongoing debate in animals. The trend towards 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 due to 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, in the present study, 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 was compared to 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 suggest 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 and therefore 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 and therefore 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 to 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 that 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 germline cells of *Lilium longiflorum* (Xu *et al.* 1998) indicates that cells of the male germ-line may have increased DNA repair compared to the female germ-line, suggesting that there could be a greater likelihood of DNA repair errors in the sperm due to 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 "be 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 due to the biochemistry of translation and the patterns of codon usage within a gene (Akashi *et al.* 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 due to 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), it was 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. In addition to these three considerations, it is important to recognize that mitochondrial and chloroplastidial DNA were examined 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 bi-parentally and therefore it is not possible to 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.

CHAPTER 3

MALE-BIASED TRANSMISSION OF DELETERIOUS MUTATIONS TO THE PROGENY IN *ARABIDOPSIS THALIANA*

INTRODUCTION

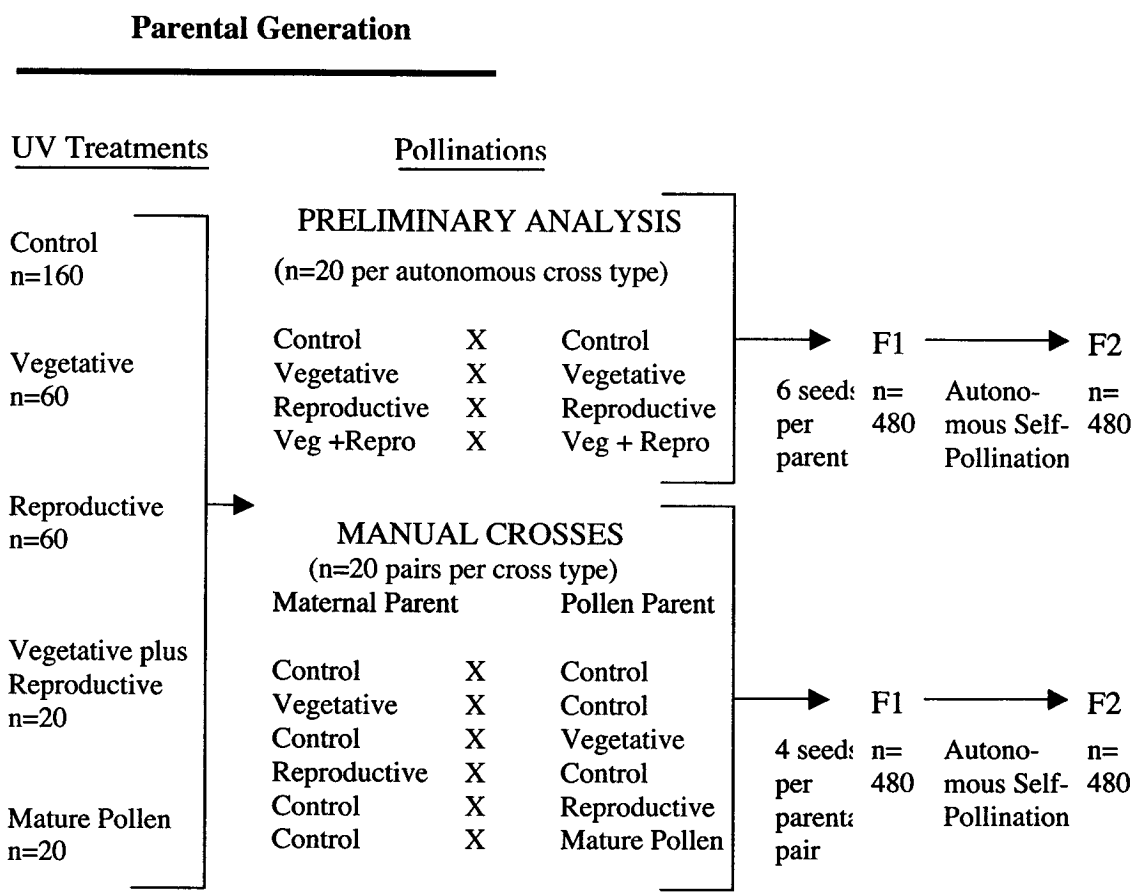
One approach to estimating sex-specific mutation rates is to compare the evolutionary substitution rate of selectively neutral DNA, which equals the mutation rate (Kimura 1983), in male- and female-inherited DNA. Using this approach, male-biased mutation rates have been suggested to exist in humans and higher primates (Miyata *et al.* 1987, Agulnik *et al.* 1997, Huang *et al.* 1997, Bohossian *et al.* 2000, Huttley *et al.* 2000, Nachman and Crowell 2000, Ellegren 2000, Makova and Li 2002,), birds (Shimmin *et al.* 1993, Kahn and Quin 1999), rodents (Chang *et al.* 1994) and sheep (Lawson and Hewitt 2002), and more recently, in gymnosperms (Whittle and Johnston 2002). Few studies, outside of human diseases (Crow 1997), however, have examined whether more deleterious mutations (non-neutral) are inherited from a sperm than from an egg. This is remarkable because deleterious mutations play a fundamental role in many evolutionary theories and have been the focus of much research (e.g., Drake *et al.* 1998, Lynch *et al.* 1999). The absence of scientific information about this topic may be due to the difficulty in developing experimental methods that can distinguish between male and female mutations and the poor suitability of DNA sequence data for such analysis due to lack of knowledge about the direction (i.e., positive, negative or neutral) and magnitude of selective effects. Other, more direct approaches, therefore need to be developed. Here, an

experimental approach to testing the hypothesis that the sperm transmit more induced deleterious mutations to the progeny than the eggs in *Arabidopsis thaliana* is described.

External mutagenic agents such as UV-irradiation are an effective tool to compare the level of deleterious mutations transmitted to the progeny by a sperm and an egg. UV-B and UV-C lead to DNA damage that includes the formation of pyrimidine dimers and 6, 4 photoproducts (Friedberg *et al.* 1995), which change the binding properties between DNA strands and can lead to mutational clusters or "hotspots" upon DNA replication (Hutchingon 1987, Jiang and Taylor 1993, Samach *et al.* 1997, Waterworth *et a*l. 2002, Kovalchuk *et al.* 2000). In addition, UV-B and UV-C can increase the production of free radicals, leading to DNA strand breaks and the formation of mutations by the misincorporation of nucleotides upon strand-break repair (Hutchingson 1987, Friedberg *et al.* 1995). These mutations can be transmitted by a sperm or an egg and have a deleterious effect on the fitness of progeny (Strid *et al.* 1994). It is therefore possible to evaluate the relative number of deleterious mutations that are transmitted by a sperm and an egg by assessing the fitness of offspring produced by UV-treated pollen parents (i.e., sperm donors) and UV-treated maternal parents (i.e., egg donors).

In the present investigation, the fitness of two generations of progeny produced from UV-B- and UV-C-treated pollen and maternal parents in *A. thaliana* are compared. Parent plants were exposed during either the vegetative stage, reproductive stage, or both, and mature pollen was also exposed. The analysis consisted of two main components (Figure 3). Firstly, a preliminary analysis was conducted to confirm that the doses of UV applied induced a sufficient level of mutations to have a detectable effect on the fitness of progeny. Subsequently, manual crosses were conducted and the fitness of the F2

Figure 3. UV-B and UV-C treatments of the parental generation and the types of crosses conducted to form the F1 and F2 generations for the preliminary analysis and for the manual crosses. The design was used separately for the UV-B and the UV-C experiments.



generation produced from UV-treated pollen and maternal parents and from UV-treated mature pollen were compared. The F2 generation was used for all comparisons for two main reasons. Firstly, the homozygous mutant genotype was more frequent in the F2 than the F1 generation, thereby making fitness effects more evident. Secondly, and more importantly, fitness differences in the F2 generation can almost entirely be attributed to deleterious mutations, unlike in the F1 generation, which may be affected by physiological effects of UV on the quality of seed produced by the parental generation.

METHODS

Genetically identical *A. thaliana* seeds produced from the self-pollination of a single plant were used as the parental generation in both the UV-C and UV-B experiments (catalog # cs907, *Arabidopsis* Biological Resource Center, Ohio). All plants were maintained in 2cm deep pots placed in trays measuring 25cm X 40cm in Schultz Cactus Mix. Fertilization was provided in deionized water using Schultz General Nutrient Solution (7drops/liter) approximately every three to four days. Plants were always watered from below by covering the bottom of trays and were well spaced to prevent shading.

UV Application

For the UV-B experiment, the plants were grown under pairs of Plant and Aquarium bulbs (GE Lighting, Cleveland, OH) spaced 42 cm apart and suspended 38 cm above the surface of the trays for 18-hour days at 25°C. Individual fluorescent sunlamps (Q-Panel, UV-B 313, Cleveland, Ohio) were evenly spaced at 21cm intervals with one

lamp located between each pair of Plant and Aquarium bulbs. At the time of UV-B application, the plants were placed under wooden frames covered with either mylar (0.13mm thick), to absorb UV-B radiation (no treatment), or with cellulose acetate film (0.075mm thick), which transmits UV-B (for the various treatments). Both the mylar and the acetate were pretreated with UV-B for 8 hours to increase their effectiveness in isolating UV-B radiation (Middleton and Teramura 1993). UV-B was measured with an Optronic OL 754 spectroradiometer with an integrating sphere that was calibrated using an Optronic OL 752-irradiance standard (Optronic, Orlando Florida). Because the biological action spectrum that is most appropriate for a particular biological process remains largely uncertain, it is important to weight irradiance by each function that could be relevant. Therefore, the biologically active radiation was calculated by weighting the spectral irradiance by both a plant action spectrum (Caldwell 1971, Green *et al.* 1974) and a DNA damage spectrum (Setlow 1974). The UV-B treatments consisted of a dose of 10.1 KJ/m^2/day according to the plant action spectrum (Green *et al.* 1974) applied over 18 hours, followed by a six-hour period of darkness, and a subsequent treatment of 4.5 KJ/m^2 over 8 hours. This was the maximum dose that could be applied without affecting plant survival. These values were 3.7 and 1.7 KJ/m^2/day, respectively, according to the DNA damage spectrum (Setlow 1974). Photosynthetically active radiation (PAR) was measured with a quantum sensor (Apogee Instruments INC, Model QMSW-SS) and was 54 $\mu mol/m^2/s$ during the light cycle. The spectroradiometer and the quantum sensor were placed at 10.5cm intervals along the bench and mean values for the room were determined.

All plants other than the parental UV-B generation were grown for 18 hours of light per day at 25°C under high pressure sodium Superbulbs (P.L. Lighting Systems, Ontario) suspended approximately 1.8m above the trays at 1.2m intervals. For the UV-C treatment, plants were irradiated with 1000J/m^2 at 254nm for 30 seconds using a Minerallight-Lamp R-52 (UV-Products, San Gabriel, CA) held 3cm above the rosette for all treatments except the pollen treatment, where it was held 3cm from the pollen-producing flower. Average photosynthetically active radiation during the light cycle was 120 μmol/m^2/s.

