DNA sequence evolution in fast evolving mitochondrial DNA *nad*1 exons in Geraniaceae and Plantaginaceae

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Previously, nucleotide substitution rates in mitochondrial DNA of Geraniaceae and Plantaginaceae have been shown to be exceptionally high compared with other angiosperm mtDNA lineages. It has also been shown that mtDNA introns were lost in Geraniaceae and Plantaginaceae. In this study we compile 127 DNA sequences from two partial exons of the mtDNA *nad*1 gene in Geraniaceae, Plantaginaceae, and other angiosperm groups for which rate accelerations have not been reported, to assess the extent and nature of the nucleotide substitution rate acceleration. Whereas *Litorella* appears to have undergone a rate acceleration comparable to that observed in *Plantago*, the Geraniacean sister group representative *Hypseocharis biloba* has not, indicating that the rate change has occurred between the split of *Hypseocharis* and the rest of the Geraniaceae. Silent/non-silent rate ratios ω have decreased threefold in the "fast mtDNA" clades compared with other angiosperms, whereas their codon usage bias is around 20% lower. Absence of RNA editing in Geraniacean and *Plantago* mtDNA genes is confirmed. Possible causes for the exceptional substitution rate accelerations observed in these lineages are discussed in terms of the retroprocessing process or the possibility of affected mitochondrial DNA polymerase γ proofreading accuracy control.

KEYWORDS: Geraniaceae, mtDNA, *nad*1, Plantaginaceae, retroprocessing, silent rate, substitution rate.

INTRODUCTION

Palmer & Herbon (1988) demonstrated a high structural diversity, yet low sequence divergence in mtDNA—even when comparing closely related congeneric angiosperm species. Because of this, mtDNA has been under-utilised as a source of phylogenetic markers in plant phylogenetics for the past decade, the re-evaluation of which situation warrants this special issue of TAXON. Studies aimed at reconstructing mitochondrial phylogenetic relationships at phylum level, including plants, have been successful and were reported by Delwiche & Palmer (1997), Bullerwell & Gray (2004), and Delsuc & al. (2005). For use of mtDNA sequences in land plant phylogenetic reconstruction, see Malek & al. (1996), Chaw & al. (2000), Nickrent & al. (2000), Pruchner & al. (2001), Turmel & al. (2003), and papers in this issue.

At the lowest taxonomic levels, mtDNA has been the genome of choice in animal phylogenetic and phylogeographic studies (e.g., Avise, 2000). More recently, its suitability for DNA barcoding has been claimed in vertebrates and some insect groups (e.g., Hebert & al., 2004; Hajibabaei & al., 2006; see further http://www.barcoding.si.edu). In angiosperms, however, published species-level studies using mtDNA sequences are relatively rare (e.g., Dumolin-Lapègue & al., 1998; Bakker & al., 2000; Freudenstein & Chase, 2001).

Palmer & al. (2000) reported on at least two unrelated angiosperm plant lineages (Geraniaceae, *Plantago*) in which mtDNA is highly divergent at the sequence level, amidst the hundreds of slowly evolving lineages. It was found that substitution rates at third codon positions were highly elevated. Indeed, using *nad*1 b/c exons Bakker & al. (2000) were able to distinguish species groups within *Pelargonium* (Geraniaceae), whereas phylogenetic resolution within the angiosperms using mtDNA regions usually stops at the family level. More recently, Cho & al. (2004) reported on a 1200–4000 fold increase in silent substitution rates in several mtDNA encoded genes in *Plantago*. The authors have hypothesized on possible causes of these increased substitution rates, suggesting that error-prone replication or altered detoxification of oxygen free-radicals could have led to the increased rates. Parkinson & al. (2005) explored substitution rates in Geraniacean mtDNA using sequences from several genes, and inferred a roughly 10-fold mtDNA rate increase in the common ancestor of the Geraniaceae, followed by a second increase in *Pelargonium*. In addition, the authors inferred several rate slowdowns to "normal" levels later on in this lineage, and suggested "mutagenic retroprocessing" followed by subsequent selection for

lower mutation rates could possibly explain this striking pattern.

This study aims at further documenting the rate acceleration in the mtDNA encoded *nad*1 partial b and c exons in Geraniaceae and *Plantago*. The sequence data matrix used here is an extension from data presented in Bakker & al. (2000), and the approach taken here is to analyse these sequences from the codon perspective as codon substitution rates give a better insight of how and to what extent silent and non-silent substitution rates have been affected (see also Ren & al., 2005). The applicability of these fast-evolving Geraniacean and *Plantago* mtDNA genomes as a source of phylogenetic markers appears to be only "local", being exceptions within all angiosperms studied so far (Palmer & al., 2000). Nevertheless, they offer a fascinating insight in the molecular evolutionary mechanisms operating upon and shaping mtDNA genomes in plants.

