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Multiple major increases and decreases in mitochondrial substitution rates in the plant family Geraniaceae

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Abstract

Background: Rates of synonymous nucleotide substitutions are, in general, exceptionally low in plant mitochondrial genomes, several times lower than in chloroplast genomes, 10–20 times lower than in plant nuclear genomes, and 50–100 times lower than in many animal mitochondrial genomes. Several cases of moderate variation in mitochondrial substitution rates have been reported in plants, but these mostly involve correlated changes in chloroplast and/or nuclear substitution rates and are therefore thought to reflect whole-organism forces rather than ones impinging directly on the mitochondrial mutation rate. Only a single case of extensive, mitochondrial-specific rate changes has been described, in the angiosperm genus *Plantago*.

Results: We explored a second potential case of highly accelerated mitochondrial sequence evolution in plants. This case was first suggested by relatively poor hybridization of mitochondrial gene probes to DNA of *Pelargonium hortorum* (the common geranium). We found that all eight mitochondrial genes sequenced from *P. hortorum* are exceptionally divergent, whereas chloroplast and nuclear divergence is unexceptional in *P. hortorum*. Two mitochondrial genes were sequenced from a broad range of taxa of variable relatedness to *P. hortorum*, and absolute rates of mitochondrial synonymous substitutions were calculated on each branch of a phylogenetic tree of these taxa. We infer one major, ~10-fold increase in the mitochondrial synonymous substitution rate at the base of the *Pelargonium* family Geraniaceae, and a subsequent ~10-fold rate increase early in the evolution of *Pelargonium*. We also infer several moderate to major rate decreases following these initial rate increases, such that the mitochondrial substitution rate has returned to normally low levels in many members of the Geraniaceae. Finally, we find unusually little RNA editing of Geraniaceae mitochondrial genes, suggesting high levels of retroprocessing in their history.

Conclusion: The existence of major, mitochondrial-specific changes in rates of synonymous substitutions in the Geraniaceae implies major and reversible underlying changes in the mitochondrial mutation rate in this family. Together with the recent report of a similar pattern of rate heterogeneity in *Plantago*, these findings indicate that the mitochondrial mutation rate is a more plastic character in plants than previously realized. Many molecular factors could be responsible for these dramatic changes in the mitochondrial mutation rate, including nuclear gene mutations affecting the fidelity and efficacy of mitochondrial DNA replication and/or repair and – consistent with the lack of RNA editing – exceptionally high levels of "mutagenic" retroprocessing. That the mitochondrial mutation rate has returned to normally low levels in many Geraniaceae raises the possibility that, akin to the ephemerality of mutator strains in bacteria, selection favors a low mutation rate in plant mitochondria.

Background

For almost 20 years, it has been widely appreciated that nucleotide substitution rates are unusually low in mitochondrial genomes of land plants. This conclusion was first reached by Wolfe et al. [1] from an examination of synonymous substitution rates for several mitochondrial genes among a small set of angiosperms. Wolfe et al. [1] estimated that synonymous substitution rates in plant mitochondrial genomes are several times lower than in chloroplast genomes, 10–20 times lower than in plant nuclear genomes, and 50–100 times lower than in mammalian mitochondrial genomes. Palmer and Herbon [2] quickly extended this inference to the entire mitochondrial genome (most of which is noncoding) through genome-wide comparative restriction site mapping among crucifers and also showed that whereas sequence evolution is abnormally slow in plant mitochondrial genome, structural evolution is quite rapid.

Subsequent studies have extended this picture of unusually low mitochondrial substitution rates to other groups of land plants [reviewed in 3–5]. Within this context of generally slow evolution, a few studies have reported moderate variation in mitochondrial synonymous substitution rates between different groups of plants [6-9]. In most of these cases, correlated rate changes have been noted for chloroplast and/or nuclear genes. For example, all three genomes of grasses exhibit several-fold higher synonymous rates than do palms [6]. These findings suggest the operation of forces, such as generation time effects [6-8], paternal transmission of organelles [9], or correlated substitution and speciation rates [10,11], acting on all three genomes or on both organellar genomes.

Beginning in the late 1990's, we have carried out a large-scale Southern hybridization survey in which numerous mitochondrial and chloroplast gene and intron probes were hybridized to filter blots containing total DNAs from 280 diverse angiosperms [12-16]. With the exception of two plants, most if not all of the mitochondrial gene probes hybridized well to all 280 plant DNAs. For these two plants, all mitochondrial probes hybridized poorly if at all, despite typically strong hybridization with chloroplast probes. This very reduced mitochondrial hybridization (relative to chloroplast hybridization) suggested either 1) greatly reduced mitochondrial genome copy number in one or both plants, 2) highly elevated mitochondrial sequence divergence, or 3) loss from the mitochondrial genome and transfer of these genes to the low copy number and high mutation rate environment of the nucleus. A preliminary report [15] provided evidence of exceptional divergence for two, normally mitochondrially-located genes for each of these two plants, *Plantago rugelii* (plantain) and *Pelargonium hortorum* (the common geranium).

In a recent study [17], we explored the *Plantago* case in some detail, showing that it represents the first example of extremely rapid sequence evolution of mitochondrial DNA in plants. This rapid evolution was confined to the mitochondrial genome and to *Plantago*, for which major increases and decreases were found in the mitochondrial synonymous rate and, underlying this, the mitochondrial mutation rate. The fastest evolution in *Plantago* was estimated to exceed even the fastest evolution in animal mitochondria by an order of magnitude. Coupled with the discovery of other angiosperms with exceptionally slow evolution (even for plants), synonymous substitution rates in angiosperm mitochondrial genomes were estimated to vary by a factor of some 4,000-fold [17].