Experimental Design

The same design was used in the separate UV-B and UV-C experiments (Figure 3). This consisted of two main components. First, a preliminary analysis was conducted to assess the fitness of progeny produced following autonomous self-pollination of individuals treated with UV during the vegetative and/or reproductive stage. Second, a series of manual crosses was conducted to evaluate the fitness of progeny produced by pollen parents (sperm donor) and maternal parents (egg donor) that were UV-treated during the vegetative or reproductive stage. Fitness of progeny produced by UV-treated mature pollen was also assessed. The vegetative treatment was applied 25 days following germination at the rosette stage and the reproductive treatment was applied on day 32, near the initiation of bolting. A total of 320 individuals was used in the parental generation. Of these, 160 were treated with UV. Specifically, 60 randomly chosen plants were treated with UV during the vegetative stage, 60 were treated during the reproductive stage, 20 were treated during both the vegetative and reproductive stages and 20 were

treated during pollen release (i.e., the pollen was treated following the opening of the anther). The 160 plants that were not treated with UV were used as controls. Treated and control individuals were randomly assigned to undergo autonomous self-pollination (n=80), for the preliminary analysis, or manual crossing (n=240, Fig. 3). Manual crosses were conducted between the controls and pollen and maternal parents from the vegetative, reproductive and mature pollen treatments to produce the F1 generation. Manual crosses were also conducted among the controls to account for any effect of emasculation on progeny fitness. For both the preliminary analysis and manual crosses, the F1 generation subsequently underwent autonomous self-pollination to form the F2 generation. Each individual in the parental generation was used only once, and thus all crosses were independent.

Offspring Measurements and Analysis

Seeds comprising the F1 generation for both the UV-C and UV-B treatments were sown in the growth room that was used for UV-C treatments. The seeds were placed in trays, each containing 24 pots. For the preliminary analysis, each tray contained six offspring from a single randomly chosen parent from the vegetative treatment, the reproductive treatment, the vegetative plus reproductive treatment and from the controls (4 types of treatments per tray). Six seeds of each parent per treatment were randomly placed among the pots and trays were randomly positioned on the bench. For the manually crossed plants, each tray contained 4 seeds per parent plant per cross type (6 types of crosses per tray, see Figure 3). Following self-pollination the seeds produced by the F1 individuals were collected and these comprised the F2 generation. One seed was

randomly chosen per individual and was sown as described for the F1 generation, with one parent (i.e., from the parental generation) per tray. For the F1 and F2 generation there were 480 individuals for the preliminary analysis and for the manual crosses, for each UV-type. Upon maturity, measurements were taken of the seed number per flower (averaged over three flowers) and the number of flowers per individual for both F1 and F2. Total fitness for each individual was determined as the number of flowers X the average number of seeds per flower. The mean total fitness was determined for each treatment per parent or grandparent for the F1 and for F2 generations, respectively.

A separate analysis was conducted for each combination of UV-type (UV-B and UV-C) and each generation category (the F1 and F2 generations). For the preliminary analysis, a one-way analysis of variance (ANOVA) was conducted with four treatment categories (control, vegetative stage, reproductive stage, and both vegetative and reproductive stages) using the mean fitness for each parent within a tray (maximum n=20 parents per treatment type per tray). For the manual crosses, a one-way ANOVA was conducted using the six treatment categories that were based on the timing of UV-application and the sex of the UV-exposed parent (i.e., control, maternal parent-vegetative stage, maternal parent-reproductive stage, pollen parent-vegetative stage, pollen parent-reproductive stage, and mature pollen) using the mean fitness for each treatment type within a tray. Pairwise comparisons of treatments were conducted using Tukey post hoc tests (Wilkinson *et al.* 1992).

Calculation of the Sperm-to-Egg Mutation Transmission Ratio

The sperm-to-egg mutation transmission ratio was estimated using fitness data from the F2 generation. When proportional reductions in fitness combine in a multiplicative fashion across loci, as appears reasonable (Willis 1993, Elena and Lenski 1997), the relative fitness of an individual with i heterozygous and j homozygous mutations is $\tilde{w} = (1-hs)^i(1-s)^j$, where h is the dominance coefficient of mutant alleles, and s is the selection coefficient against a homozygous mutant locus (e.g., Charlesworth and Charlesworth 1992). Although the F2 was produced by self-fertilization of the F1, in which any new mutations were presumably heterozygous, the number of heterozygous (i) and homozygous (j) mutations in the F2 was unknown. Therefore relative fitnesses in the F2 was treated as $\tilde{w} = (1-hs)^i(1-s)^j = (1-x)^n$, where $n = i + j$ is the combined number of heterozygous and homozygous mutant loci. 1-x equals $(1-hs)^{i/(i+j)}(1-s)^{j/(i+j)}$ and is therefore a measure of the geometric mean relative fitness based on the two types of loci. It is thus straightforward to calculate the sperm-to-egg mutation transmission ratio as

$$\frac{n_{male}}{n_{female}} = \frac{log(\tilde{w}_{male})}{log(\tilde{w}_{female})} = \frac{log(w_{male}/w_{control})}{log(w_{female}/w_{control})},$$

where w_k is the mean absolute fitness of treatment k. This ratio was calculated for both the vegetative and reproductive treatments within each UV type for cases where mean control > mean maternal treatment > mean paternal treatment. Confidence intervals (95%) were estimated for the mean and median using a bootstrap program written in Mathematica (Wolfram 1999). For each treatment (control, treated maternal, treated

paternal) F2 individuals were randomly resampled with replacement within trays, and the mean (or median) was obtained. The mean and median over trays were then used in the male-bias ratio, and the procedure was iterated 10 000 times.

RESULTS

The preliminary analysis was conducted to assess whether there was a detectable effect of deleterious mutations on the progeny in *A. thaliana*. Fitness of the F1 and the F2 generation produced from UV-B and UV-C exposed parents differed among treatment categories (one-way ANOVA's, Table 7). Specifically, the fitness of the vegetative, the reproductive and the vegetative plus reproductive treatments were each statistically significantly lower than the controls (Table 8). No statistically significant differences were detected between the vegetative, reproductive and the vegetative plus reproductive treatments for either the F1 or F2 generation.

For the manual crosses, total fitness of the F1 generation differed among the six treatment categories for the UV-B but not for the UV-C exposures (one-way ANOVA's, Table 7). Only two of the pairwise comparisons were near statistical significance, however, namely the vegetative UV-B treatment of pollen parents and of mature pollen were lower than the controls (Table 9). In contrast, for the F2 generation, mean fitness differed among treatment categories for both UV-B and UV-C (Table 7), and many pairwise differences were evident (Table 9). Specifically, the mean fitness was statistically significantly lower for the progeny produced by UV-treated pollen parents than by treated maternal parents for both the vegetative and the reproductive treatments

Table 7. ANOVA's for the effect of UV on the fitness of the F1 and F2 generations for the preliminary analysis and for the manual crosses. Model: Fitness = Treatment category + Constant + Error.

	df	Mean Square	*F*-Ratio	*P*<	R^2
Preliminary Analysis					
UV-C, F1	3	4 803 757	6.8	0.003	0.22
UV-B, F1	3	8 172 055	14.7	0.0000003	0.38
UV-C, F2	3	3 285 750	14.1	0.0000003	0.38
UV-B, F2	3	2 021 530	8.3	0.0001	0.29
Manual Crosses					
UV-C, F1	5	412 136	2.0	0.091	0.13
UV-B, F1	5	3 546 860	2.9	0.021	0.23
UV-C, F2	5	4 186 215	7.5	0.000013	0.37
UV-B, F2	5	5 784 270	14.8	0.00000001	0.60

Table 8. Mean, standard errors (SE) and *P*-values of Tukey pairwise comparisons for the preliminary analysis. Bold indicates statistically significant ($\propto$=0.05) *P*-value after Tukey correction for multiple comparisons.

	UV-C				UV-B			
	Mean Total Fitness (SE)	*P*-value			Mean Total Fitness (SE)	*P*-value		
F1 Generation		Veg. Stage	Repro. Stage	Veg. + Repro.		Veg. Stage	Repro. Stage	Veg. + Repro.
Controls	2491 (211)	**0.0007**	**0.0015**	**0.0143**	2664 (170)	**0.0002**	**0.0002**	**0.0032**
Vegetative Stage	1274 (206)		0.9925	0.7007	1283 (170)		0.9998	0.9442
Reproductive Stage	1355 (206)			0.8533	1301 (166)			0.9576
Veg. + Repro.	1586 (200)				1466 (281)			
F2 Generation								
Controls	1853 (116)	**0.0002**	**0.0002**	**0.0002**	1590 (113)	**0.0062**	**0.0022**	**0.0029**
Vegetative Stage	898 (113)		0.8890	0.6820	917 (113)		0.959	0.935
Reproductive Stage	1014 (110)			0.9801	996 (110)			0.774
Veg. + Repro.	1074 (110)				788 (186)			

Table 9. Mean, standard errors (SE) and *P*-values of Tukey pairwise comparisons for the manual crosses for the F1 and F2 generations. Bold indicates statistically significant ($\propto$=0.05) *P*-value after Tukey correction for multiple comparisons.

	UV-C						UV-B					
	Mean Fitness (SE)	*P*-value					Mean Fit-Ness (SE)	*P*-value				
		Maternal Parent Veg.	Maternal Parent Repro.	Pollen Parent Veg.	Pollen Parent Repro.	Mature Pollen Parent		Maternal Parent Veg.	Maternal Patent Repro.	Pollen Parent Veg.	Pollen Parent Repro.	Mature Pollen Parent
F1 Generation												
Controls	1403 (126)	0.1386	0.5034	0.2056	0.1677	0.1913	1846 (122)	0.1312	0.3513	0.3320	0.0576	0.0715
Maternal Parent Veg.	936 (137)		0.9228	1	0.9998	0.9999	1207 (219)		0.9291	0.9769	0.9999	0.9999
Maternal Parent Repro.	1109 (110)			0.9419	0.9694	0.9556	1460 (147)			0.9999	0.9330	0.8768

Table 9

Pollen Parent Veg.	931 (161)				0.9998	0.9999	1412 (173)				0.9845	0.9550
Pollen Parent Repro.	980 (121)					0.9999	1253 (163)					0.9989
Mature Pollen Parent	954 (143)						1185 (200)					
F2 Generation												
Controls	1898 (207)	0.6739	0.9683	**0.0316**	**0.0377**	0.6389	2516 (156)	0.9032	0.9999	**0.0001**	**0.0001**	**0.0036**
Maternal Parent Veg.	2353 (225)		0.966	**0.0008**	**0.0006**	0.0633	2185 (278)		0.9492	**0.0071**	**0.0086**	0.2471
Maternal Parent Repro.	2118 (186)			**0.0029**	**0.0022**	0.2041	2482 (188)			**0.0002**	**0.0002**	**0.0096**
Pollen Parent Veg.	852 (264)				0.9956	0.6624	873 (219)				0.9999	0.7222
Pollen Parent Repro.	1021 (199)					0.8442	924 (207)					0.7905
Mature Pollen	1398 (249)						1347 (254)					

with UV-C and with UV-B (Table 9). Mean fitness of the UV-B and UV-C vegetative and reproductive treatments was statistically significantly lower for pollen parents than for the controls. No statistically significant differences, in contrast, were detected between the controls and the UV-treated maternal parents. Several differences were detected involving the treatment of mature pollen. The fitness of progeny produced from UV-B mature pollen was statistically significantly lower than the controls (Table 9). In addition, UV-B exposed mature pollen produced less fit F2 progeny than the maternal parents treated during the reproductive stage. Overall, the pattern of significant treatment effects is similar in the UV-B and UV-C experiments. The sperm-to-egg mutation transmission ratio (and the 95% confidence interval (CI)), calculated using mean fitnesses of the F2 generation was 7.5 (CI, -67 to 70) for the UV-B vegetative treatment and 25 (CI, -251 to 270) for the reproductive treatment. Using median fitnesses, the ratios were 5.0 (CI, 1.7 to 9.0) for the UV-B vegetative treatments and 11.1 (CI, -162 to 190) for the reproductive treatment. Ratios were not calculable for the UV-C treatments.