MATERIAL AND METHODS

Material used and collection details are given in the Appendix. For the purpose of this study, 31 mtDNA *nad*1 b/c sequences were generated; 51 sequences were obtained from Bakker & al. (2000), 30 from Merckx & al. (in press), and 40 from GenBank, including *Amborella*, *Welwitschia*, and *Gnetum nad*1 b/c sequences as outgroups. Standard DNA extraction, PCR and DETT terminator sequencing protocols were applied (see Bakker & al., 2000, for further details). Primers used for both PCR and cycle sequencing were *nad*1 b (5'-GCA TTA CGT CTG CAG CTT CA-3') and *nad*1 c (5'-GGA GCT CGA TTA GTT TCT GC-3') described by Demesure & al. (1995). Sequences were assembled from ABI electropherograms in the STADEN package (Staden, 1996) and manually aligned in MacClade 4 (Maddison & Maddison, 1992) using the amino acid level as a guide and applying the *Drosophila* mitochondrial genetic code. Any IUPAC coded polymorphic nucleotide, as well as a few incompletely sequenced triplets were changed into "missing data" in order to prevent polymorphic amino acids to occur in the translation.

Parsimony analyses were conducted using PAUP* version 4.0b10 (Swofford, 2002) for Macintosh (ALTIVEC) on a Macintosh G5 with 1 GB of RAM. Maximum Parsimony search included using the parsimony ratchet (Nixon, 1999) implemented for PAUP* as PAUPRat (Sikes & Lewis, 2001) in order to quickly find shortest trees, with number of perturbed characters set to 25%. A set of most parsimonious reconstructions (MPRs), obtained from one round of PAUPRat were then used as starting trees for a subsequent heuristic search in PAUP* involving TBR branch swapping, "swap on best only" and "steepest descent off". Jackknife resampling analysis was performed using settings as in Freudenstein & al. (2004), i.e. TBR branch swapping, 10 replicates of random addition sequence and saving 1 tree per replicate. (See Farris & al., 1996 and Lee, 2000 for justification of the use of jackknife instead of the bootstrap.)

Best-fitting nucleotide substitution model was selected using ModelTest v. 3.7 for Mac OSX (Posada & Crandal, 2001). ModelTest analyses were conducted on either the unpartitioned, or partitioned data matrix ($1^{st}+2^{nd}$ versus 3^{rd} codon position). Model testing was also performed on separate taxon sets representing main clades in order to check whether different models apply.

Codon analysis was conducted using CodeML from the PAML package (version 3.15; Yang, 1997) and was run on a Linux PC using SUSE 10. The CodeML analyses involved calculation of a codon usage table, and assessment of ω rates in individual (main) branches of the *nad*1 b/c phylogenetic tree using the following CodeML settings: "yeast mitochondrial genetic code" (no *Drosophila* mtDNA code was available in the programme), "CodonFreq = F3×4", (codon) "model = two" i.e., two or more d_N/d_S ratios are optimised for separate clades, whereas for the site models a single ω rate ratio was assumed across sites ("NSsites = one"). Ambiguity data was not used in any of the counts. In addition, d_N and d_S trees, i.e., showing branch lengths in terms of silent and non-silent nucleotide substitutions per codon (not nucleotide position) respectively, were constructed based on the same settings.

Variation in the rate of synonymous substitution among genes may be related to codon use (Sharp & Li, 1986). Therefore, in addition to the codon usage table, several parameters related to codon usage bias, such as the codon bias index (CBI; Morton, 1993), G+C content at second and third positions, and the effective number of codons (ENC; Wright, 1990) were estimated using DnaSP version 4.10.7 (Rozas & al., 2003).

Inference of the occurrence of RNA editing would provide additional evidence that the region under study actually resides in the mitochondrial genome, as RNA editing is so far only known from mitochondria and plastids (Bowe & dePamphilis, 1996; Knoop, 2004; Tillich & al., 2005). RNA editing usually takes place as post-translational C→U edits (e.g., Araya & al., 1994; Yokobori & Pääbo, 1995), i.e., at the mRNA level C's are changed back to U's to correct for amino acid changes which could otherwise affect functionality of the translated peptide (see for examples Bock & al., 1994; Zito & al., 1997; Sasaki & al., 2001). Hence, this process tends to increase protein conservation across species by "correcting" codons that specify unconserved amino acids (Mower, 2005). Although, ideally, evidence for edited sites is assessed using cDNA sequencing, this option was

not available to us and we therefore applied PREP-Mt (Mower, 2005), which is especially designed for predicting RNA editing in plant mitochondrial genes. The method is based on identification of sites that would require correction "towards" a consensus conserved amino acid sequence. Sites in the *nad*1 b/c matrix that could be prone to RNA editing were predicted using PREP-Mt with cut-off value C set to 0.

RESULTS

The final *nad*1 b/c sequence alignment consisted of 127 taxa × 66 codons, twenty seven from the 3' end of *nad*1 exon b and the first 39 codons from exon c. Whereas no intron from any Geraniacean and *Plantago* taxa could be amplified, interestingly, only *Pelargonium odoratissimum* produced two fragments using the *nad*1 b and c primers indicating that the intron is still present (not shown). It was assessed that sequences translated into functional proteins by applying a *Drosophila* mitochondrial genetic code in MacClade 4. The alignment (including *Amborella*) comprised 26 variable and 51 parsimony-informative characters.

Parsimony analysis and resampling statistics. — The semistrict consensus of 778,000 MPRs of 168 steps is shown in Fig. 1 with jackknife frequencies indicated at corresponding nodes. The tree topology obtained comprised a basal polytomy including a representation of several angiosperm orders, as well as three main clades comprising i) *Pelargonium*, ii) *Erodium*/*Geranium*/*Sarcocaulon*, and iii) *Plantago*. The *nad*1 sequences of *Pelargonium anethifolium*, *P. lobatum*, *P. crassicaule*, *Hypseocharis biloba*, and *Licania heteromorpha* (Chrysobalanaceae) group with these three main clades in a polytomy. The proximity of the Geraniacean and *Plantago* clades is almost certainly a long-branch attraction artefact. Note that, in order to avoid further such artefacts, *nad*1 sequences from *Welwitschia* and *Gnetum* could not be used and *Amborella* was used instead as single outgroup.