Here we explore the *Pelargonium* situation and report a second, entirely independent case of exceptionally rapid and variable sequence evolution in plant mitochondria, involving the Geraniaceae and especially *Pelargonium*. The Geraniaceae and *Plantago* cases are similar in overall aspect, involving initial, stepwise major increases in the rate of mitochondrial synonymous substitutions, followed by major rate decreases in certain descendant lineages. There are, however, differences between the two cases, including striking evidence from the Geraniaceae

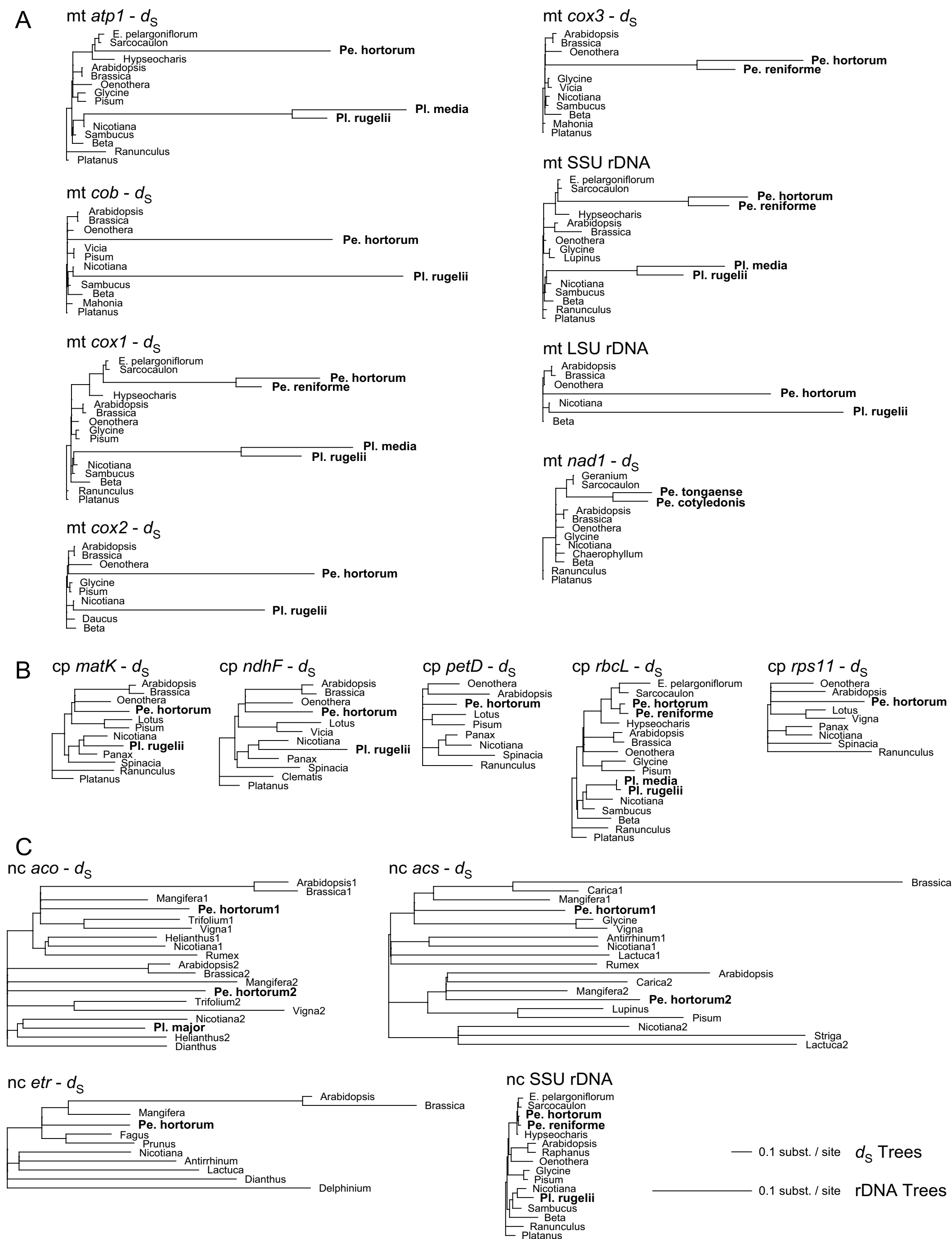

Figure 1

Extreme divergence of mitochondrial genes in *Pelargonium hortorum*. Shown are ML trees based on synonymous (d_S) sites for protein genes or all sites for rRNA genes. All tree topologies were completely constrained as described in Methods. All d_S trees are drawn to one scale, while all rDNA trees are drawn to a different scale (see bottom right). Abbreviations: Pe, *Pelargonium*; Pl, *Plantago*; E, *Erodium*. (**A**) Mitochondrial gene trees are based on 1,275 (*atp1*), 1,119 (*cob*), 1,413 (*cox1*), 723 (*cox2*), 690 (*cox3*), 805 (LSU rDNA), 1,395 (SSU rDNA), and 198 (*nad1*) NT. (**B**) Chloroplast gene trees are based on 1,497 (*matK*), 2,172 (*ndhF*), 483 (*petD*), 1,377 (*rbcL*), and 417 (*rps11*) NT. (**C**) Nuclear gene trees are based on 945 (aco, 1-aminocyclopropane-1-carboxylate oxidase), 1,245 (acs, 1-aminocyclopropane-1-carboxylate synthase), 2,226 (etr, ethylene receptor), and 1,667 (SSU rDNA) NT.