DISCUSSION

Preliminary Analysis

For the preliminary analysis, parent plants were exposed to UV-B or UV-C during different stages of development, specifically the vegetative stage, the reproductive stage, or both (Fig. 1). Individuals thus became +/- at certain loci for each individual, where "+" denotes the wild type and "-" represents the mutant allele. Subsequently, all parental individuals underwent autonomous self-pollination to form the F1 generation, which, upon maturity, underwent self-pollination to form the F2 generation. Accordingly, the predicted genotypic frequencies for any locus experiencing mutation

were approximately 1/4 +/+, 1/4 -/-, and 1/2 +/- for the F1 generation and approximately 3/8 +/+, 3/8 -/-, and 1/4 -/*m* for the F2 generation. That statistically significantly lower fitness, measured as total seed production, was found for the F2 generation produced from plants treated during each developmental stage relative to the controls (Table 8) indicates that a detectable level of deleterious mutations induced in the parental generation were transmitted to the progeny, such that the main analysis using manual crosses could be conducted.

Fitness of Progeny of Pollen Versus Maternal Parents

The UV-treated parents that were manually crossed with untreated plants, to form the F1 generation, underwent autonomous self-pollination to form the F2 generation. The estimated genotype for any mutant locus in the F1 generation were thus entirely +/-, which following self-pollination, produced approximately 1/4 +/+, 1/4 -/- and 1/2 +/- in the F2 generation. The data from the F2 generation, containing some homozygous mutants and minimal/no residual physiological effects from the UV exposures, demonstrate that more deleterious mutations were transmitted to the progeny by sperm than by eggs. For the UV-C and the UV-B treatments, the mean total fitness was statistically significantly lower for F2 progeny of exposed pollen parents than the maternal parents, for both the vegetative and reproductive treatments (Table 9). In fact, the fitness of progeny of UV-treated pollen parents was statistically significantly lower than maternal parents in all pairwise comparisons, even when the timing of UV application differed. The finding of statistically significantly lower fitness of the progeny of UV-treated pollen parents than UV-treated maternal parents for the vegetative

treatment indicates, remarkably, that more somatic mutations, which arose long before sexual differentiation, and even before formation of the reproductive apex, are transmitted to offspring by the sperm than the eggs. Further supporting evidence for sperm-biased transmission of mutations includes the statistically significantly lower total fitness of the F2 generation produced from UV-B- and UV-C-treated pollen parents than the controls (Table 9). In addition, the fitness of progeny produced by UV-B-treated mature pollen was statistically significantly lower than the maternal parents that were UV-treated during the reproductive stage as well as the controls. That the results were consistent for the UV-B and UV-C exposures strengthen the results beyond those which would have been possible by using only a single UV type.

Although the fitness differences show evidence that male-bias exists in these plants, the magnitude of this effect is less certain. This is largely because the sperm-to-egg mutation transmission ratios were estimated using the ratio of the logarithm of two ratios (and thus have large errors). Using mean fitnesses, the sperm-to-egg mutation transmission ratio for the F2 generation was estimated to be between 7.5 (95% *CI*, -35 to 79) and 25 (95% *CI*, -17 to 375) for the UV-B treatments. Use of median fitness, however, gave less variable results: the transmission bias ratio was 5.0 (*CI*, 1.7 to 9.0), which excludes unity, for the UV-B vegetative treatment and was 11.1 (*CI*, -162 to 190) for the reproductive treatment. The ratios were not calculable for UV-C because the mean fitness of the controls in the F2 generation was smaller than the female treatments, largely resulting from unusually low control values in two trays. It is probable that larger sample sizes within each treatment would have allowed calculation of the ratios for the UV-C treatments and narrowed the each of the confidence intervals for UV-B treatments

to exclude unity (approximately 1900 plants were maintained per generation, the maximum permitted in the growth facilities). Nevertheless, it is notable that the estimates of the sperm-to-egg mutation ratio, of between 5 and 25, are consistent with the sex-specific mutation ratios predicted using male- and female- inherited mitochondrial and chloroplastidial DNA sequences in gymnosperms (Whittle and Johnston 2002), which were between two and ten. One would not necessarily expect these ratios to be similar, however, because, in the gymnosperm study, the sperm-to-egg mutation ratio represents naturally occurring and selectively neutral mutations, whereas in the present study it represents the transmission of induced deleterious mutations to progeny. Further study will be needed to narrow the confidence intervals of the sperm-to-egg mutation transmission ratio of deleterious mutations in plants.

Our results indicate that the differential transfer of mutations largely involves nuclear genes, for the following reason. Because mitochondrial and chloroplastidial DNA is maternally inherited in *A. thaliana*, UV-induced mutations in these organelles would reduce fitness in the F2 progeny of UV-treated maternal parents. It was observed, however, that the fitness of the F2 generation produced by UV-treated maternal parents was higher than for UV-treated pollen parents, despite the effects of any deleterious mutations in the organellar DNA. This suggests that more mutations in nuclear genes are transmitted by the sperm than the eggs. This result for nonneutral, nuclear mutations thus extends the previous findings that sperm transmit more selectively neutral chloroplastidial and mitochondrial mutations to progeny in plants (Whittle and Johnston 2002). Further study is needed to determine whether different levels of deleterious

mutations in the chloroplastidial and mitochondrial DNA are transmitted by the sperm than the eggs.

What Causes Male-Biased Mutation Transmission?

There are at least four plausible explanations for the increased transmission of deleterious mutations through sperm than egg. Firstly, fewer UV-induced somatic mutations may be removed by cell lineage selection (selection against mutant diploid cells during development, Otto and Hastings 1998) for a sperm than for an egg. Secondly, the egg (or megagametophyte) may experience stronger selection than the sperm (or pollen). Thirdly, there may be no difference in mutation number, but rather the deleterious mutations have greater effect on progeny produced by mutant sperm than mutant eggs. Although this could partially explain a sex-specific bias in the F1 generation, it is an unlikely explanation for our results because a mutation in the F2 generation, which was examined here, would have the same effect regardless of whether it was inherited from a sperm or from an egg. Fourthly, it is possible that UV radiation has different physiological effects on sperm and egg production, causing a different number of mutations to arise during gametogenesis. This also seems unlikely, because the results were consistent for two kinds of UV radiation which were applied well before gametogenesis, at different stages of development. It therefore appears most likely that the sperm transmission bias is caused either by greater cell lineage selection or stronger selection at the gametophyte stage for an egg than for a sperm.

CHAPTER 4

IS G1 ARREST IN PLANT SEEDS INDUCED BY A P53-RELATED PATHWAY?

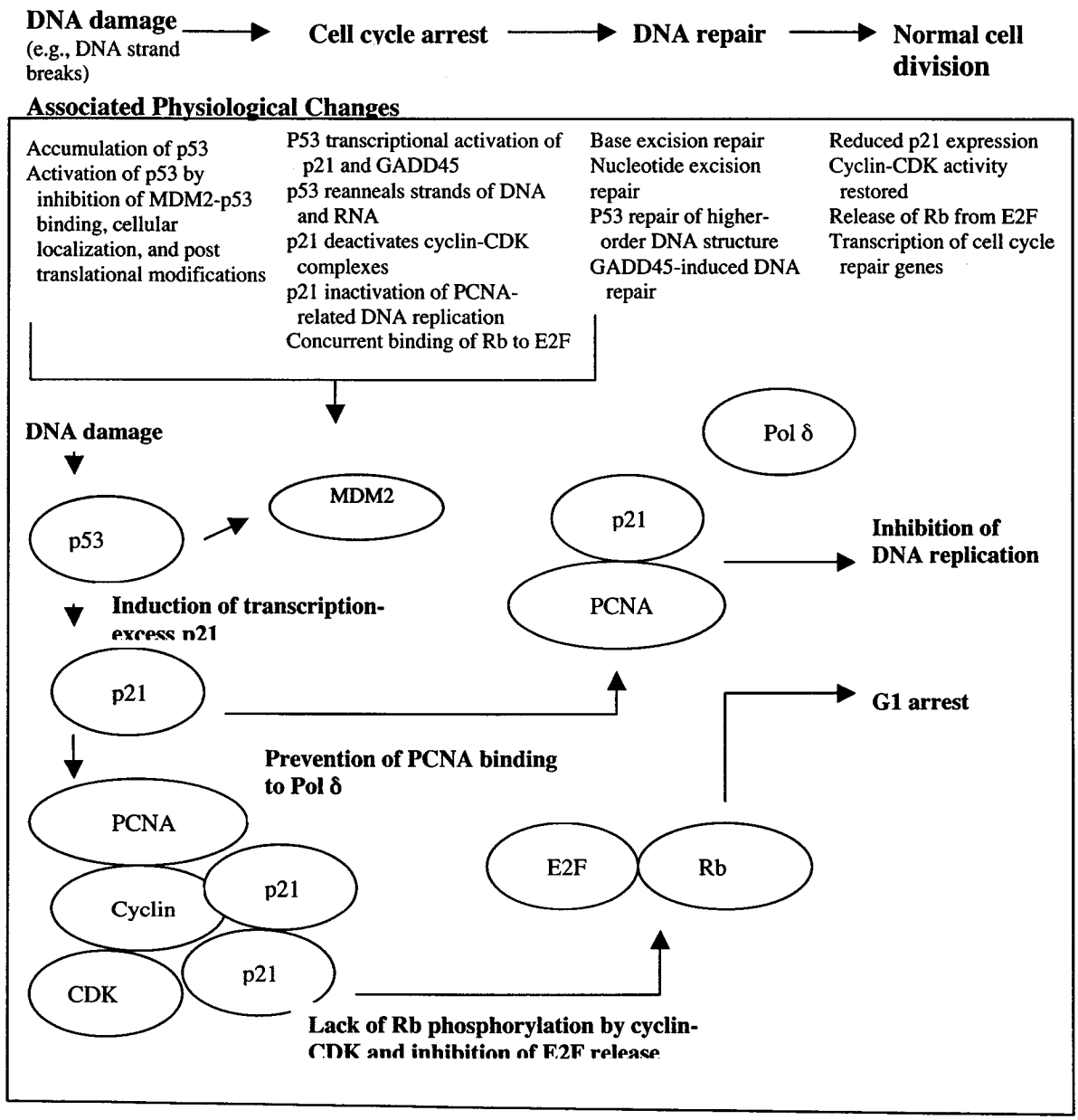
INTRODUCTION

Although p53 in mammals is known to be critical for the induction of genes leading to G1 arrest following DNA damage (to allow DNA repair), there has been minimal consideration of the possibility of such a DNA-damage response system in plants. Even though some cDNA sequences sharing partial homology to p53 have recently been reported within the Plantae (that are available in Genbank), there has been little analysis of how these molecules may relate to DNA damage. Aged seeds are known to contain high levels of DNA damage. That many aged seeds can survive and develop into adult plants suggests that the embryo has an ability to respond to and reduce DNA damage. There are remarkable parallels between the germination of aged seed (i.e., from germination up to the first cell divisions) containing DNA damage and the p53-mediated-G1-arrest in mammals. Based on these parallels, it may be inferred that aged seed may be an especially useful model system from which to evaluate the possibility that a p53-related pathway could exist in plants.