Model testing. — ModelTest analyses (Posada & Crandal, 2001) were set up as outlined in Table 2: the matrix was analysed unpartitioned, or partitioned in a $1^{st}+2^{nd}$ versus 3^{rd} codon position. These analyses were also conducted for the four main clades to check, given the apparent large differences in substitution rates, whether different models apply. From the results given in Table 1 it appears that different models do indeed apply to different partitions and that the "fast" clades tend to fit parameter-less models (e.g., JC) better.

Codon models. — The unusually long branches leading to *Pelargonium, Geranium/Erodium/Sarcocaulon*, and *Plantago* were further explored, especially with respect to whether their ω rates differed from those in the other angiosperm sequences. The results from the CodeML analyses indicate that the overall ω rates were up to 3 times lower in the fast clades compared with those in the other angiosperms (Table 2). A d_N and d_S tree was constructed in CodeML (Fig. 2) showing branch lengths in terms of silent and non-silent nucleotide substitutions per codon (not nucleotide position). It can be concluded that the *Plantago* clade has a silent substitution rate d_S ranging from 0.026–0.100 substitutions per codon (Table 2), which is outside the range observed for the other angiosperms. The *Pelargonium* clade (especially its terminal branches) exhibits a d_S range that is comparable to that in the angiosperms, but a d_N range that is far lower. Yet, the *Pelargonium* ω rate ratio is about 3-fold lower compared with the angiosperms.

A codon usage table was calculated using CodeML (Table 3) and, in addition, several parameters related to codon usage bias, such as the codon bias index (CBI; Morton, 1993) and the effective number of codons (ENC; Wright, 1990) were estimated using DnaSP (Rozas & al., 2003), and are given in Table 4. It appears that whereas the ENC is comparable between the four clades, the CBI is around 20% lower in all three "fast" clades compared with the other angiosperms.

RNA editing. — Whereas up to five sites were predicted by PREP-Mt to occur in all other angiosperms (including *Hypseocharis biloba*), just a single site was predicted in all *Plantago* sequences (not *Litorella*), and none in the main Geraniaceae clade. One site, actually the same position as in the *Plantago* sequences, was predicted in the *Pelargonium lobatum* sequence which groups outside the main Geraniaceae clade (Fig. 1).

DISCUSSION

The dramatic increase in silent substitution rate in mtDNA encoded *nad*1 b/c exons reported here largely supports the findings in Geraniaceae by Bakker & al. (2000), Palmer & al. (2000) and Parkinson & al. (2005), and *Plantago* by Cho & al. (2004). The acceleration seems to be restricted to the Geraniaceae excluding its sistergroup taxon *Hypseocharis biloba.* In contrast, within the *Plantago* clade the sister taxon *Litorella uniflora* is included in the rate acceleration. More inclusive taxonomic sampling in these clades will enable assessment of to what extent rate acceleration has occurred. Unfortunately, the next sister lineage of Geraniaceae beyond *Hypseocharis* has not yet been resolved, in fact the phylogenetic position of Geraniales in either rosid I or II clades is not stabilised yet (Soltis & Soltis, 2004). Interesting exceptions to the rate acceleration pattern outlined above are represented by the *nad*1 sequences of *Pelargonium anethifolium*, *P. lobatum*, *P. crassicaule*,