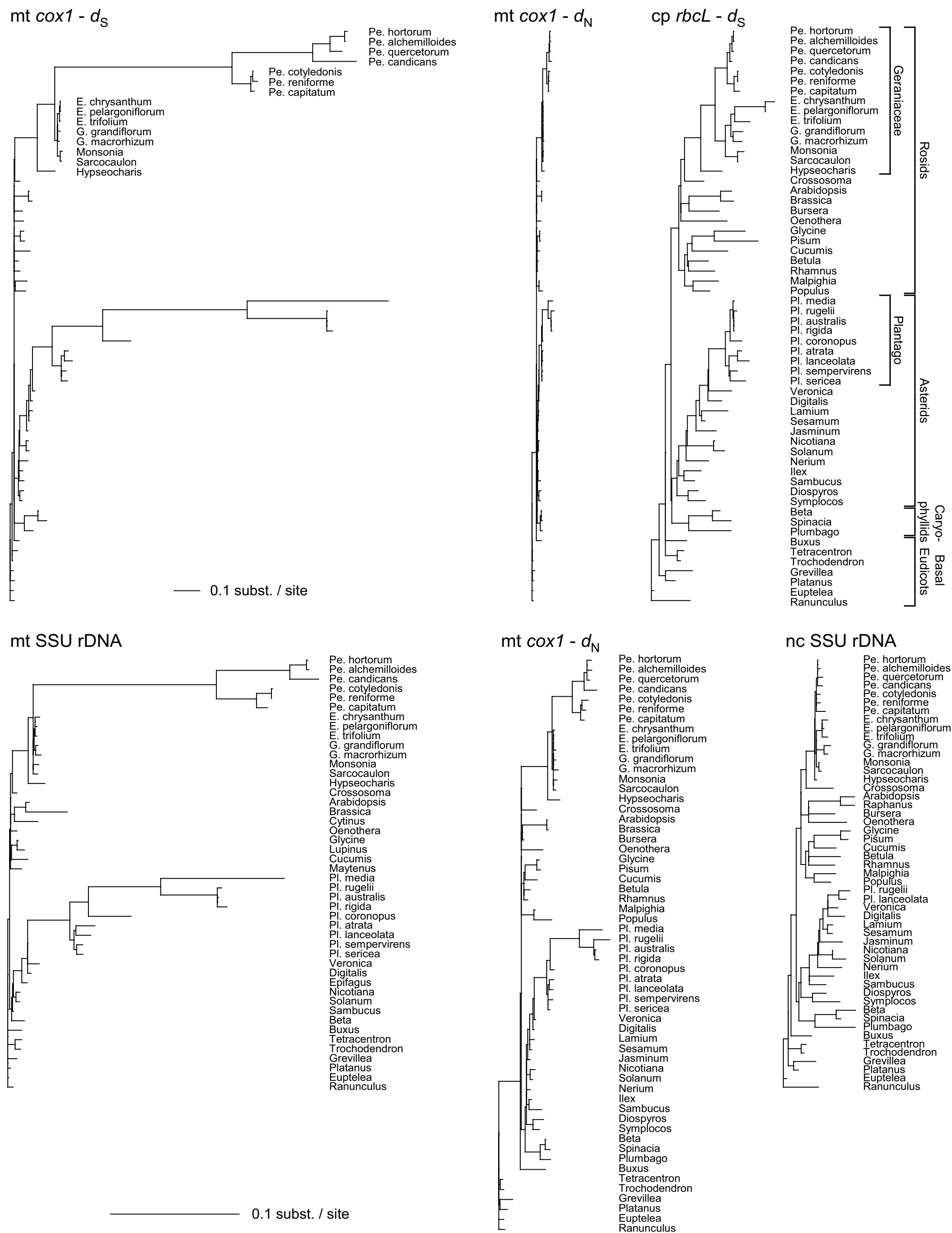

Figure 2

Extensive rate variation in Geraniaceae mitochondrial genes. Shown are ML trees based on synonymous (d_S) or non-synonymous (d_N) sites for protein genes or all sites for rRNA genes. All tree topologies were completely constrained as described in Methods. To emphasize branch length disparities, taxon names were moved to the right of each tree. Unlabeled branches in the *cox1* trees are the same species shown for *rbcL*. The top three trees are drawn to the same scale, while the bottom three trees are drawn at five times that scale (see scale bars). Abbreviations: Pe, *Pelargonium*; Pl, *Plantago*; E, *Erodium*; G, *Geranium*. Gene trees are based on 1,413 (cox1), 1,377 (rbcL), 1,471 (mitochondrial SSU rDNA), and 1,667 (nuclear SSU rDNA) NT.

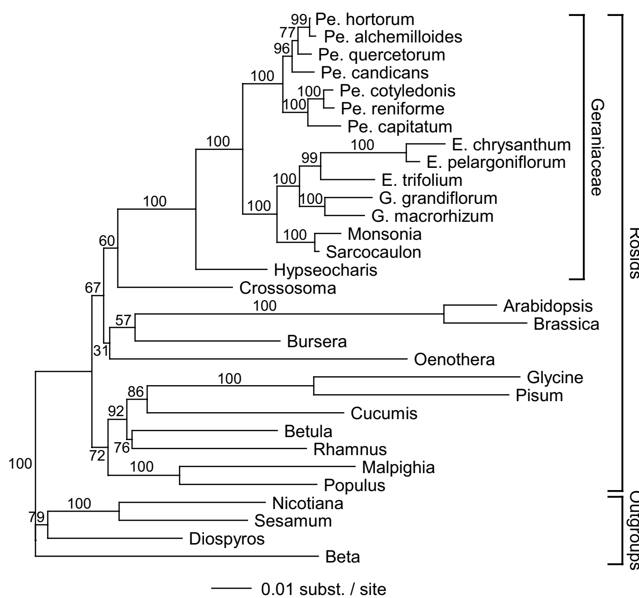

Figure 3
Multigene phylogeny of Geraniaceae and other rosids. Shown is a ML tree based on a 6,226 NT alignment comprising three chloroplast genes (*atpB*, *matK*, *rbcL*) and the nuclear SSU rDNA. Numbers on branches are ML bootstrap values. The tree is rooted using one caryophyllid (*Beta*) and three asterids. Abbreviations: Pe, *Pelargonium*; E, *Erodium*; G, *Geranium*.

that high mitochondrial mutation rates may be deleterious in plants, at least in the long run.