P53-MEDIATED ARREST IN HUMANS AND MICE

To understand why a p53-mediated-G1-arrest may exist in plant seed, it is first necessary to consider the key events enforcing arrest in humans and mice (Figure 4). Following DNA damage in mammalian cells, p53 levels increase via post-translational

Figure 4. Pathway leading to p53-induced G1 arrest in humans and mice and the physiological changes associated with each stage (DNA damage, G1 arrest, DNA repair, and cell division). Following DNA damage, p53 induces transcription of p21 which binds cyclin-cyclin dependent kinase (CDK) complexes and to proliferating cell nuclear antigen (PCNA, a trimer) inducing G1 arrest and preventing DNA replication, respectively.



modifications (or by modification of its regulator protein, murine/human double minute (MDM2/HDM2)) and by its translocation from the cytoplasm into the nucleus, each of which circumvents the need for p53 transcription in the presence of a damaged DNA template. Subsequently, p53 may be activated by post-translational modifications (phosphorylation and/or acetylation), thereby allowing the protein to bind DNA and induce transcription of the cyclin dependent kinase inhibitor (CKI) *p21* (reviewed by Burns and El-Deiry 1999). p21 is critical for inducing G1 arrest following DNA damage because it: i) binds to complexes containing cyclin and cyclin-dependent-kinases (CDK's) thereby inhibiting the modification of the retinoblastoma (Rb) protein and preventing its release from the E2F transcription factors (a step that is necessary for cell-cycle progression) and ii) adheres to the proliferating cell nuclear antigen (PCNA), a DNA polymerase δ processivity factor, and prevents DNA replication (but does not affect pre-replicative DNA repair, reviewed by Boulaire *et al.* 2000). Pre-replicative DNA repair conducted during this enforced G1 arrest may be partially induced by p53 as it has been shown to play a role in base excision repair, nucleotide excision repair, the removal of damage to higher-order DNA structure and the transcriptional activation of other DNA repair genes (Burns and El-Deiry 1999, Offer *et al.* 1999, Armando *et al.* 1999, Smith *et al.* 2000). Nevertheless, it is believed that the extension of the G1 arrest by p53 is critical for allowing both p53-dependent and independent repair to occur, and therefore may be the ultimate determinant of whether a DNA-damaged cell can undergo normal cell division.

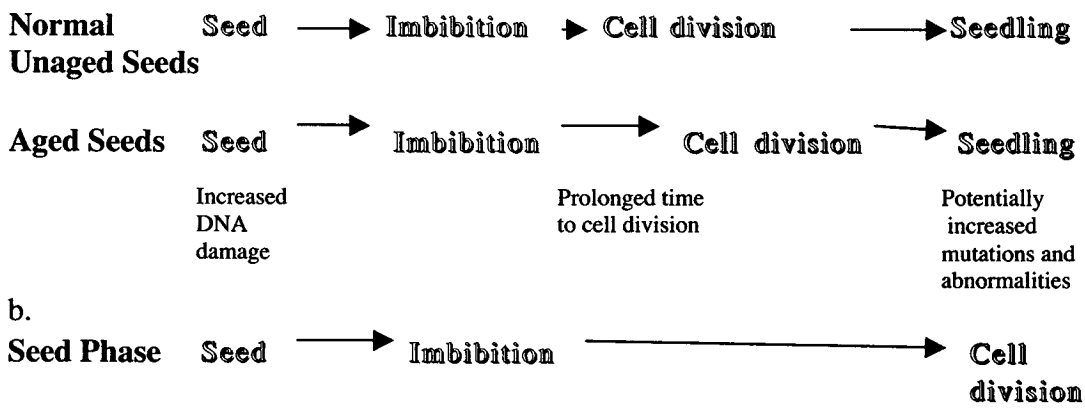
INDICATORS OF A P53-RELATED PATHWAY IN PLANT SEEDS

Prolonged G1 Arrest in Aged Seed

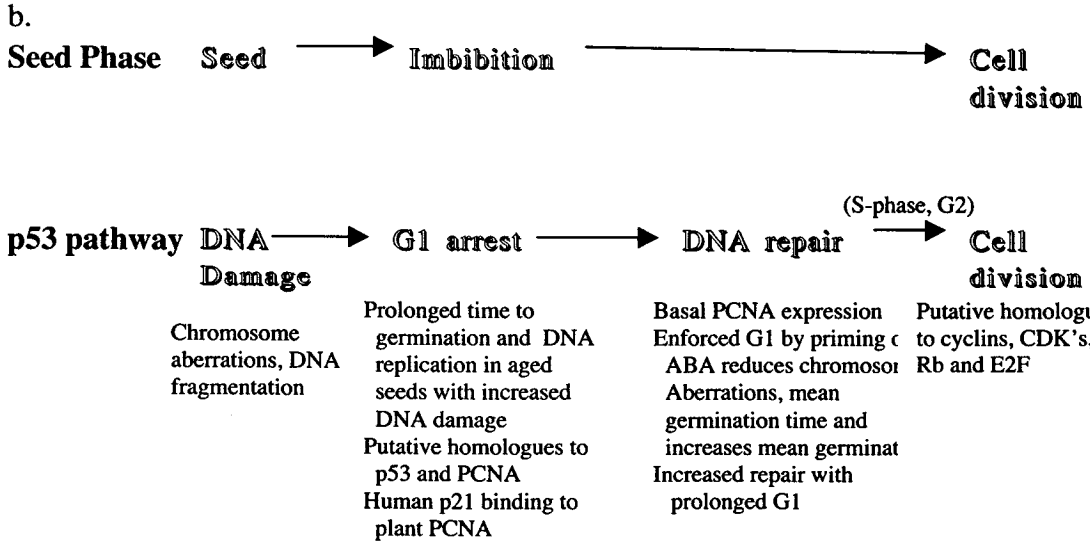
If a p53-related-DNA-damage-response-system exists during process of seed germination, then it may be expected that older seed containing increased levels of DNA damage (Cheah and Osborne 1978) will require an extended time period to undergo cell division following imbibition. Evidence indicates that non-dormant seed from older seedlots do have an increased mean germination time (Figure 5a, Dell-Aquila and Trito 1990) relative to their unaged counterparts, a characteristic that may primarily be due to a delay in the onset of DNA replication (i.e., an extension of G1 for those cells in G1 within the dry seed, Osborne 1983). Possible factors causing this prolonged G1 in older seed include: i) the prevention of cells from proliferating due to the effects of a damaged DNA template during early imbibition (thereby impeding effective transcription), ii) the active process of arresting cells in G1 allowing pre-replicative DNA repair and, iii) other unknown causes. Of these, the first possibility may be unlikely because evidence in irradiated and mutagen-treated mammalian cells indicates that the presence of damaged DNA template in itself does not impede the ability of cells to progress through the cell cycle (Kastan *et al.* 1991). Given that cells containing DNA damage can divide, it is likely that many aged plant embryonic cells are actively arresting in G1. Nevertheless, since other plant-specific factors, genes, or intracellular conditions (e.g., damaged membranes or rRNA) may be causing a prolonged G1 arrest, further molecular evidence is necessary to determine whether a p53-related pathway may be responsible.

Figure 5.a. Steps inherent to cell division of non-dormant unaged and aged viable plant seed. Shortly following imbibition (during G1 in most cells) seed undergo all processes necessary for cell division including synthesis of new mRNA tRNA, rRNA and proteins, as well as respiration, mobilization of storage reserves, and membrane stabilization. During this early developmental period DNA repair occurs. For aged seed, with increased levels of DNA damage, the time period between imbibition and cell division is prolonged suggesting that these seeds may have a greater time period in which to conduct DNA repair. b. Evidence consistent with the presence of a p53-related pathway in plant seed during three progressive developmental stages of seed emergence including seed quiescence, imbibition, and cell division, and the parallels with p53-mediated-G1-arrest associated with each stage (DNA damage, G1 arrest, DNA repair, cell division).

a.



b.



DNA Repair Occurs During Prolonged G1 in Imbibed Seed

Since DNA repair can occur shortly following seed imbibition (for both aged and unaged seed, Osborne 1983, Wang and Berjak 2000, Roberts 1988), the extended time period required for aged seed to germinate may be an active process of prolonging G1 arrest to accommodate the greater levels of pre-replicative repair needed in these embryos. The importance of a prolonged G1 for induction of such repair may be indicated by the artificial enforcement of G1 arrest on aged seed containing DNA damage by priming (exposing seed to an osmoticum) or by abscisic acid (ABA) treatment, each which may extend the time period between the start of imbibition and DNA replication (i.e., G1 for many cells, Sivritepe and Dourado 1995). Such priming and ABA treatment have been shown to reduce the levels of chromosomal aberrations in aged seed and to enhance the capability to germinate (as indicated by an increase in the percentage of normal germination and a reduction in the mean germination time (Dell' Aquila and Tritto 1990, Sivritepe and Dourado 1995). In this regard, extension of G1 arrest in aged seed may be directly related to the level of pre-replicative DNA repair, a feature consistent with the p53-mediated-G1-arrest in human and mouse cells. Nevertheless, because it may be possible that ABA and priming could reduce DNA damage by independent pathways, the levels of DNA repair following imbibition among seed of different ages will need to be determined before the precise relationship between elongation of G1 and the level of DNA repair can be ascertained.

Putative Homologues

While the presence of a prolonged G1 and DNA repair following imbibition of aged seed could be indicative of an active process of G1 arrest, the immunodetection of numerous putative cell cycle proteins within plants indicates that a p53 homologous pathway could be responsible for this arrest (Figure 5b). For example, using a human p53 antibody (Pab-240), a putative p53 homologue has been detected at remarkably high levels in unaged *Zea mays* L. seed, with successively lower levels occurring during imbibition and after the start of replicative DNA synthesis (Georgieva *et al.* 1994a, Cruz-Garcia *et al.* 1998), a pattern consistent with the trend observed in mammalian p53. The tendency of the putative p53 protein to exist prior to DNA replication suggests that it could be an active enforcer of G1 arrest in both the imbibed embryo and the quiescent seed (suggesting the protein may be formed during seed maturation). Furthermore, its presence in the quiescent seed suggests that the need for transcription upon imbibition may be circumvented (from a damaged template), a characteristic consistent with the post-translational regulation of p53 inherent to humans and mice. Nevertheless, while the changes in the abundance of this putative p53 protein are consistent with p53-mediated arrest in mammals, the studies published to date only demonstrate that a protein region consistent with the human p53 epitope exists in seed. Further evaluation of the constitution of this protein by either protein sequencing and/or protein-protein interaction studies, as well as the use of antibodies containing other epitope affinities within both aged and unaged seeds will be essential for the determination of whether a true p53-related homologue exists.

Evidence of a conserved binding site between human p21 and plant PCNA (Ball and Lane 1996) suggests that at least some of the downstream components of a p53-induced-G1-arrest could be conserved between plants and mammals (Figure 5b). Because plant PCNA has been shown to function as a cofactor of DNA synthesis in rice (*Oryza sativa,* Matsumoto *et al.* 1994), similar to its function in humans and mice, it is likely that inhibition of this function (possibly by a p21 homologue, other CKI's have been detected in plants, den Boer and Murray 2000) would have the same effect in plants as in mammals. A putative PCNA homologue (detected using an anti-human antibody) in maize seed indicates that this protein occurs at maximum levels during the first S-phase, a feature consistent with the PCNA role in mammalian DNA replication, while occurring at basal levels during the initial hours of imbibition, consistent with its role in pre-replicative DNA repair in mammals (Georgieva *et al.* 1994b). In addition to characterization of these potential homologues important for p53-mediated-G1-arrest in seeds, the identification of putative homologues of proteins essential for releasing cell cycle arrest including E2F, Rb, cyclins and CDK's (Georgieva *et al.* 1994b, Cruz-Garcia *et al.* 1998, Murray *et al.* 1998) suggests that it may be possible that the complete pathway between DNA damage, p53-induced-G1-arrest, DNA repair, and the re-initiation of the cell cycle could be inherent to the process of seed germination.

