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Broad-Scale Analysis Contradicts the Theory That Generation Time Affects Molecular Evolutionary Rates in Plants

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Abstract. Several studies of plant taxa have concluded that generation time, including annual/perennial life history, may explain molecular evolutionary rate variation in selectively neutral DNA. Unlike in animals, there is little theoretical basis for why generation-time effects would exist in plants. Furthermore, previous reports fail to establish the generality of a generation-time effect in plants because of the small size of the datasets, a large proportion of which compared very widely divergent taxa differing in many characteristics other than generation time. Using 24 phylogenetically independent species pairs, each containing a species with an annual and a species with a perennial life history, and nine species pairs, each containing a tree species with a short and a long minimum generation time, we found no evidence that generation time is related to molecular evolutionary rate variation of the nuclear 18S *ITS1* and *ITS2* regions. This analysis strongly contradicts the growing belief that evolutionary rates are affected by generation time in plants. Possible reasons for the absence of generation-time effects are discussed, including an evaluation of the cell-division theory.

Key words: Generation time — Life history — Evolutionary rate — 18S — *ITS* — Mutation — Plant — Cell division

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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 and Wright 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 hypothesized 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 to 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, we provide a generically extensive analysis, 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.

Materials and Methods

Evaluation of Life-History and Minimum Generation-Time Effects

We chose species pairs based on availability of the complete DNA sequences of 18S *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, we used the following approach 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 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, we examined the appropriate phylogeny 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 between 182 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, we conducted the following supplemental analyses. 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, we 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 least 18 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

Table 1. Species pairs, outgroups, 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 membranaceus*	AF121675	Fabaceae	*Glycyrrhiza echinata*	U56000 U55999	Wojciechowsk et al. 1999[b]
Poaceae	*Bromus briziformis*	U83356 U83357	*Bromus racemosus*	U83372 U83373	Poaceae	*Brachypodium arbuscula*	AF019783	Aïnouche and Bayer 1997[b]
Portulacaceae	*Claytonia pelforiata*	AF084152 AF084173	*Claytonia megarhiza*	L78027	Cactaceae	*Pereskia aculeata*	L78035	Downie and Palmer 1994[a]
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	*Machaeranthera canescens*	U97622	*Machaeranthera tanacetifolia*	AF251567	Asteraceae	*Erigeron annuus*	AF118489	Morgan 1997[b]
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 and Soltis 1999[c]

Table 1. Continued

Family	Species 1	Accession number	Species 2	Accession number	Family of reference species	Reference species	Accession number	Phylogeny citation
Caryophyllaceae	*Silence gallica*	U30959 U30985	*Silene vulgaris*	U30969 U30996	Amaranthaceae	*Amaranthus albus*	AF210918	Reference from another family
Apiaceae	*Smyrnium olusatrum*	AH003553 U30595	*Myrrhis odorata*	AH003481 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 tetrasperma*	AF335210	*Vicia cracca*	AF335189 AF335190	Fabaceae	*Arachis batizocoi*	AF203553	Raina and Ogihara 1994[c]; Gimenes et al. 2000[d]
Violoaceae	*Viola arvensis*	AF097242 AF097288	*Viola calcarata*	AF097243 AF097289	Euphorbiaceae	*Macaranga angulata*	AF361112	Reference from another family
Asteraceae	*Volutaria lippi*	L35870	*Cheirolophus arboreus*	AF021147 AF021164	Asteraceae	*Gazania krebsiana*	U84770	

Comparisons of minimum generation time (MGT)

	Short MGT		Long MGT					
Aceraceae	*Acer macrophyllum*	AF020367	*Acer saccharum*	AF401152	Aceraceae	*Dipteronia sinensis*	AF020386	Suh et al. 2000
Betulaceae	*Betula pendula*	AJ006445	*Betula alleghaniensis*	X68133	Betulaceae	*Alnus maritima*	X68135	Chen et al. 1999[a]
Juglandaceae	*Carya illinoinensis*	AF303825	*Carya cordiformis*	AF303820	Juglandaceae	*Juglans nigra*	AF179579	Manos and Stone 2001[a]
Myrtaceae	*Eucalyptis grandis*	AF390471	*Eucalyptis globulus*	AF058467	Myrtaceae	*Angophora costata*	AF190356	Steane et al. 1999[a]
Oleaceae	*Fraxinus excelsior*	AH004997 AH004996	*Fraxinus ornus*	AH004981 U82893	Oleaceae	*Syringa amurensis*	AF297074	Wallander and Albert 2000[d]
Juglandaceae	*Juglans nigra*	AF179579	*Juglans microcarpa*	AF179577	Juglandaceae	*Alfaroa costaricensis*	AF303803	Stanford et al. 2000[b]
Pinaceae	*Larix decidua*	AF041343	*Larix laricina*	AF041348	Pinaceae	*Picea abies*	AJ243166 AJ243167	Govindaraju et al. 1992[a]
Rosaceae	*Prunus besseyi*	AF318732	*Prunus cerasitera*	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.