Results

We used PCR to isolate nearly full-length segments of seven mitochondrial genes from *Pelargonium hortorum* and three genes from *P. reniforme*. All seven *P. hortorum* mitochondrial genes show exceptional divergence relative to other angiosperms. This finding suggests that exceptionally rapid mitochondrial evolution – rather than reduced mitochondrial DNA copy number or mitochondrial gene loss/nuclear transfer – is the explanation for the very poor hybridization of heterologous mitochondrial gene probes to *P. hortorum* total DNA in the survey blots described in Background. Several other lines of evidence support the conclusion that these genes are located in the mitochondrial genome and are functional: 1) Two of the seven genes were tested by Southern blots and shown to hybridize preferentially to purified mitochondrial DNA compared to total DNA from *P. hortorum* (data not shown). 2) Both genes tested by Northern blots hybridized preferentially to poly (A)- RNA compared to poly (A)+ RNA (data not shown), and all three genes examined for RNA editing are transcribed. 3) Four of the seven genes are invariantly mitochondrially-located in all examined eukaryotes (except in those rare organisms where the gene function has been dispensed with entirely). 4) The five protein genes examined all contain intact open reading frames. 5) In comparisons with homologs from other Geraniaceae (see below), the protein genes all display very low ratios of nonsynonymous to synonymous substitutions, indicating that they are functional and evolving under strong purifying selection.

Because a disproportionate amount of the enhanced divergence of the protein genes was at synonymous sites, we chose to present their divergence graphically in the form of phylogenetic trees constructed from synonymous sites only (Fig. 1). The level of divergence in *P. hortorum* is generally comparable, at both synonymous sites and all sites for ribosomal RNA genes, to divergences of *Plantago rugelii* (Fig. 1), which has one of the most divergent mitochondrial genomes in *Plantago* [17] (*P. media* is the most divergent *Plantago*). For the three genes available, *Pelargonium reniforme* is also quite divergent but consistently less so than *P. hortorum* (Fig. 1). For the eighth mitochondrial gene shown in Fig. 1, *nad1*, only a relatively short sequence was available, and this only from different species of *Pelargonium* [18]. To illustrate synonymous site divergence for this gene, we chose *P. tongaense* and *P. cotyledonis*, as these are close relatives of *P. hortorum* and *P. reniforme*, respectively. The less exceptional apparent divergence of *nad1* for these two taxa (Fig. 1) is probably a function of the very short region analyzed (198 NT in total, and only about 50 synonymous sites) rather than gene-specific differences in substitution rates. Phylogenetic analysis of synonymous sites for five chloroplast and three nuclear genes shows, unlike the mitochondrial situation, no evidence of exceptional divergence in *P. hortorum* (Fig. 1).

An expanded data set (Fig. 2) was generated for two mitochondrial genes, *cox1* and small subunit ribosomal DNA (SSU rDNA), in order to explore the extent and pattern of mitochondrial rate heterogeneity within *Pelargonium* and other genera in the Geraniaceae, and to allow comparison with rate variation in other rosids and across eudicots, including the previously described case of rapid mitochondrial evolution in *Plantago* [17]. Seven species of *Pelargonium* were examined, as were eight other Geraniaceae species representing the five other genera in the family.

The fifteen Geraniaceae taxa all show enhanced divergence at *cox1* synonymous sites compared to all other rosids examined, but to markedly different extents, falling into three divergence groups (Fig. 2). The five non-*Pelargonium* genera are divergent compared to other rosids, members of *Pelargonium* subgenus *Pelargonium* (comprising *P. cotyledonis* through *P. capitatum*) are much more divergent, and those of the other subgenus, *Ciconium* (comprising *P. hortorum* through *P. candicans*), are even

more divergent. The mitochondrial SSU rDNA gene shows much the same pattern, which is striking considering that all sites are included for this gene compared to only synonymous sites for *cox1*. The magnitude and overall aspect of enhanced mitochondrial sequence divergence in Geraniaceae – different levels of divergence according to taxonomic group within the family – mirrors the pattern seen within *Plantago* (Fig. 2) [17]. Consistent with the results shown in Fig. 1 for *Pelargonium hortorum*, levels of chloroplast synonymous site divergence and nuclear SSU rDNA divergence are unexceptional in Geraniaceae (except for somewhat enhanced *rbcL* divergence in *Erodium*).

To put the synonymous site divergence data on a quantitative footing, we calculated absolute rates of *cox1* synonymous substitutions along all branches in the rosid part of the *cox1* trees shown in Fig. 2. Absolute rates were calculated for each branch by dividing its synonymous branch length by the estimated divergence time of the branch. Divergence times within rosids were calculated using the 4-gene (three chloroplast and one nuclear), 6,226 NT character set used to estimate rosid phylogeny in Fig. 3. Support for the relationships in this phylogenetic tree is mixed at basal nodes within rosids. Critically, though, support is strong at the node leading to Geraniaceae and throughout the family (96–100% bootstrap support at 13 of 14 nodes; Fig. 3).

A chronogram (time-based tree) of rosids is shown in Fig. 4, with R_S, the absolute rate of *cox1* synonymous substitutions, marked on each branch in units of substitutions per site per billion years (SSB units). The dearth of inferred synonymous site changes on the relatively short branches at the base of rosids is reflected in the $R_S = 0$ estimates on most of these branches. R_S is comparably low (0.31 SSB) on the H-L branch leading to the common ancestor of Geraniaceae and *Crossosoma* as throughout the rest of the non-zero rosid branches (R_S = 0.14–0.74 SSB). On the next four branches leading from the H-L branch towards the "top" of tree, R_S becomes progressively higher, from 0.3→2.4→4.1→30→38 SSB, before diminishing on subsequent branches. There is no reason to think that rate changes should coincide in timing with cladogenetic events. Given this, and wishing to minimize the number of inferred rate changes, we can very provisionally model these *branch-wise* rate increases with a minimum of two actual rate changes: an ~10-fold increase in R_S (from 0.3 to 4 SSB) in the common ancestor of the Geraniaceae (L-M branch) and a further ~10-fold increase (from 4 to 38) in the common ancestor of *Pelargonium* (N-U; Fig. 4). Thus, we provisionally infer at least (see Discussion) a 100-fold overall increase in R_S from near the base of rosids to the period of putatively fastest evolution in *Pelargonium.*

The terminal branches leading to all examined species of *Pelargonium* have estimated R_S values considerably below the genus' peak inferred value of 38 SSB. Likewise, the terminals leading to all non-*Pelargonium* Geraniaceae have R_S values considerably lower than 4 SSB. In fact all of this latter set of terminals have values comparable to the inferred ancestral rosid value of roughly 0.3 SSB, as do even some of the *Pelargonium* terminals (Fig. 4). Thus, there must have been multiple decreases in the synonymous substitution rate, some of considerable magnitude, following its early increases. We are confident of at least three rate decreases in the Geraniaceae: one each in the two subgenera of *Pelargonium* and one in the common ancestor of the clade comprising *Erodium* through *Sarcocaulon*. Furthermore, Fig. 4 raises the possibility of additional rate decreases, e.g, in *P. hortorum*, in *Erodium*, and in *Hypseocharis*.