PERSPECTIVES AND FUTURE DIRECTIONS

Determination of whether a p53-mediated-G1 arrest pathway (or a downstream component of the pathway) may truly be inherent to aged seed will require investigation beyond the use of human probes to include the study of interaction between putative

homologues, protein sequencing, and a greater focus on the identification of genes. An important component of future analysis will be determination of the cell cycle phase distribution in aged-seeds and during germination (providing information regarding the relative number of cells in G1 (2C) and G2 (4C) associated with molecular changes). Furthermore, it may be prudent to examine the meristematic regions of the aged seed embryo, because these cells are necessary for normal seedling growth and development (and thus where DNA damage may have the greatest effects, and the need for repair should be highest). Because a definitive p53 homologue has not yet been identified in plants despite the fact that the entire genome of *Arabidopsis thaliana* has now been sequenced (Walbot 2000), suggests that if a p53 plant gene exists, it may share little sequence homology with its human counterpart. Nevertheless, given that plant seed have such remarkable parallels to the p53-mediated-G1-arrest pathway in humans and mice, the germination of aged seed may be an especially useful model system to identify possible *p53* RNA and protein homologues, to characterize downstream gene and protein homologues involved in a possible p53-related-pathway, and/or to develop an understanding of possible novel processes underlying DNA damage, G1 arrest, and DNA repair in plants.

CHAPTER 5

HOW DO MOST MUTATIONS ARISE?

INTRODUCTION

Mutations cause diseases and play an important role in many evolutionary processes including sexual reproduction, population extinction and evolution of mating systems. Currently, it is widely believed that almost all germline point mutations (i.e., base pair changes) are copying mistakes made during S-stage-DNA replication (Li *et al.* 1996, Makova and Li 2002). This viewpoint has mostly been based on two types of findings. Firstly, the finding that higher mutation rates exist in male- than in female-carried DNA in some animals is believed to be caused by the higher number of cell divisions, and therefore DNA replications (and errors), in the male germline. Secondly, the higher substitution rate of selectively neutral DNA, which equals the point mutation rate (Kimura 1983), in organisms with shorter generation times is considered evidence that most mutations are replication dependent. This is because taxa with shorter generation times undergo more germline cell divisions per unit time. Here, a synopsis of the argument for, and a review of the weaknesses of the belief that most point mutations are caused by mistakes during synthesis (S)-stage DNA replication are provided. It is described why other factors, including DNA damage and mutations arising during the resting stage (G0 and/or G1) of the cell cycle, may play a important role. The lack of attention on the potential importance of DNA damage in determining the mutation rate is surprising because it has long been known that the DNA of all organisms is continually under the threat of attack from environmental and cellular mutagens (Friedberg *et al.* 1995). In addition, there is considerable information suggesting that there is a positive

relationship between substitution rates at silent sites and the factors known to induce DNA damage such metabolic rate, salinity and ultraviolet radiation. Another phenomenon that contradicts the theory that all mutations are S-stage replication errors is that many point mutations can occur in non-dividing cells.

THE ARGUMENT THAT MOST MUTATIONS ARE S-STAGE REPLICATION ERRORS

Male-Mutation Bias

Synopsis of the Evidence for Male-Mutation Bias

The notion that most point mutations are replication errors has largely been based on the existence of a higher substitution rate in male-carried DNA (Table 10). Analyses of the nucleotide substitution rates in selectively neutral DNA in the male and female sex-chromosomes (Miyata *et al.* 1987) indicate that male-carried DNA evolves faster in humans (Nachman and Crowell 2000), other higher primates (Shimmin *et al.* 1993, Chang *et al.* 1996, Makova and Li 2002), birds (Ellegren and Fridolfsson 1997, Kahn and Quinn 1999), rodents (Chang *et al.* 1994) and sheep (Lawson and Hewitt 2002). Male-mutation bias has also been shown to exist by the quantification of diseases inherited from normal male and female parents. For example, from large-scale population analysis, males have been shown to have a higher point-mutation rate for Hemophilia B (Haldane 1947, Green *et al.* 1999) and Apert syndrome (Moloney *et al.* 1996). The male-to-female ratio of selectively neutral substitution rates has been shown to be similar to the ratio of cell divisions in mice and in rats, where each is approximately two (Chang *et al.* 1994, Li *et al.* 1996), in *Drosophila*, where a one-to-one relationship has been reported.

Table 10. The ratio of male-to-female point mutations, the ratio of male-to-female cell divisions, and conclusions about the S-stage-replication-based theory of mutation for studies in a range of organisms. *K* represents methods that use nucleotide substitution rates.

Organism(s)	Male-to Female Mutation Ratio	Male-to-Female Cell division Ratio	Methods and Gene/DNA region Assessed	Conclusions/Postulations made about replication-based mutation	Reference
Humans	2.5	None	*K* for the *ZFX* and *ZFY* introns	Mutation rate largely depends on number of DNA replications	Erlandsson *et al.* 2000
Humans	4	None	*K* of X-linked and autosome pseudogenes	None	Nachman and Crowell 2000
Humans and primates	5.1	3-6, age 15-20	*K* for *AMGX* and *AMGY* intron	Point mutations in nuclear DNA is largely DNA-replication dependent	Huang *et al.* 1997
Humans and primates	5.25	None	*K* for noncoding Y-linked and autosomal DNA	DNA replication errors are the primary source of germline mutation	Makova and Li 2002
Humans and chimpanzees	3	None	*K* for X- and Y-linked DNA and autosomal DNA	Mutation may be replication dependent; other unknown factors also important	Ebersberger 2002
Humans	8.64	None	Male and female mutations for Hemophilia B, includes both point mutations and a small number of indels	Mutation rate is not largely dependent on DNA methylation	Green *et al.* 1999

Table 10

Humans	None	Ratio at least: 8, age 20 10, age 25-30 32, age 45	None	Mutation bias should increase with paternal age nonlinearly	Carlson *et al.* 1994
Higher Primates	1.7	None	*K* for 38kb X-linked and Y-linked noncoding DNA	Mutation ratio not parallel to cell divisions; Replication errors may account for minority of mutations	Bohossian *et al.* 2000
Higher Primates	3-6,	2.8, age 15 6, age 20	Summary of various data from X and Y chromosomes	Mutation is largely DNA replication- dependent	Li *et al.* 1996
Higher Primates	6	6	*K* for ZFX and ZFY introns and of SMCX SMCY introns. Other estimate of male-to-female ratio of 12 deemed unreliable	Mutation is largely replication dependent	Chang *et al.* 1996
Higher Primates	6	None	*K* for *ZFX* and *ZFY* intron	Replication-independent mutagenic factors are likely not negligible	Shimmin *et al.* 1993
Humans. Mice and horses	3	None	*K* for *SMCX* and *SMCY* genes (silent sites)	Male-bias is low; Replication-independent mutagenic factors are likely not negligible	Agulnik *et al.* 1997
Mice	2	2, age 5 months	*K* at introns	Mutation is largely DNA replication-dependent	Li *et al.* 1996

Table 10

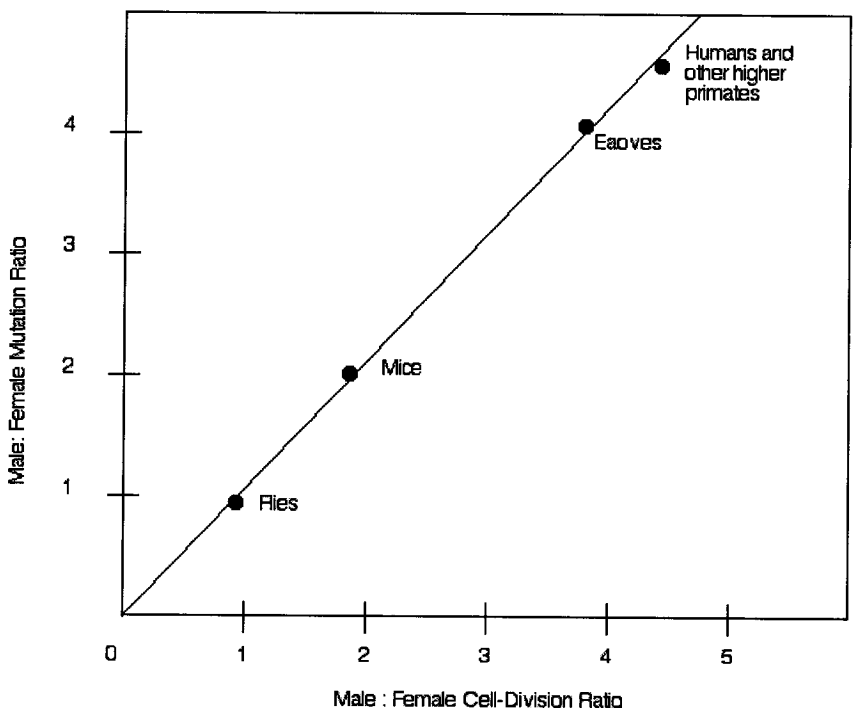
Mice and Rats	2	2	*K* for *ZFX* and *ZFY* intron	Replication errors are the primary source of mutations	Chang *et al.* 1994
Birds	4.1	4.4	*K* for W- and Z-linked *CHD* exon (silent sites) and introns, Z-male, W-female chromosome	DNA replication errors are a primary source of mutation	Kahn and Quinn 1999
Birds	3.9-6.5	None	*K* for W- and Z-linked CHD exon (silent sites) and introns	Mutation is replication-dependent; mutations caused other factors cannot be excluded	Ellegren and Fridolfsson 1997
Birds	1.8-5	None	*Ks* for W and Z-linked *ATP5A1* genes	None	Carmichael *et al.* 2000
Sheep and Goats	2.9-3.9	None	*K* for *ZFX* and *ZFY* introns	None	Lawson and Hewitt 2002
Cats	4.38	None	*K* for *ZFX* and *ZFY* introns	None	
Flies	1	1	*K* for introns for genes X and autosomal chromosomes DNA	Mutation ratio parallels cell division	Bauer *et al.* 1997
Gymnosperms	2-10	None	*K* for silent sites and introns	None	Whittle and Johnston 2002

(Bauer *et al.* 1997), and in humans and other higher primates, where each is between three and six (Shimmin *et al.* 1993, Makova and Li 2002). This proportionality has been considered evidence that most mutations are replication errors (Table 10). Four of the species groups studied to date have reliable estimates of the male-to-female cell division ratio including flies, higher primates, eoaves (birds), and mice (Table 10). Using these four data points, it appears that there is a strong correlation between cell division rates and mutation rates (Figure 6), consistent with the theory that most mutations are replication errors. There are, however, several issues that one should consider in deciding whether the male-mutation bias provides evidence that most point mutations are replication errors.