Table 2. Comparisons of annual/perennial life history[a]

		ITS1 Branch length			*ITS2* Branch length		
Annual	Perennial	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	*Calytonia 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)	−
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[b]	*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 (0.225)	−	**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)	−	–	–	
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

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

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 ($r = 0.570$, $p = 0.007$) and for 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 × number of comparisons = $0.05 \times 24 +$

Table 3. Comparisons of minimum generation time[a]

Family	Taxon with shorter generation time	Taxon with longer generation time	Minimum generation time (*MGT*)								Vertical growth rate (*VGR*)			
					ITS1			*ITS2*						
			MGT_S[b]	MGT_L[b]	Branch *S*	Length *L*	Sign	Branch *S*	Length *L*	Sign	VGR_S (m/year)[c]	VGR_L (m/year)[c]	Sign *ITS1*[d]	Sign *ITS2*[d]
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	*Eucalyptus grandis*	*Eucalyptit globulis*	2	4	0.039	0 (0.222)	+	0.010	0.028	–	21.5	5.75	+	–
Oleaceae	*Fraxinus excelsior*	*Fraxinus ornus*	15	20	0.067	0.102 (0.406)	–	0.070	0.057 (0.575)	+	0.78	*0.30*	–	+
Juglandaceae	*Juglans nigra*	*Juglans microcarpa*	12	20	**0.016**	**0** **(0.048)**	+	0.009	0 (0.125)	+	1.15	0.09	+	+
Pinaceae	*Larix decidua*	*Larix laricina*	10	20	0.001	0.029 (0.571)	–	0.009	0 (0.135)	+	1.20	0.6	–	+
Rosaceae	*Prunus besseyi*	*Prunus cerasifera*	2	6	0.005	0.012 (0.446)	–	0.008	0.023 (0.243)	–	NA[f]	NA[f]	NA[f]	NA[f]
Fagaceae	*Quercus acutissima*	*Quercus robur*	5	20	**0**	0.098 **(0.015)**	–	0.089	0 (0.412)	+	0.90	0.51	–	+
Ulmaceae	*Ulmus americana*	*Ulmus rubra*	15	15	0.023	0.011 (0.715)	NA[e]	0	0.043 (0.482)	NA[e]	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			0.508			1.0	0.508

[a] *S* and *L* represent 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).

[b] *MGT* provided by Young and Young (1992).

[c] The average heights, provided by Young and Young (1992) and Burns and Honkala (1990), used to calculate *VGR* are: *A. macrophyllum* 9.2 m, *A. saccharum,* 9.2 m, *B. pendula* 6 m, *B. alleghaniensis* 9.3 m, *C. illinoensis* 13 m, *C. cordiformis* 9.2 m, *E. grandis* 43 m, *E. globulus* 23 m, *F. excelsior* 11.7 m, *F. ornus* 6 m, *J. nigra* 13.8 m, *J. microcarpa* 1.8 m, *L. decidua* 12 m, *L. laricina* 12 m, *Q. acumtissima* 4.5 m, *Q. robur* 10.2 m, *U. americana* 11.1 m, *U. rubra* 6.6 m.

[d] The sign was determined as the branch length difference between VGR_S versus VGR_L.

[e] Not applicable, there is no difference in *MGT*.

[f] Not applicable, *P. besseyi* is not a vertical plant.

$0.05 \times 22 = 2.3$). 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 we examined only very closely related taxonomic groups. 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 role of replication-dependent versus replication-independent factors remains unresolved (Shimmin et al. 1993; McVean and Hurst 1997; Hurst and Ellegren 1998; Smith and Hurst 1999; Bohosslan et al. 2000; Kumar and Subramanian 2002; Whittle and Johnston 2002).

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 soma is the overwhelming source of gametic mutations (Klekowski 1988; Klekowski and Godfrey 1989; Klekowski 1998) and the other which suggests that germ-line mutations 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. 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 inter-

ested 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.

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