The large standard errors on many of the R_S values within the family (Fig. 4) pose a particular problem in formulating rate-change scenarios with any certainty. But what is clear is that there have been multiple increases and decreases in the rate of mitochondrial synonymous substitutions within the Geraniaceae, and that the overall magnitude of these changes has been quite large.

Divergence at nonsynonymous sites in *cox1* was also analyzed phylogenetically and is plotted both at the same scale as synonymous site divergence (Fig 2, middle top) and at a five-fold expanded scale (Fig. 2, middle bottom). Although replacement sites also show enhanced divergence in *Pelargonium* and other Geraniaceae relative to other rosids, the effect is considerably muted compared to the pattern of synonymous site divergence. Absolute rates of nonsynonymous substitutions (R_N) were calculated within the rosids and are given in Additional File 1, as are R_N/R_S values. Among the very lowest R_N/R_S values are those on the branches with the highest R_S values (R_N/R_S = 0.02 for the N-U branch of R_S = 30, and R_N/R_S = 0.03 for the U-X branch of R_S = 38).

One notable feature of the very rapidly evolving mitochondrial DNAs of mammals is a pronounced (ca. 20-fold) transition/transversion (ti/tv) bias in favor of transitions [19]. The most divergent Geraniaceae *cox1* genes, in *Pelargonium*, show only a modest increase in ti/tv (= 1.91 averaged across all within-*Pelargonium* comparisons) relative to all non-Geraniaceae (ti/tv = 0.82). Base composition and the overall mutational spectrum are not significantly different in *Pelargonium* than in other plant lineages (data not shown).

Although only limited data are available, there seems to be relatively little RNA editing of mitochondrial genes in the Geraniaceae (Table 1). We directly assessed RNA edit-