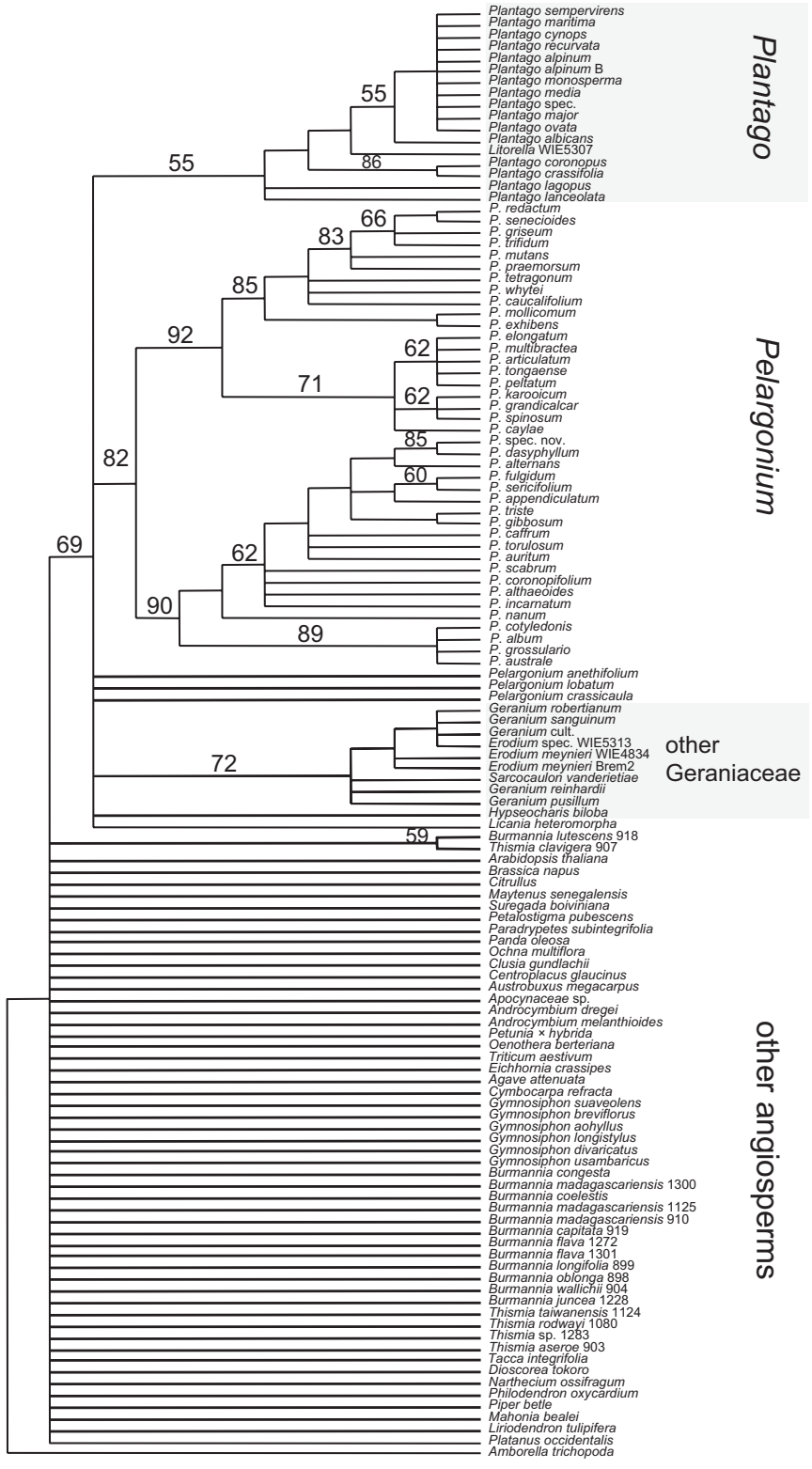

Fig. 1. mtDNA *nad*1 b/c exons—semistrict consensus of 778,000 MPRs of 168 steps resulting from a heuristic parsimony search using *Amborella* as outgroup. A set of 201 most parsimonious reconstructions (MPRs), obtained from one round of PAUPRat and with length 168 steps (CI = 0.61, RI = 0.94), were used as starting trees for heuristic search in PAUP* involving TBR branch swapping, "swap on best only" and "steepest descent off", and run until the RAM was depleted. Jackknife support values (10,000 jack replicates, TBR) are indicated at the nodes. Clades (and the angiosperm grade) are labelled as referred to in the text.