Weaknesses of the Argument that Male-Mutation Bias Supports Replication Errors

The correlation between the ratio of male-to-female mutations and the ratio of male-to-female cell divisions is striking. The degree to which this correlation supports the replication hypothesis, however, is limited by the uncertainty of each of these estimates. The ratio of male-to-female mutations varies with the nucleotide composition of the gene, the species, and the methods used to estimate the male bias (Table 10). For example, in humans and other higher primates, the male-to-female mutation ratio using DNA sequence analysis has often been estimated to be between three and six (Table 1, e.g., Shimmin *et al.* 1993, Makova and Li 2002). Higher estimates have been derived from diseases including Hemophilia B (8.6 for point mutations plus indels, Green *et al.* 1999). Mutations for endocrine neoplasia Type B and Type A (Carlson *et al.* 1994) and Apert syndrome (Moloney *et al.* 1996) have been shown to arise entirely from males,

Figure 6. Relationship between the male-to-female mutation ratio and the male-to-female cell-division ratio for four species where each measure is known. For humans and primates the cell- division ratio was estimated as 4.5, the mean of the commonly cited range of 3 to 6. The mean value for the male-to-female ratio of mutations was obtained using references: Shimmin *et al.* 1993, Nachman and Crowell 2000, Ebersberger 2002, Agulnik *et al.* 1997, Bohossian *et al.* 2000, Carmichael *et al.* 2000, Makova and Li 2002. For the other species groups male-to-female ratio of cell divisions and mutations were obtained as follows: mice, Chang *et al.* 1994, Li *et al.* 1996; flies, Bauer *et al.* 1997; and eoaves, Kahn and Quinn 1999.



suggesting the male-to-female mutation ratio is even higher. In addition to the inherent noise among studies, another concern is that each of these estimates has enormous errors, with the upper range of the 95% confidence interval including values as high as 30, 80 and sometimes infinity (e.g., Shimmin *et al.* 1993, Chang *et al.* 1996, Makova and Li 2002). Similar to the ratio of mutations, the values of the ratio of the number of male-to-female germline cell divisions also vary considerably and depend on the assumed mean age of reproduction. For example, the male-to-female cell division ratio in humans has been estimated to be both 8 (Crow 1997, Huttley *et al.* 2000) and 6.2 (Crow 1997) assuming an average reproductive age of 20, to be 2.8 at age 15, 18 at age 30 (Crow 1997), 23 at age 35 (Huttley *et al.* 2000) and 32 at age 45 (Crow 1997). Another issue is that for species other than humans and mice, including higher primates, birds, sheep and cats, there is relatively little information about the number and pattern of germline cell divisions, and thus the relationship between the number of cell divisions and the point-mutation rate is even more uncertain. These variations in estimates of the ratio of cell divisions and the ratio of mutations are important because only a two fold difference between them would indicate that most mutations (more than 50%) are caused by factors other than replication errors.

In addition to difficulties in establishing a relationship between cell divisions and point-mutation rates, there are several commonly cited concerns about using substitution rates of selectively neutral DNA on sex chromosomes to estimate the male-to-female mutation rates. For example, McVean and Hurst (1997) and Smith and Hurst (1999) have suggested that there is a selective reduction of the mutation rate on the X chromosome due to the pressure of hemizygous expression, and that there is no true

male-mutation bias. There has been some evidence against this viewpoint, including the higher substitution rates for male-carried DNA in birds, where the males are the homogametic sex (Ellegren and Fridolfsson 1997), and the detection of male-mutation bias using autosomal and Y-linked DNA, without the use X-linked DNA (Makova and Li 2002). Nonetheless, one cannot rule out the possibility that selection may affect the estimates of male-mutation bias obtained using DNA sequence analysis, even those not using the X chromosome. For example, background selection and/or selective sweeps may affect substitution rates on the sex chromosomes (Charlesworth and Charlesworth 1993). In particular, lower, rather than higher levels of genetic variation have been detected on the Y chromosome, a finding commonly attributed to selective sweeps (Charlesworth and Charlesworth 1993, Whitfield *et al.* 1995, Hammer *et al.* 1995).

Another concern with using male-mutation bias as evidence that most point mutations are replication errors is that there are many, possibly yet unidentified, differences other than the number of cell divisions that could cause the bias. The more compact packaging of DNA in the sperm than the eggs is just one factor that could affect the mutation rate, resulting in male-mutation bias. An additional issue is that male-mutation bias may be caused by the higher methylation in male germlines (a type of DNA damage which leads to point mutations, mostly C to T) rather than replication errors (Hurst and Ellegren 1998, McVean 2000, Huttley *et al.* 2000). Methylation has been associated with the ten-fold higher point-mutation rate in male-inherited human pseudogenes (Friedberg *et al.* 1995). It has been argued, however, that DNA methylation likely has little effect on substitution rates in selectively neutral DNA (Hurst and Ellegren 2002), and the higher mutation rate for males for Hemophilia B was shown not to be

caused by a higher mutation rate at methylated sities (Green *et al.* 1999). Nevertheless, there is insufficient evidence to exclude DNA methylation as an important factor underlying at least some cases of male-mutation bias. As in DNA sequence analysis, there are also inherent problems with estimating the male-to-female mutation rates using the more direct approach of measuring the onset of human diseases. In these cases, most mutations occur at only a few sites which have unusually high mutation rates, which are often methylated (Li *et al.* 2002), and not all mutations have a detectable effect. In addition, as reviewed by Hurst and Ellegren (2002), the frequency of achondroplasia mutations was shown to be tenfold higher in children even when the frequency of the causative point mutation was only two fold higher in the sperm. It is possible that selection favours the transmittance of deleterious mutations to offspring by the sperm (Hurst and Ellegren 2002), a phenomenon that has been shown to exist in some plants (Whittle and Johnston 2003a). This suggests the analysis of male- and female-inherited diseases does not accurately reflect gender-specific mutation rates.

One means to evaluate whether male-mutation bias can be caused by factors other than replication errors is to examine organisms in which the number of cell divisions between generations is similar for the male and female gametes. Any realized differences in mutation rate between male- and female-inherited DNA would therefore result primarily from sex-specific differences in the point mutation rates during gamete production. Cosexual seed plants fit this description. Seed plants produce both sperm and eggs during each reproductive bout, with many vegetative cell divisions between each bout. Eggs and sperm therefore do not probably differ much in number of cell divisions for each generation. Despite the likely similarity in the number of cell divisions, there is

evidence that plant sperm have more point mutations than eggs. Specifically, there are higher substitution rates in selectively neutral male-inherited mitochondrial and chloroplastidial DNA in gymnosperms (Whittle and Johnston 2002), a result that, unlike many other studies of male bias, cannot be explained by differences between the sex chromosomes in selection or methylation levels. It thus appears that, at least in some gymnosperms, male-biased-point-mutation rates exist in the absence of a difference in number of DNA replications. Nevertheless, if there were a tendency of maternally inherited organelles to undergo fewer organellar replications per cell division (which seems unlikely), replication errors could underlie these differences in the point-mutation rate.

It is notable that, in addition to point mutations, male-mutation bias has been reported for other types of mutations. Specifically, a higher mutation rate has been reported for microsatellite mutations (McVean and Hurst 1997, Ellegren 2000). In these cases, the ratio of male-to-female mutation was reportedly lower than what would be expected based on the number of DNA replications (Table 10). Male-mutation bias has also been shown to exist for deletions (of a few base pairs) leading to Pelizaeus-Merzbacher disease (Mimault *et al.* 1999). The uncoupling of the ratio of cell divisions and indel mutation rates suggests either that the number of strand slippage events is uncoupled with the number of DNA replications or that replication-independent events govern the level of indels (Huttley *et al.* 2000). Male-mutation bias has also been reported for transposable elements (Erlandsson *et al.* 2000), a result that cannot be explained by replication errors. Another relevant issue is that the male-mutation bias is sometimes clearly absent. In particular, the mutation rate of microsatellite mutations has

been found to be between 2.5 and five times higher in female than in male birds (Brohede *et al.* 2002). These results suggest that the mutation rate, at least for mutations other than point mutations, are not replication dependent.

Generation-Time Effects

Evidence for Generation-Time Effects

Another widely accepted finding that supports the notion that most point mutations are DNA replication errors is an association between mutation rates and generation time. The generation-time effect predicts that taxa with shorter generation times have a higher mutation rate per unit time because they undergo more rounds of germline cell divisions, and therefore more DNA replications, per unit time than taxa with longer generation times (Wu and Li 1985). A wide range of studies has found generation-time effects (Laird *et al.* 1969, Kohne 1970, Wu and Li 1985, Ohta 1993). For example, the selectively neutral DNA in rodents evolves substantially faster than in primates (Li *et al.* 1996). One of the most commonly cited pieces of evidence for the generation-time effect is the faster evolutionary rate of the old world monkey lineage than humans ("hominoid slowdown", Seino *et al.* 1992, Li *et al.* 1996), which is consistent with the theory that most mutations are S-stage replication errors.

When one examines the generation-time effect using DNA sequence analysis, it is possible that generation time and the number of germline cell divisions per generation has changed many times over the phylogenetic histories of the compared species. One can assess whether the generation-time hypothesis holds over the short term, when the generation time and the number of germline cell divisions is known with some certainty.

By assessing the currently available information, Drake *et al.* (1998) estimated the point-mutation rate per DNA replication in the germline per generation for four well-studied organisms, *Homo sapiens* (generation time 30 years), *Mus musculus* (2 months), *Caenorhabditis elegans* (5 days) and *Drosophila melanogaster* (12 days). Using this information, the number of mutations per year and the number of cell divisions per year was calculated for each of the four taxa (Table 11). It was found that the annual mutation rate per bp per year increased in the following order: *H. sapiens* < *M. musculus* < *C. elegans* < *D. melanogaster*. The annual mutation rate was, as expected by the generation-time hypothesis, generally higher in the organisms with shorter generation times with the exception of *D. melanogaster* and *C. elegans* which were opposite what was expected. In addition, the annual mutation rate and cell division rate seem to be positively correlated (Figure 7) suggesting that S-stage replication errors cause most mutations.

Weaknesses of the Argument for Replication Errors Based on The Generation-Time Effect

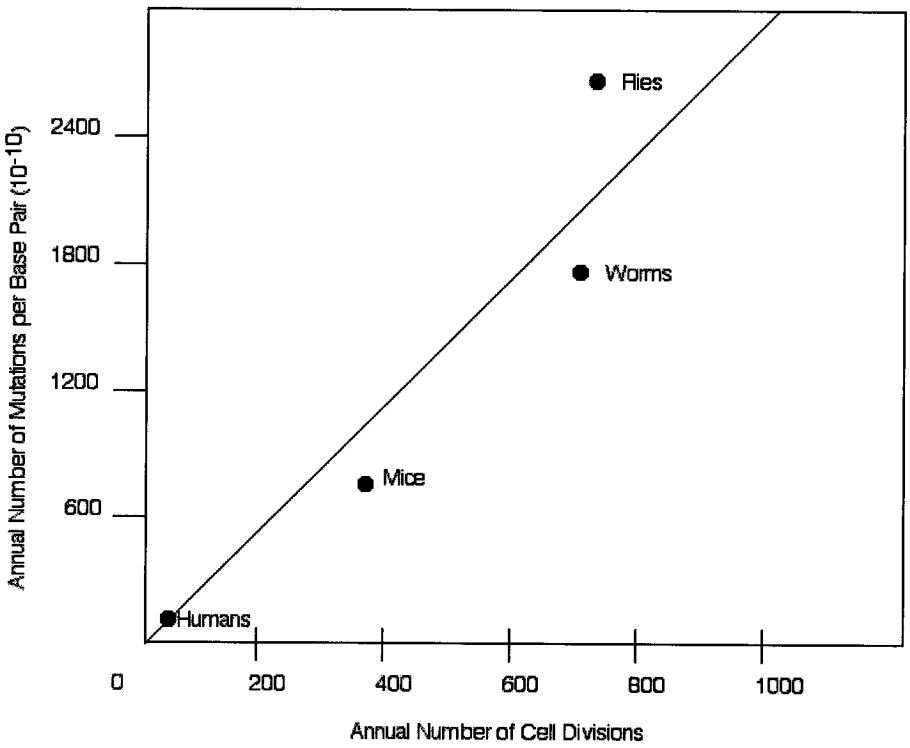
The validity of the generation-time effect using DNA sequence analysis has been considered uncertain as the sample size used in many studies is very small, includes few genes and often involves highly phylogenetically divergent comparisons, and therefore many species-specific factors other than the number of replication errors could account for the rate variation (Bromham *et al.* 1996). Moreover, some generation-time studies are plagued by uncertain fossil dates and outgroups (Easteal and Collett 1995). One of the most taxonomically complete analyses conducted to date that has shown the presence of

Table 11. Estimate of the annual number of cell divisions and number of mutations for four well-studied organisms.