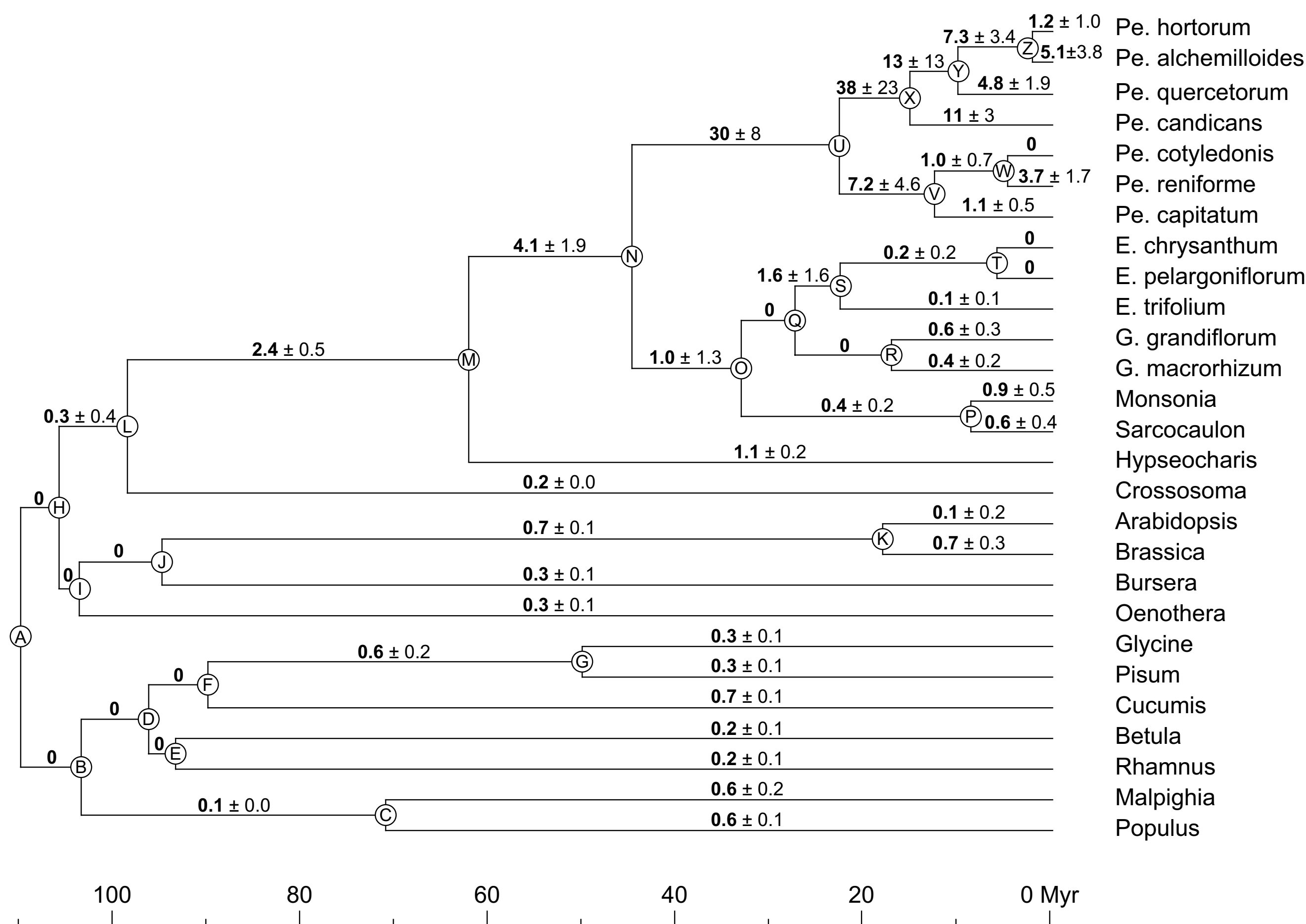

Figure 4

Variable rates of synonymous substitutions in Geraniaceae *cox1* genes. Shown is a chronogram based on the topology of Fig. 3. Nodes are labeled A-Z (also see Additional File 1). Shown above each branch are *cox1* R_S values (R_S = absolute rate of synonymous substitution per branch) in SSB (sub/site/byr). Abbreviations: Pe, *Pelargonium*; E, *Erodium*; G, *Geranium*. See Additional File 1 for the full set of R_S values taken to two digits after the decimal point, plus full sets of R_N, d_S,d_N,R_N/R_S, and divergence time values.

ing, by cDNA sequencing, for three mitochondrial genes in *P. hortorum*. Only a single, C→U edit site was found among the three genes, and very few edits were predicted (Table 1) for the various other sequenced Geraniaceae mitochondrial genes using a sensitive program [20] for predicting C→U editing of angiosperm mitochondrial genes. In comparison, other rosids (*Crossoma* through *Pisum* in Table 1), as well as the caryophyllid *Beta* and the grasses *Oryza* and *Triticum*, generally have much more RNA editing of these same five protein genes, with many edit sites conserved among angiosperms.

Discussion

Tempo and pattern of rate variation

We have discovered dramatic variation in rates of mitochondrial synonymous substitutions within the angiosperm family Geraniaceae. Our principal findings are three-fold: 1) The synonymous rate increased hugely during the evolution of the Geraniaceae, by at least a factor of 100. 2) This overall rate increase occurred via multiple (at least two, possibly more) stepwise and temporally separated rate increases. 3) Following these rate increases, at least three, and quite possibly more, rate decreases occurred, with many lineages reverting to the very low synonymous rates typical of most angiosperms.

Table 1: Observed and predicted number of RNA editing sites

Organism	*atp1*	*cob*	*cox1*	*cox2*	*cox3*	Editing Site Source
Pe. hortorum	1	**1**	**0**	**0**	1	this study
Pe. alchemilloides	-	-	1	-	-	
Pe. quercetorum	-	-	0	-	-	
Pe. candicans	-	-	0	-	-	
Pe. cotyledonis	-	-	0	-	-	
Pe. reniforme	-	-	0	-	2	
Pe. capitatum	-	-	0	-	-	
E. pelargoniflorum	0	-	0	-	-	
G. macrorhizum	-	-	0	-	-	
Sarcocaulon	0	-	0	-	-	
Hypseocharis	0	-	1	-	-	
Crossosoma	-	-	22	-	-	
Arabidopsis	**5**	**7**	**0**	**15**	**8**	GenBank: Y08501, Y08502
Brassica	**5**	**8**	**1**	**13**	**7**	GenBank: AP006444
Oenothera	**4**	**14**	17	**15**	12	GenBank: X04023, X07126, X00212
Glycine	1	-	17	13	11	
Pisum	1	15	18	**13**	-	GenBank: X52866
Beta	**3**	**13**	**0**	**9**	**4**	JPM and JDP, unpublished
Oryza	**5**	**19**	**4**	**19**	**1**	GenBank: BA000029
Triticum	**6**	**18**	4	**14**	**13**	GenBank: X80470, X02352, X52867, X52539

Numbers in bold indicate that editing sites were identified experimentally. All other reported numbers were predicted with PREP-Mt [20] using a cutoff value of 0.5.

Our estimates for the highest and lowest rates of synonymous substitutions, and of the number of rate changes, are inherently conservative ones, being constrained by the limited taxonomic sampling of this study. The R_S values by definition average the substitution rate across an entire branch, the length/time of that branch being a function of the number of extant Geraniaceae taxa and our sampling of those taxa. Any one R_S value may in fact average across periods of substantially higher and lower absolute substitution rate than would be indicated by the R_S value itself. For example, the highest branch-wise (R_S) rate estimated in this study, of 38 SSB, may turn out to be a significant underestimate of the fastest period of mitochondrial evolution within *Pelargonium*. We sampled only 7 of ~280 species in the genus, and thus there is ample opportunity to more finely dissect the pattern of rate variation and thereby discover greater peaks (and valleys) of substitution rates, as well as detect a larger number of rate changes.

If synonymous rates are more or less equal across genes within a mitochondrial genome (as seems to be the case for *P. hortorum*; Fig. 1), then sampling multiple protein genes (and among many taxa) should provide another opportunity to better elucidate the tempo and pattern of rate changes. In particular, this should significantly reduce the standard errors on the estimates of R_S, many of which are quite high for *cox1* alone (Fig. 4 and Additional File 1). Sequencing additional non-mitochondrial genes, to reduce the standard errors on the divergence time estimates (see Additional File 1), should also be helpful in this regard.

The mitochondrial rate variation in Geraniaceae uncovered in this study is similar in several respects to that recently described in *Plantago* [17]. In both cases, 1) there is dramatic variation in synonymous substitution rates; 2) taxa fall into multiple (three or four) groups with respect to the amount of accumulated sequence divergence (Fig. 2); 3) the most divergent groups have accumulated comparable levels of synonymous site divergence (Fig. 2); 4) at least two successive major increases in the synonymous rate occurred relatively early on; 5) multiple rate decreases occurred subsequent to the initial increases; and 6) rates of chloroplast and nuclear evolution are not elevated, such that in the fastest lineages mitochondrial rates significantly exceed those of the chloroplast and nuclear genomes (Fig. 2; ref. [17]).