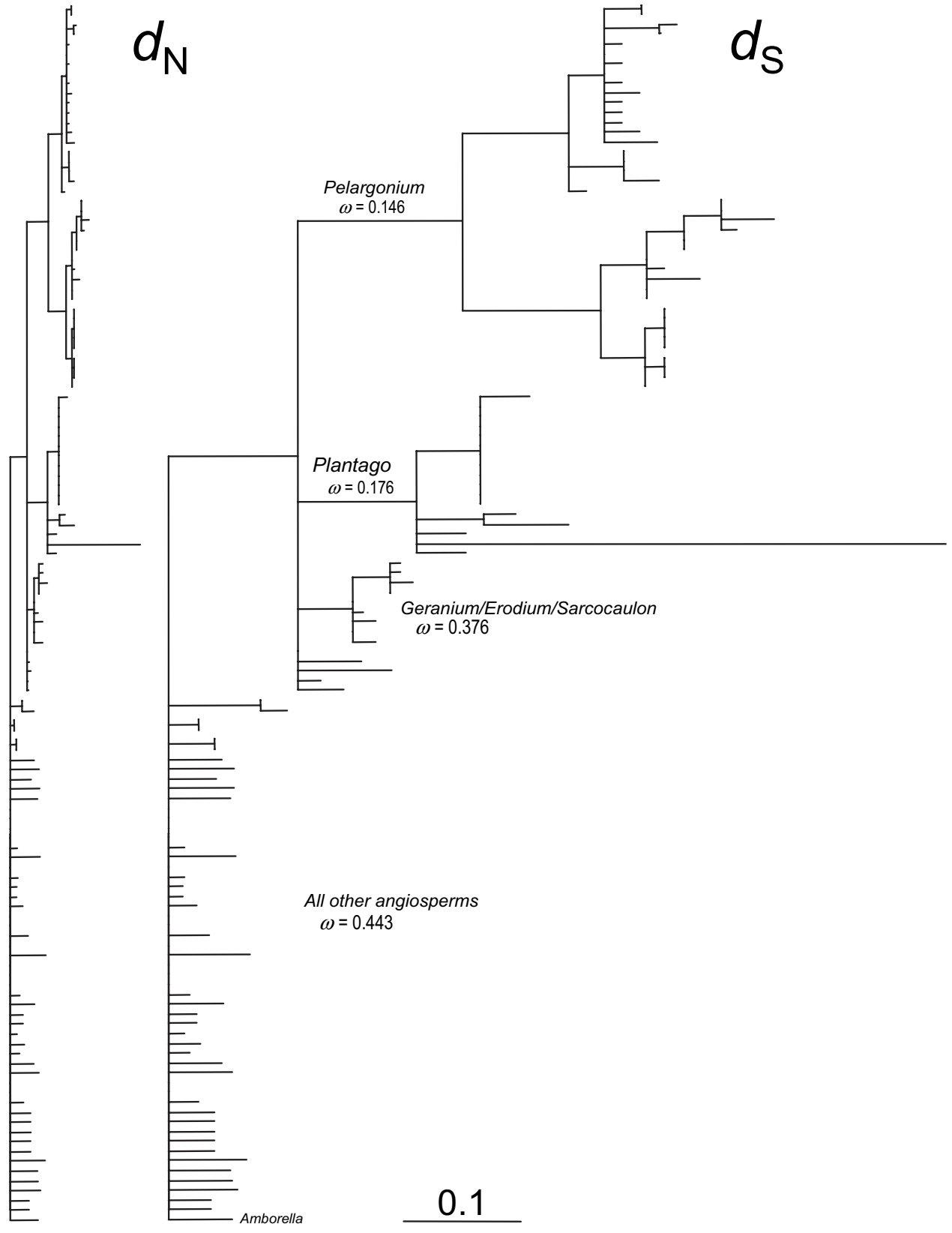

Fig. 2. mtDNA *nad*1 b/c exons—d_N (left) and d_S (right) tree representation of the *nad*1 b/c exon sequences. The scale-bar depicts 0.01 (silent or non-silent) substitution per codon. Clades are labelled as referred to in the text, with average reconstructed ω rate ratios indicated.

Hypseocharis biloba, and *Licania heteromorpha*, which occupy short branches but group together with the three long branch clades. Possibly the phylogenetic position of these, apparently fully functional, sequences reflects their status as processed paralogs that somehow did not undergo the substitution rate acceleration observed in the long branch clades. The fact that *Pelargonium lobatum* appears to be the sole Geraniacean representative in our sampling for which RNA editing is predicted, indeed suggests that it may have undergone an evolutionary pathway differing from the other, fast-evolving Geraniacean *nad*1 sequences. The fact that RNA editing was not predicted for the other slow-evolving Geraniacean sequences (i.e., *Pelargonium anethifolium*, *P. crassicaule*, *Hypseocharis biloba*) may be due to the short sequences (66 codons) on which this analysis is based.

Hypseocharis is a small genus endemic to the Andes. Apparently, the dramatic substitution rate acceleration in mitochondrial genomes of the remainder of what we now recognise as Geraniaceae, occurred after the split from the geographically isolated *Hypseocharis*. A rough comparison of branch lengths in the d_S tree (Fig. 2) suggests that within Geraniaceae the *Pelargonium* clade has undergone an additional substitution rate acceleration when compared with the *Erodium*, *Geranium*, and *Sarcocaulon* clades. This pattern is largely in agreement with the results of Parkinson & al. (2005) based on significantly larger data sets.

Within the *Plantago* clade, the *nad*1 b/c sequence for *Litorella* is on a very long branch indicating an additional, separate acceleration in d_S. Cho & al. (2004) concluded that *Plantago* mtDNA may have undergone an up to 4000-fold acceleration in substitution rate compared with other angiosperms, based on a dated "forced clock" tree and several thousand bp of sequence data. Parkinson & al. (2005) inferred a peak rate of up to 38 substitutions

Table 1. ModelTest results by dataset partitions and main clade; selected models plus associated –lnL values, based on hierarchical Likelihood Ratio Test (hLRT) or Akaike Information Content (AIC) test criteria, are given.

→ Codon position	1st +2nd		3rd	
Clade ↓	hLRT / AIC	–lnL	hLRT / AIC	–lnL
Pelargonium	JC+I/HKY+I	240.0335/233.3038	HKY/K81uf	263.7152/260.6030
Geraniaceae	JC/F81	208.1135/204.3934	F81/TrN	114.6555/111.4449
Plantago	JC/F81+I	273.6246/269.4033	JC/HKY	215.2412/207.9480
All other angiosperms	TrN+G/SYM+I	310.1556/309.0499	F81/TrN	189.8883/184.8157

Table 2. Comparison of d_N, d_S and ω in main *nad*1 b/c clades in the jackknife tree topology depicted in Fig. 1; d_N (non-synonymous substitutions per codon) and d_S (synonymous substitutions per codon) are given as ranges of rates measured over all branches included in the *Pelargonium*, Geraniaceae, *Plantago* or "All other angiosperms" main clade; overall values for ω (d_N/d_S) were optimized for the four designated main clades separately; settings used were: "yeast mitochondrial genetic code", "CodonFreq = F3×4", (codon) "model = two" (two or more d_N/d_S ratios optimised for each of the four main clades separately), and for the site models one ω rate was assumed across sites ("NSsites = one").

Clade	d_N	d_S	ω
Pelargonium	0.0021–0.0065	0.0146–0.0447	0.146
Geraniaceae	0.0073	0.0194	0.376
Plantago	0.0046–0.0176	0.0261–0.0997	0.176
All other angiosperms	0.0082–0.0242	0.0185–0.0546	0.443

Table 3. Sums of codon usage counts (according to the yeast mitochondrial genetic code) for the entire matrix used in this study. Usage counts are given following the three- and single-letter amino acid codes and their associated codons.