Species	Mean generation time (years)* *(A)*	Cell divisions per generation+ *(B)*	Mutations per bp per cell division+ *(C)*	Number of mutations per bp per generation *(CXB)*	Annual number of cell division *(B/A)*	Annual number of mutations per bp *CX(B/A)*
Homo sapiens	30	400	5.0×10^{-11}	2.0×10^{-8}	13.3	6.6×10^{-10}
Mus musculus	0.16 (60 days)ψ	62	1.8×10^{-10}	1.1×10^{-8}	388	6.9×10^{-8}
Drosophila melanogaster	0.033 (12 days)	25	3.4×10^{-10}	8.5×10^{-9}	758	2.6×10^{-7}
Caenorhabditis elegans	0.013 (5 days)	9	2.3×10^{-10}	2.0×10^{-9}	692	1.6×10^{-7}

*, *H. sapiens* (Huttley *et al.* 2000), *M. musculus* (Linzey 1995), *C. elegans* (Sulton 1988), *D. melanogaster* (Ashburner 1989)
+, Drake *et al.* (1998)
ψ, Average reproductive age of mice differs among studies, sometimes suggested to be as high as 5 months (Li *et al.* 1996)

Figure 7. Relationship between annual cell division rates and annual mutation rates for humans (*Homo sapiens*), mice (*Mus musculus*), flies (*D. melanogaster*) and worms (*C.elegans*). Data from Table 11.



generation-time effects (61 mammalian species) was based on mitochondrial genes (*cytochrome b* and *beta-globin*, Bromham *et al.* 1996). This analysis unfortunately provides little information about the cause of most point mutations because of the uncertainty between mitochondrial DNA replication rates and cell division.

Although there has been broad acceptance of the generation-time theory (Li *et al.* 1996), many exceptions have been identified, suggesting that, at least in certain cases, something besides or in addition to replication errors could be a major factor affecting the point-mutation rate. For example, no relationship was detected between evolutionary rates and generation time among species of hummingbirds (Bleiweiss 1998). Species of mammals and species of reptiles with shorter generation times have been shown to have slower, rather than faster, rates of nucleotide evolution for nuclear and mitochondrial genes, respectively (Hasegwawa 1989, Avise *et al.* 1992). One of the more complete analyses of generation-time effects conducted to date (Kumar and Subramanian 2002) was based on analysis of fourfold degenerate sites for 5 669 genes across more than 326 mammalian species. This study indicated that the point-mutation rate is approximately constant per year (Kumar and Subramanian 2002) and it was concluded that there is no evidence that generation time affects molecular evolutionary rates and that most point mutations are likely caused by replication-independent factors (see also ref. Huttley *et al.* 2000).

In addition to animals, the generation-time theory has been applied to wide range of plants, where the selectively neutral DNA of taxa with shorter generation times has sometimes been found to evolve faster (e.g., Gaut *et al.* 1992, Gaut *et al.* 1996, Laroche and Bousquet 1999). Similar to animals, there are exceptions (e.g., Whittle and Johnston

2003b). In a broad-taxonomic study of more than 40 phylogenetically independent species pairs of seed plants it was found that generation time does not generally affect the mutation rate (Whittle and Johnston 2003b). Regardless of the effect of generation time, it seems reasonable to hypothesize that if point mutations arising during somatic cell divisions are transmitted to the gametes in plants (Klekowski 1998), then plants with higher annual growth rates should exhibit a higher annual point-mutation rate. Analysis of substitution rates of the 18S *ITS1* and *ITS2* regions in nine phylogentically independent species pairs, however, provided no evidence that the point-mutation rate differs among plants with different growth rates (Whittle and Johnston 2003b), suggesting S-stage replication errors are not the main cause of mutations. Further information on the pattern of somatic cell divisions is needed to make strong conclusions about the role of replication errors in determining the point-mutation rate in plants.

Summary of Weaknesses of Argument that Most Mutations are Replication Errors

It has been widely accepted, based on the existence of male-mutation bias and generation-time effects, that copying mistakes during DNA replication are the major source of point mutations. There are, however, many issues that need to be considered. Firstly, male-mutation bias and generation-time effects have been studied in very few species and genes. Secondly, there have been many exceptions identified for each of these hypotheses. Thirdly, there are uncertainties about the use of DNA sequence analysis to estimate the male-to-female point-mutation ratio. Fourthly, the most extensive analyses of generation-time effects conducted to date in animals and plants (Kumar and Subramanian 2002, Whittle and Johnston 2003b), suggest that that annual point-mutation

rates are similar among lineages with very different generation lengths. Fifthly, there has not been any independent evidence to date that the point mutations reported in many studies were caused by replication errors. As noted by several authors (e.g., Britton 1986, Crow 1997, Easteal and Hervert 1997, Huttley *et al.* 2000, McVean 2000, Hurst and Ellegren 2002) both male-mutation bias and generation-time effects could equally be explained by other causes, including DNA damage.

WHAT IS THE EVIDENCE FOR DNA-DAMAGE-INDUCED POINT MUTATIONS?

Some of the most common types of DNA damage include DNA stand breaks and crosslinks, abasic sites, modified nucleotides, including bases and sugars, UV-photoproducts, including bonds between adjacent pyrimidines (dimers), and methylation of bases, each of which can lead to point mutations during DNA replication or repair (Friedberg *et al.* 1995). Although there has been some support for the idea that DNA methylation substantially affects point-mutation rates, which could account for male-mutation bias (Hurst and Ellegren 2000, McVean 2000), the role of DNA damage in determining the point-mutation rate has received relatively little attention. This is remarkable because DNA is under constant attack by metabolic byproducts and environmental agents and there is substantial evidence that DNA damage accumulates in resting cells in a time-dependent manner, as found in seeds and bacteria (Cheah and Osborne 1978, Friedberg *et al.* 1995, Whittle *et al.* 2001). A major mechanism for the formation of point mutations is the replication of DNA damage that has escaped repair. This process, known as translesion replication, provides a certain tolerance to DNA damage at the expense of mutations (also known as translesion bypass or translesion synthesis, Advin *et al.* 2002). Through this mechanism, the DNA polymerase bypasses

DNA damage (Friedberg *et al.* 1995), a process known to occur in bacteria, yeast as well as mammalian cells (Friedberg *et al.* 1995, Advin *et al.* 1995). Most studies of the types of point mutations that are induced by specific types of DNA damage during translesion replication have been based on examination of UV-photoproducts, which often lead to C to T mutations, and it has been shown that a range of nucleotide substitutions can occur at abasic sites (Friedberg *et al.* 1995). Further studies of the relationship between translesion replication of DNA lesions caused by other mutagenic agents, including metabolic byproducts, will likely reveal that many point mutations result from other types of DNA damage.

Metabolic-Rate Effects

The importance of DNA damage in causing point mutations is also evidenced by DNA sequence analysis. The metabolic-rate hypothesis predicts that the selectively neutral DNA of taxa with higher metabolic rates evolves faster because they have more mutations, resulting from a greater level of DNA-damaging byproducts from oxygen metabolism (Kohne 1970, Martin 1999). Considerable evidence supports the metabolic-rate hypothesis, including a magnitude lower synonymous substitution rate at the mitochondrial *cytochrome b* and nuclear *dls*, *hsp70* and *RAG-1* genes in sharks than in mammalian species, which have similar generation times (Martin 1999). In addition, metabolic rate was shown to explain the many exceptions to the generation-time hypothesis for some nuclear and mitochondrial genes among species of animals (Martin and Palumbi 1993). Metabolic rate was correlated with DNA hybridization analysis among 26 hummingbird species, in which generation time was shown to have no effect (Bleiweiss 1998). An advantage of this study is that the species were closely related and

thus the authors were able to exclude generation time as a potential factor, and thus, strongly suggest that mutation rates are affected by the level of DNA damaging metabolic byproducts.

Similar to studies about male-biased mutation and generation-time effects, there are some exceptions to the relationship between substitution rates and metabolic rates. For example, slow evolutionary rates have been reported among birds that have high metabolic rates (Mindell *et al.* 1996), and mitochondrial DNA evolves faster in alligators than in birds, which have the higher metabolic rate (Janke and Arnason 1997). Other evidence includes the lack of association between metabolic rate and substitution rates of mitochondrial and nuclear DNA in a broad-scale analysis across 61 mammalian species using phylogenetically independent comparisons (Bromham *et al.* 1996). The lack of effects, however, may be due to the difficulty in isolating metabolic rate from other factors that may affect the mutation rate (Easteal *et al.* 1995). It is not surprising that there are exceptions to the detection of metabolic-rate effects, because this type of analysis is based on interspecific comparisons, and thus there are many interacting factors that may affect the level of DNA damage, including species-specific physiology and DNA repair as well as susceptibility to environmental agents (Hebert *et al.* 2002). In this regard, the findings of higher selectively neutral substitution rates among species with higher metabolic rates in some studies (Bleiweiss 1998, Martin 1999, Advin *et al.* 2002), suggests that metabolic rate plays an especially important role in determining the point mutation rate in the organisms examined. Note that this is contrary to the generation-time effect, where exceptions would not be expected for interspecific comparisons, based on the notion that replication errors are the only cause of mutations. Other evidence cited

for the metabolic-rate theory is the faster evolutionary rate of mitochondrial than nuclear DNA in animals, which presumably is caused by greater exposure to metabolic byproducts (Martin 1999).

Other Factors Associated with Mutation Rates

In addition to metabolic rate, there are many other factors that affect the level of DNA damage and cause point mutations. Increased substitution rates in halophilic crustaceans has been attributed to a reduced point-mutation rate in saline conditions (Hebert *et al.* 2002). In addition, there have been many studies about the induction of DNA damage by UV-B that includes the formation of pyrimidine dimers, DNA protein crosslinks and DNA strand breaks, which can lead to point mutations upon replication or repair (Friedberg *et al.* 1995). That UV causes substantial levels of point mutations has been demonstrated by the increased occurrence of reversion mutations in the *B-glucuronidase* gene for transgenic plants (Kovalchuk *et al.* 2000) and the accelerated substitution rates in fungal lineages exposed to UV-B (Lutzoni and Pagel 1997), as well as innumerable other studies. Again, that these effects were detected despite the potential for such interaction suggests that they each play an important role in determining the mutation rate in the particular cases investigated.

Information from Microbes

It is worthwhile to consider the mutation rate among from microbes to assess whether this may provide insight about how most mutations arise. The point-mutation rate per DNA replication per genome for seven well-studied species of microbes has been

shown to be within the relatively narrow range of 0.0025 to 0.0046 (Bacteriophages M13, λ, T2 and T4, *Escherichia coli*, *Saccaromyces cerevisiae*, and *Neurospora crassa*, reviewed by Drake *et al.* 1998). One may expect that if mutation depended almost entirely on replication errors, then, on average, the mutation rate per genome per DNA replication would be higher in microbes with larger genome sizes. The information for mutation rates among microbes analyzed by Drake *et al.* (1998) does not support this trend. For example, the mutation rate per genome per generation was reported to be 0.0046 for Bacteriophage M13 with a small genome size of $6.4X10^3$ bp, ($7.2X10^{-7}$ mutation per bp per DNA replication) and to be 0.0030 for *Neurospora crassa*, with the relatively large genome size of $4.2X10^7$ bp ($7.2X10^{-11}$ mutations per bp per DNA replication). Thus, there does not seem to be a positive relationship between the amount of DNA that is copied and the number of mutations (largely because the mutation rate per bp is lower in taxa with larger genomes). Mutations in bacteria and some other microbes can be induced by a range of DNA damage-induced mechanisms including heat exposure, creation of mutator phenotypes by inhibiting a protein necessary for accurate DNA repair or replication, spontaneous DNA damage that forms mutations upon replication, and/or hypermutability resulting from an SOS response to DNA damage (Drake *et al.* 1998). It is not known how DNA damage or DNA repair may differ among these species of microbes that may explain the mutation rate differences. In addition, it is possible that selection has favoured a particular genomic mutation rate for each species (Drake 1991, Drake *et al.* 1998). Thus, it is not possible to make any definitive inference about the cause of most mutations based on the information from these microbes.