The Geraniaceae and *Plantago* situations also differ in several ways: 1) The Geraniaceae situation encompasses a broader group taxonomically. 2) The initial speed-up occurred much earlier in the Geraniaceae (ca. 80 Myr) than in *Plantago* (ca. 30 Myr). 3) The peak rates estimated in *Plantago* (ca. 200 SSB and possibly even 700 SSB) far exceed those in Geraniaceae (38 SSB), although this difference could in large part be a sampling artifact (i.e., if the fastest period of mitochondrial evolution extended throughout a single sampled branch in *Plantago*, whereas that in *Pelargonium* was averaged, on the fastest measured branch, with periods of significantly slower evolution). 4)

There seems to be less of a tendancy to revert to a typically low substitution rate (of ca. 0.5 SSB) in *Plantago* compared to Geraniaceae. Overall, then, the Plantago speed-up is more recent, apparently more intense, and apparently less reverted. Like *Pelargonium*, *Plantago* is a large genus (in fact the two are almost exactly the same size), and it will be interesting to see how the two situations compare once more taxa and genes (and even some complete genomes) are sampled from both groups.

Geraniaceae and *Plantago* are, to our knowledge, the only two cases of such dramatic variation in synonymous substitution rates – both in magnitude and rollercoaster-like behavior – documented in any group of plant mitochondrial genomes, or, for that matter, in any genome/group whatsoever. However, several published, mostly phylogenetic studies have identified other plants for which mitochondrial divergence is disproportionately high compared to chloroplast divergence. These long-branched mitochondrial clades include *Ephedra* in the Gnetales [21-23], the monocots *Acorus* and Alismatales [24], the lycophyte *Selaginella* [25], the asterids *Goodenia* and *Polemonium* [17,26], and the simple thalloid and leafy liverworts [27]. With the exception of Goodenia [17], none of these cases has been quantitatively treated, and the published trees include all sites rather than just synonymous sites. Nonetheless, in each case, the accumulated divergence appears to be lower than in *Pelargonium* or *Plantago*. However, in most of these cases, only one or two species were examined, and thus there is plenty of opportunity to find even more divergent lineages in these groups that may approach or even exceed *Pelargonium* and *Plantago* in amount of divergence and peak substitution rate.

Mutation rates, mechanisms, and selection

Because the extensive variation in substitution rates in the Geraniaceae is restricted to the mitochondrial genome and because nonsynonymous rates are proportionately less elevated than synonymous rates (Fig. 2, Additional File 1), the root cause of this rate variation is almost certainly changes in the mitochondrial mutation rate. The same conclusion was reached for *Plantago* mitochondrial rate variation [17], whereas most other cases of (much more modest) synonymous site variation in plant mitochondrial DNA have been ascribed to factors that would affect all three plant genomes, such as generation time, population size, and/or speciation rate [6-8].

By analogy to the extensive literature on mutators (and antimutators) in bacteria and yeast, mutations affecting a broad range of processes that impinge on the mutation rate could be responsible for the extensive variation in mitochondrial mutation rates in Geraniaceae. The only mitochondrial mutators characterized thus far, in lab mutants of the yeast *Saccharomyces cerevisiae*, affect DNA replication and mismatch repair [28]. These are also the most common mutators in microorganisms [29,30]. In plants and virtually all other mitochondrial systems, these processes are controlled entirely by nuclear genes. Thus, the major increases in the mitochondrial mutation rate that took place early in the evolution of the Geraniaceae and then *Pelargonium* could be the consequence of nuclear mutations resulting in error-prone replication or defective mismatch repair, as postulated for other cases of synonymous rate variation in plant organelle genomes [7,17,31]. Subsequent rate decreases could reflect direct reversal or compensatory suppression of these mutator mutations. Mitochondrial-specific forces could also be at work, e.g., major increases in the amount of oxygen free-radical damage to mitochondrial DNA [32,33].

That Geranicaeae mitochondrial genes seem, based on limited samping (Table 1), to be relatively bereft of RNA editing, raises the possibility of a novel molecular mechanism, which we term "mutagenic retroprocessing" (see also [34]). Reverse transcriptases are extremely error-prone polymerases, and so exceptionally high levels of reverse transcription in Geraniaceae mitochondria, coupled with high levels of homologous recombination, could in principle be responsible for at least part of the elevation in mitochondrial mutation rate seen in the family. If this hypothesis is correct, we would expect nontranscribed regions to be much less divergent than genes. Unfortunately, identifying and amplifying homologous intergenic spacer DNA is not straightforward given the high rate of rearrangement in plant mitochondrial genomes [2], and our attempts to do so in *Pelargonium* have been unsuccessful. According to this hypothesis, the existence of limited RNA editing in extant Geraniaceae (Table 1) would reflect mutational drift to unedited C residues following the evident reversals in mutation rate, i.e, cessation of the hypothetical mutagenic retroprocessing activity.

In all 15 examined Geraniaceae, the mitochondrial mutation rate has dropped from its peak levels, with at least three (and probably more) separate rate decreases implied by the R_S patterns of Fig. 4. In many taxa, the mutation rate appears to have dropped more or less to the low rates (ca. 0.3 SSB) typical of most rosid lineages (Fig. 4). And even those taxa (four species of *Pelargonium*) whose terminal branches possess relatively high R_S values may nonetheless have more fully reverted mutation rates, the present-day rates being averaged with potentially higher rates earlier on these branches.

Such a pervasive local pattern of rate decreases, combined with pervasively slow mitochondrial evolution across land plants in general, suggests that high mutation rates

are selected against in the evolution of plant mitochondrial genomes. Chance mutations that directly reverse or otherwise suppress the earlier mutator-like mutations in Geraniaceae may be favored and fixed by natural selection. From this perspective, plant mitochondrial mutators may be viewed as ephemeral, like bacterial mutators but on a much longer time scale.

If most or all Geraniacease have reverted to low mitochondrial mutation rates, then it may be difficult if not impossible to figure out the nature of the mutator mutations that caused the rapid periods of mitochondrial evolution. Mutagenic retroprocessing, admittedly a long-shot hypothesis, is nonetheless attractive in this regard because it would leave obvious signatures in the genome (see above).