Phe-F	TTT	128	Ser- S	TCT	354	Tyr-Y	TAT	96	Cys-C	TGT	289
	TTC	226		TCC	77		TAC	1		TGC	6
Leu-L	TTA	123		TCA	1	*** *	TAA	0	Trp-W	TGA	13
	TTG	204		TCG	90		TAG	0		TGG	110
Thr-T	CTT	145	Pro-P	CCT	69	His-H	CAT	0	Arg-R	CGT	28
	CTC	41		CCC	212			0		CGC	0
	CTA	164		CCA	74	Gln-Q	CAA	208		CGA	153
	CTG	105		CCG	14		CAG	108		CGG	0
Ile-I	ATT	625	Thr-T	ACT	201	Asn-N	AAT	185	Ser-S	AGT	120
	ATC	43		ACC	0		AAC	21		AGC	1
Met-M	ATA	209		ACA	2	Lys-K	AAA	40	Arg-R	AGA	1
	ATG	284		ACG	0		AAG	78		AGG	1
Val-V	GTT	170	Ala-A	GCT	158	Asp-D	GAT	1	Gly-G	GGT	269
	GTC	213		GCC	0		GAC	0		GGC	3
	GTA	317		GCA	245	Glu-E	GAA	158		GGA	59
	GTG	73		GCG	107		GAG	139		GGG	20

per site per billion years (SBB) for the fastest Geraniacean branch (i.e., the one leading to the *Pelargonium* clade), and assert this to be much lower than the fastest rate in *Plantago*, i.e., possibly up to 700 SSB as estimated by Cho & al. (2004). However, as the authors indicate, this estimate could have been affected by the limited taxon sampling of 15 sequences representing the Geraniaceae. Given the highly differing rates present within our data set, as well as its limited size of only 66 codons, we felt it not appropriate to try dating our overall tree. Interestingly, likelihood ratio tests did however not reject clockwise accumulation of substitutions for the separate *Pelargonium*, Geraniaceae, and *Plantago* clades ($p < 0.001$), data not shown), calling for follow-up rate-assessment studies based on longer sequences. In any case, we conclude that the *Pelargonium nad*1 b/c clade may exhibit substitution rates comparable to those in *Plantago*, which ranks them as unprecedented high for the angiosperms. Note that the previously observed increased substitution rates in mtDNA of achlorophyllous taxa (Petersen & al., 2006) are not confirmed here for our *Burmannia* samples, but that could well represent a character sampling artefact.

What could have caused the accelerated substitution rates in Geraniaceae and Plantaginaceae mtDNA poses an interesting question from the perspective of molecular evolution. At first one might assume low or absent functional constraints on these sequences. However, as is clear from the d_S plots, virtually all substitutions that contribute to the rate acceleration are silent which we take as indication that functional constraints are unlikely to have been affected and remain in place in all clades.

Two alternative hypotheses can be put forward, one of them emphasizing transfer of the mtDNA encoded genes (including) *nad*1 to the nuclear genome where it might then reside in a region with high substitution rates. The observation that all introns are lost from Geraniacean and *Plantago* mtDNA (Bakker & al., 2000; Palmer & al., 2000) would lend support for this "nuclear transfer hypothesis" because it is characteristic of a processed paralog (for examples of organelle genes relocated to the nucleus see e.g. Blanchard & Schmidt, 1995; Adams & al., 1999, 2000, 2002; Daley, 2002; Bergthorsson & al., 2004). However, the gene for NADH dehydrogenase subunit 1 is considered to have been retained in the mitochondrial genome during evolution in land plants (Palmer, pers. comm.; Parkinson & al., 2005; Petersen & al., 2006; see Cho & al., 2004 for *cox*1 and *cob* genes). For instance, *nad*1 is divided in 5 exons in *Petunia hybrida* (Conklin & al., 1991; Dombrovska & Qiu, 2004), scattered throughout the mtDNA. That these genes have not been transferred to the nucleus could possibly be explained by the polarity of their products, which may prevent insertion back into the inner mitochondrial membrane (e.g., Popot & Devitry, 1990; Thorsness & al., 1996; Palmer, 1997). NADHase subunits are hydrophobic and as such not easily transported through the cytoplasm and mitochondrial membranes (Gray & al., 1998; Gray, 1999).

Table 4. Codon characteristics of mtDNA *nad*1 b/c exons in the four main clades as found in this study. Codon usage measures (ENC, CBI), and base composition at second and third codon positions are indicated for each clade.

Pelargonium	46.382	0.577	0.39	0.30
Geraniaceae	37.378	0.608	0.39	0.27
Plantago	42.217	0.519	0.41	0.34
All other angiosperms	41.320	0.700	0.39	0.36