Point Mutations in Resting Cells

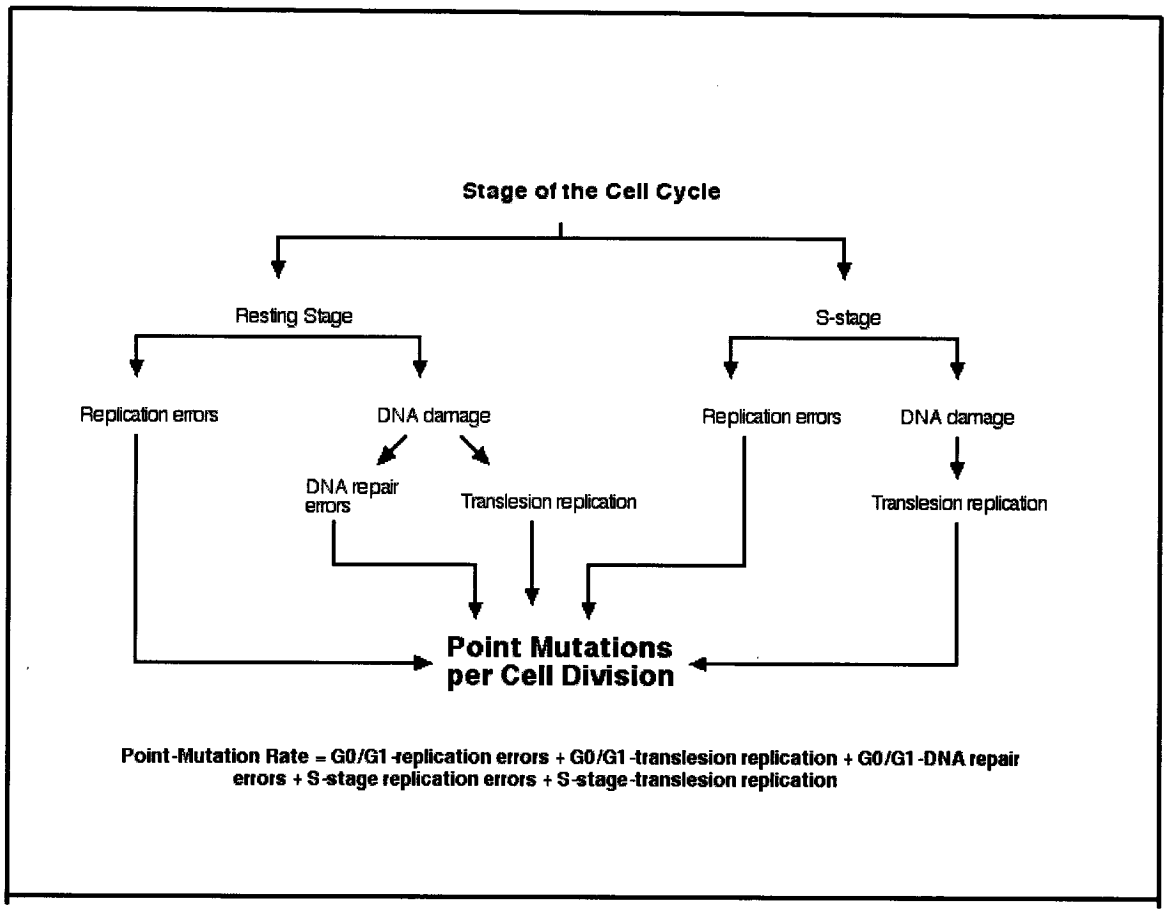
The hypothesis that most point mutations are caused by replication errors is based on firstly, the findings of proportionality between the number of cell divisions and the mutation rate, and secondly, the assumption that DNA replication errors occur during the S-stage of the cell cycle. There is evidence, however, from the study of rat neurons, human oocytes, cork cells in plants (*Quercus suber*) and bacteria indicating that many point mutations arise in non-dividing cells, without transition into the S-stage (Foster and Rosche 1990, Evans *et al.* 1994, Evans *et al.* 1995, Bridges 1997, Pla 2000). These mutations accumulate in a time-dependent manner. How do these mutations arise? Recent findings in bacteria indicate that DNA turnover occurs in resting cells (the resting stage represents either the G0 or G1 of the cell cycle, reviewed by Bridges 1998), which may lead to polymerase errors. In this regard, replication errors during the resting stage could be an important cause of point mutations (and one would not expect male-mutation bias or generation-time effects). It is possible that non-S-stage replication also occurs in eukaryotes. In addition to resting-stage replication errors, mutations occurring in non-dividing cells may also be explained by other factors. Although translesion replication has been studied only during the S-stage, it is possible that translesion replication also occurs in DNA-damaged regions during DNA synthesis in the resting stage. Alternatively, some of these mutations could be DNA-damage-induced mutations, arising from errors occurring during DNA repair. To date, the role of point mutations in non-dividing cells on the mutation rate has largely been ignored. Instead most mutagenesis research has focused on actively dividing cells, where the mechanism of mutation, and therefore the mutation rate, may differ from cells that spend considerable periods in the

resting stage. The focus on actively dividing cells is remarkable given that the majority of cells spend most of their lifespan in the resting stage. Mutations arising during this time period could cause substantial variation in the mutation rate per cell division, and be responsible for the exceptions to the male-mutation bias and the generation-time effect. In sum, it may be concluded that point mutation rate per cell division likely depends on several events. Specifically, point mutations may be caused by replication errors and translesion replication during the S-stage as well as replication errors, translesion replication, and erroneous repair of DNA damage in the resting stage (Figure 8).

CONCLUSIONS AND FUTURE DIRECTIONS

It has been widely accepted, based on the detection of male-mutation bias and generation-time effects, that S-stage replication errors are the major source of point mutations. Although it is likely that some fraction of point mutations is replication errors occurring during S-stage, there is growing evidence that many mutations arise from S-stage translesion replication, which is dependent on the level and types of DNA damage, and from mutations arising in the resting stage (Figure 8). It is probable that both models of point mutation apply to most organisms. The proportion of each type of point mutation in a gamete probably depends on the fidelity of the DNA polymerase and the physiological conditions and the environmental stresses in which an organism exists. This may explain some of the inconsistent results reported to date. In particular, it is possible that a correlation between the number of cell divisions and mutation rates, based on male-mutation bias and generation-time effects, can only be detected in cases where S-stage translesion errors and point mutations during the resting stage are relatively infrequent.

Figure 8. Summary of the potential factors affecting the point mutation rate during the resting stage and the S-stage of the cell cycle.



Accordingly, a correlation between environmental agents or other measures of DNA damage (e.g., metabolic rate) and the mutation rate may only be detected when they result in large levels of point mutations relative to the number of S-stage replication errors. It is currently not possible, based on the available information, to make definitive conclusions about the relative frequency of each category of mutations in the gametes. For this, new and more direct approaches, beyond that of detecting an association between mutation rates and gender, generation time, metabolic rate and/or environmental mutagens need to be developed. Increased study of mutation rates in dividing and non-dividing cells, containing different levels of DNA damage, will likely provide valuable insight into the role of resting-stage-replication errors and the level of DNA-damage induced mutations that arise during the resting stage. One potentially useful model system for such analysis may be plant seeds, where the transition from non-dividing to dividing cells is easily regulated and where the level of DNA can be readily controlled.

SUMMARY AND CONCLUSIONS

Altogether, the analyses provided here have several implications about the types of factors that influence the mutation rate, particularly for plants. Firstly, contrary to the widely accepted opinion that generation time affects evolutionary rates of selectively neutral DNA, it was reported that when one uses phylogentically independent comparisons of species pairs, where the differences other than generation time are minimal, there is no evidence that generation-time effects exist in plants. This suggests that the previous reports of an inverse relationship between generation time and mutation rates in plants may have been caused by the very small number of species that were compared, and/or by the large phylogenetic distance between compared taxa. Secondly, the potential for gender to affect evolutionary rates of selectively neutral DNA in plants had not been previously examined, possibly due to the difficulty in developing experimental systems to distinguish between male and female mutations and/or because it has been assumed that such effects cannot exist. The approach described in Chapter Two was designed to address this issue. The results suggest that, despite the fact that the germline separates late in development in plants, that gender does influence the mutations rate. Specifically, the faster evolutionary rates of selectively neutral DNA in male-inherited organellar DNA in gymnosperms indicates that more mutations are present in a sperm than an egg. If one assumes that the egg and sperm undergo similar numbers of pregametic cell divisions, then this would indicate that male-mutation bias in plants is caused by factors other than a higher number of germline cell divisions, and therefore replication errors, in sperm. Because it is not possible to know for certain whether the

egg and sperm are preceded by similar numbers of cell divisions in gymnosperms, further study will be necessary to ascertain the precise cause of the male bias. Thirdly, the importance of gender in influencing inheritance of mutations is supported by the finding, reported in Chapter Three, that more deleterious mutations are transmitted by a sperm than an egg in *Arabidopsis thaliana*. This suggests either that greater selection acts on an egg than on a sperm or that more mutations are removed by cell lineage selection for an egg. In either case, the results show that more deleterious mutations, those that have the greatest evolutionary consequences, are transmitted by a sperm than an egg. Fourthly, the many parallels between the process of the germination in aged seeds and the p53 pathway, described in Chapter Four, suggests that replication-independent mutations may be regulated by a similar molecular process in plants and in animals. In other words, the mutation rate in plants may largely depend on the level of expression of p53 or p53 downstream genes or may depend on the activity of molecules that inhibit these genes or the translated proteins. This suggests that future research should focus on the identification and characterization of genes and proteins involved in a putative p53 pathway in plants by using aged seeds. Fifthly, as described in Chapter Five, there has been considerable uncertainty about whether the mutation rate depends almost entirely on S-stage replication errors or whether many mutations are caused by DNA damage or occurring during the resting stage. To date, the balance of the information suggests that many mutations are caused by factors other than S-stage replication errors. This may account for the large level of noise inherent to the identification of factors associated with the mutation rate including generation time and gender.

Overall, the results presented here suggest that, contrary to widely accepted opinion, generation time does not affect mutation rates in plants. Considerable evidence indicates, however, that gender influences plant mutation rates and the transmission of deleterious mutations to progeny. Although many mutations are likely caused by replication errors, there is also much evidence that many mutations are caused by DNA damage, the level of which may depend on a p53-related pathway. Future studies should focus towards the identification of the cause(s) of male mutation bias in plants and the cause(s) of the increased transmittance of deleterious mutations by a sperm than by an egg, possibly including an assessment of role of S-stage and resting stage mutations. Such information will not only provide valuable insight about why mutation rates vary among genes, cells, and species, but will also provide a better understanding of the dynamics underlying evolutionary processes which largely depend on the mutation rate.

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