Methods

Molecular techniques and sources of DNA sequences

Methods for plant DNA isolation, Southern and Northern blot hybridization, PCR isolation, DNA cloning, cDNA preparation, and DNA sequencing are as in [12,13,35]. Additional sequences used in this study were taken from GenBank and are listed in Additional File 2, as are GenBank numbers for the sequences generated in this study. PCR primer sequences and aligned data sets are available upon request.

Phylogenetic analysis

Phylogenetic relationships within the rosids were determined from a concatenated data set consisting of nuclear SSU rDNA and the chloroplast genes *atpB*, *matK*, and *rbcL*. Total aligned length was 6,226 NT. Poorly alignable regions and regions with gaps in most taxa were excluded from the analyses. A maximum likelihood (ML) tree was constructed with PAUP*, version 4.0b10 [36], by using the general time-reversible model, a gamma distribution with four rate categories, and an estimate of the proportion of invariant sites. The rate matrix, base frequencies, shape of the gamma distribution, and proportion of invariant sites were estimated before the ML analysis from a parsimony tree constructed from the data. Support for the ML tree was evaluated by the bootstrap procedure with 500 replicates using parameters estimated from the ML tree.

Divergence time estimates

Divergence times for all nodes within rosids were calculated using a penalized likelihood approach as implemented in the r8s program [37]. A fixed time constraint of 110 million years [38,39] was used for the crown group age of rosids. The ML tree from the phylogenetic analysis of rosids (Fig. 3) was used as the starting tree. A smoothing factor of 18 was determined by using the r8s cross-validation procedure [37]. Different starting points of initial age estimates and reanalysis after perturbation of the final age estimates had no effect on the results. Standard errors for the divergence time of each node were calculated by rerunning the divergence time analyses on 500 bootstrapped data sets.

The elapsed time along each branch, T_{ij}, was calculated as the difference in divergence times of the starting node, T_i, and the ending node, T_j, that define the branch. Standard errors for T_{ij} were calculated according to the formula

$$\sigma_{T_{ij}} = \sqrt{(\sigma_{T_i})^2 + (\sigma_{T_j})^2}$$

Branch length estimates

Branch lengths, representing the number of substitutions per synonymous site (d_S) or number of substitutions per nonsynonymous site (d_N), were determined for protein genes using codeml in the PAML package, version 3.14 [40]. The Muse-Gaut (MG94) codon model was used with separate d_N/d_S ratios for each branch. Codon frequencies were computed by using the F3 × 4 method. The transition/transversion rate ratio and d_N/d_S ratios were estimated during the analysis with initial values of 2 and 0.4, respectively. Standard errors for total branch lengths (t) were reported by PAML, and these values were propagated to calculate standard errors for their corresponding d_S and d_N branch lengths according to the formulas

$$\sigma_{d_N} = \frac{(N+S)\sigma_t}{N + \frac{S}{\omega}}$$

$$\sigma_{d_S} = \frac{(N+S)\sigma_t}{N\omega + S}$$

where ω is the d_N/d_S ratio and N and S are the number of nonsynonymous and synonymous sites, respectively, as reported by PAML. Branch lengths representing total substitutions per site for rRNA genes were estimated with baseml in PAML. The general time-reversible nucleotide model was used with a gamma distribution for rate variation with four categories. The rate matrix, nucleotide frequencies, and shape of the gamma parameter (initial value of 0.5) were estimated during the analysis.

All branch length analyses employed user-defined topologies. Within rosids, topologies were constrained according to the phylogenetic analysis carried out in this study (Fig. 3). Relationships in the Plantaginaceae were constrained according to [17]. All other relationships were constrained according to the Angiosperm Phylogeny Website [41].

Absolute substitution rate estimates

Absolute rates of synonymous substitution per branch (R_S) were calculated by dividing the synonymous branch

length, d_S, by the length of time, T, for that branch. Standard errors for R_S were determined by propagating the errors associated with branch length and time according to the formula

$$\sigma_{R_S} = R_S \sqrt{\left(\frac{\sigma_{d_S}}{d_S}\right)^2 + \left(\frac{\sigma_T}{T}\right)^2}$$

R_N values and their standard errors were calculated similarly.

Abbreviations

d_S – number of substitutions per synonymous site; d_N – number of substitutions per nonsynonymous site; R_S – absolute rate of synonymous substitution per branch; R_N – absolute rate of nonsynonymous substitutions per branch; ML – maximum likelihood; Myr – million years ago; NT – nucleotides; SSB – substitutions per site per billion years; SSU rDNA – small subunit ribosomal DNA; ti/tv – transition/transversion

Authors' contributions

CLP generated much of the data newly reported in this study, conducted intial analyses, and helped with tables and manuscript revision in response to reviewers' comments. JPM generated some of the data, carried out all final analyses, prepared the figures and tables, and drafted part of the manuscript. YLQ, AJH, KS, and NDY contributed some of the data for this study. CWD guided some of the data generation and contributed to the manuscript preparation. JDP guided the entire study and drafted most of the manuscript. All authors read and approved the final manuscript.

Additional material

Additional File 1

Absolute substitution rates per branch. *This table gives a complete list of estimates for divergence times, number of substitutions per synonymous site* (d_S)*, absolute rate of synonymous substitution* (R_S)*, number of substitutions per nonsynonymous site* (d_N)*, absolute rate of nonsynonymous substitution* (R_N)*, and* R_N/R_S *ratios for all branches shown in figure 4.*
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[http://www.biomedcentral.com/content/supplementary/1471-2148-5-73-S1.pdf]

Additional File 2

Sequences used in this study. *This table lists the GenBank accession numbers for all sequences used in this study. 2a) List of mitochondrial sequences used for figure 1. 2b) List of chloroplast sequences used for figure 1. 2c) List of nuclear sequences used for figure 1. 2d) List of sequences used for figures 2, 3, 4.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2148-5-73-S2.pdf]

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