Assuming residence of *nad*1 exons in mtDNA, which is also supported by the observed phylogenetic congruence between nuclear, mitochondrial, and plastid phylogenetic trees for *Pelargonium* (Bakker & al., 2004), we can therefore reject the "nuclear transfer" hypothesis outlined above. The observed loss of introns in Geraniacean and *Plantago* mtDNA genomes supports a "processed paralog" hypothesis (Bowe & dePamphilis, 1996). Only the absence of RNA edited sites in Geraniacean mtDNA, but typical of most other angiosperm mitochondrial genes, poses an interesting question. Parkinson & al. (2005) hypothesise "mutagenic retroprocessing", suggesting that the high replication error rate of reverse transcriptase (RT) may have caused high substitution rates during copying RNA into cDNA, prior to re-integration into the mtDNA genome. The high silent substitution rates d_S, as well as the three-fold downshifts in ω ratios observed, would then have resulted from subsequent purifying selection. The mutagenic retroprocessing mechanism (Bowe & dePamphilis, 1996; Parkinson & al., 2005) could then perhaps also explain the observed absence of RNA editing in extant Geraniaceae, as this would "reflect mutational drift to unedited C residues following […] the cessation of the hypothetical mutagenic retroprocessing activity." Possibly, in the evolutionary direct future, things could get back to "normal" again and RNA editing would resume in these lineages. Indeed, the authors did infer a slowdown of substitution rates in extant Geraniacean lineages, which is partly supported by our findings presented here.

As an alternative explanation (assuming the *nad*1 genes are indeed residing on the mitochondrial genome), not RT but the mitochondrial DNA polymerases γ in Geraniaceae and *Plantago* may have been affected with regards its proofreading accuracy control. However, whereas structure, function, and proofreading accuracy control of animal mitochondrial DNA polymerases γ is reasonably well understood (Kaguni, 2004), the enzyme operating in the plant mitochondrion remains to be investigated. Given the monophyletic origin for animal and plant mitochondria (Palmer & al., 2004) it is likely that

DNA polymerase γ is also present in plants. Polymerase γ is the sole DNA polymerase present in mitochondria in all animals "and appears to be uniquely responsible for all DNA synthetic reactions in replication, repair and recombination" (Kaguni, 2004). It consists of a polymerase activity subunit and a smaller subunit associated with 3'→5' exonuclease activity, and hence, proof reading fidelity control (Kunkel & Mosbaugh, 1989). Vanderstraeten & al. (1998) reported a 1500-fold increase in mutation frequency, as well as a significant decrease in exonuclease activity, in a yeast DNA pol γ that had been mutated in conserved amino acid residues in exons coding for the active sites of its exonuclease motifs. Perhaps such a mutation has occurred in the fast mitochondrial clades described here, and it would be interesting to explore the possibility of the presence of mutated DNA pol γ exonuclease function genes.

Given the relatively small data set used in this study (66 codons), the observed difference in Codon Bias Index (Table 4) between the "fast" clades and all other angiosperms could be an artefact of codon sampling. Larger datasets, ideally complete Geraniacean and *Plantago* mtDNA genomes, will have to be explored in order to accurately assess this. If the observed lower codon bias would persist, however, this would pose an interesting question as to the actual constraints in availability and relative abundance of iso-accepting tRNAs in these mitochondria. Since long, strong codon usage bias is considered to reflect translational selection (e.g., Ikemura, 1985). Purifying selection would then act to eliminate codons that do not correspond with abundant tRNAs. Perhaps the tRNA population in Geraniacean and *Plantago* mitochondria is sufficiently diverse and abundant to overcome the lower codon usage bias caused by the exceptionally high d_S rates observed.

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Appendix. Taxa used, along with citation of specimens, herbarium acronym or GenBank accession number. BR = National Botanic Garden of Belgium; HO = Tasmanian Museum & Art Gallery; K = Royal Botanic Gardens, Kew; LV = Katholieke Universiteit Leuven (Belgium); MCN = McNeese State University; NCU = University of Noth Carolina; NHN = Nationaal Herbarium Nederland (L = Leiden University branch, U = Utrecht University branch, W = Wageningen University branch); PPI = National Pingtung University of Science and Technology; SIU = Southern Illinois University; US = Smithsonian Institution; WBG = Wageningen Botanical Gardens. WAG numbers indicate BRAHMS database entries.

Agave attenuata Salm-Dyck (Agavaceae), AY832132; ***Amborella trichopoda*** Baill. (Amborellaceae), AY279682; ***Androcymbium dregei*** Presl (Colchicaceae), DQ091230; ***A. melanthioides*** Willd., DQ091229; **Apocynaceae sp.** *Hicks 8455* (Apocynaceae), AY788285; ***Arabidopsis thaliana*** (L.) Heynh. (Brassicaceae), Y08501; ***Austrobuxus megacarpus*** P. I. Forst. (Picrodendraceae), AY788276; ***Brassica napus*** L. (Brassicaceae), AP006444; ***Brassica*** sp., WBG, DQ887985; ***Burmannia capitata*** (Walt. ex Gmel.) Mart. (Burmanniaceae), DQ786128; ***B. coelestis*** Don, DQ786123; ***B. congesta*** (Wright) Jonk., DQ786120; ***B. flava*** Mart., DQ786131; ***B. flava*** Mart., DQ786130; ***B. juncea*** Sol. ex R.Br., DQ786143; ***B. longifolia*** Becc., DQ786138; ***B. lutescens*** Becc., DQ786144; ***B. latialata*** Pobég., DQ786125; ***B. madagascariensis*** Don, K.V. Merckx 1300; ***B. madagascariensis*** Mart., DQ786126; ***B. oblonga*** Ridl., DQ786140; ***B. wallichii*** (Miers) Hook.f., DQ786141; ***Centroplacus glaucinus*** Pierre (Phyllantaceae), AY788277; ***Citrullus lanatus*** (Thunb.) Matsum. & Nakai (Cucurbitaceae), AF453650; ***Clusia gundlachii*** A. Stahl (Clusiaceae), AY788278; ***Cymbocarpa refracta*** Miers (Burmanniaceae), DQ786095; ***Dioscorea tokoro*** Makino. ex Myabe (Dioscoreacaee), DQ786158; ***Eichhornia crassipes*** (Mart.) Solms (Pontederiaceae), AY832131; ***Erodium cicutarium*** (L.) L'Hér. (Geraniaceae), WBG brema002 X111111; ***E. meynieri*** Maire, NHN-W, WAG170966 X111111; ***E. moschatum*** (L.) L'Hér., WBG WAG201941 X111111; ***Geranium* "brookside"** (***G. clarkei* × *G. pratense***) (Geraniaceae), WBG, X111111; ***G. pusillum*** L., AF167131; ***G. reinhardii*** Trautv., WBG, X111111; ***G. robertianum*** L., AF167132; ***G. sanguinum*** L., WBG, X111111; ***Gnetum africanum*** Welw. (Gnetaceae), AY230287; ***Gymnosiphon aphyllus*** Bl. (Burmanniaceae), DQ786102; ***G breviflorus*** Gleason, DQ786098; ***G divaricatus*** (Benth.) Benth. and Hook., DQ786105; ***G. longistylus*** (Benth.) Hutch. and Dalziel, DQ786103; ***G. suaveolens*** (Karst.) Urb., DQ786097; ***G. usambaricus*** Engl., DQ786113; ***Hypseocharis biloba*** Killip (Geraniaceae), X111111; ***Licania heteromorpha*** Benth (Chrysobalanaceae), AY788279; ***Liriodendron tulipifera*** L. (Magnoliaceae), AY832125; ***Litorella uniflora*** (L.) Aschers. (Plantaginaceae), NHN-W, WAG201694; ***Mahonia bealei*** (Fortune) Carrière (Berberidaceae), AY832122; ***Maytenus senegalensis*** (Lam.) Exell. (Celastraceae) AY788286; ***Narthecium ossifragum*** Huds. (Nartheciaceae), DQ786163; ***Ochna multiflora*** DC. (Ochnaceae), AY788280; ***Oenothera berteroana*** Spach (Onachraceae), M63033; ***Panda oleosa*** Pierre (Pandaceae), AY788281; ***Paradrypetes subintegrifolia*** G. A. Levin (Phyllantaceae), AY788282; ***Pelargonium*** sp. nov. ined. (Geraniaceae), AF256582; ***P. album*** J.J.A. van der Walt, AF256577; ***P. alternans*** Wendl., AF167126; ***P. althaeoides*** (L.) L'Hér., AF256590; ***P. anethifolium*** (Eckl. & Zeyh.) Steud., AF256579; ***P. appendiculatum*** (L. F.) Willd., AF256584; ***P. articulatum*** (Cav.) Willd., AF167105; ***P. auritum*** (L.) Willd. var. ***carneum***, AF256583; ***P. australe*** Willd., AF167129; ***P. caffrum*** (Eckl. & Zeyh.) Harv., AF256587; ***P. caucalifolium*** Jacq., AF167114; ***P. caylae*** Humbert, AF167106; ***P. coronopifolium*** Jacq., AF256591; ***P. cotyledonis*** (L.) L'Hér., AF256578; ***P. crassicaule*** L'Hér., AF256586; ***P. dasyphyllum*** E. Mey. ex R. Knuth, AF256581; ***P. elongatum*** (Cav.) Salisb., AF167108; ***P. exhibens*** Vorster, AF167112; ***P. fulgidum*** (L.) L'Hér., AF167124; ***P. gibbosum*** (L.) L'Hér. ex Aiton, AF256588; ***P. grandicalcaratum*** Knuth, AF167110; ***P. griseum*** Knuth, AF167119; ***P. grossularioides*** (L.) L'Hér. ex Aiton, AF167130; ***P. incarnatum*** (L'Hér.) Moench, AF256589; ***P. karooicum*** Compton, AF167111; ***P. lobatum*** (Burm. F.) L'Hér., X111111; ***P. mollicomum*** Fourcade, AF167113; ***P. multibracteatum*** Hochstetter ex A. Rich., AF167107; ***P. mutans*** Vorster, AF167122; ***P. nanum*** L'Hér., AF167128; ***P. odoratissimum*** … , X111111; ***P. peltatum*** (L.) L'Hér., AF167103; ***P. praemorsum*** Andr. (Dietr.), AF167121; ***P. redactum*** Vorster, AF167120; ***P. scabrum*** L'Hér., AF167127; ***P. senecioides*** L'Hér., AF167117; ***P. sericifolium*** J.J.A. van der Walt, AF167123; ***P. spinosum*** Willd., AF167109; ***P. tetragonum*** (L.f.) L'Hér., AF167116; ***P. tongaense*** Vorster, AF167104; ***P. torulosum*** E.M. Marais, AF256585; ***P. trifidum*** Jacq., AF167118; ***P. triste*** (L.) L'Hér., AF167125; ***P. whytei*** Bak., AF167115; ***Petalostigma pubescens*** Domin (Picrodendraceae), AY88283; ***Petunia* × *hybrida*** Hort. ex E. Vilm. (Solanaceae), X60401; ***Philodendron oxycardium*** Schott (Araceae), AY832130; ***Piper betle*** L. (Piperaceae), AY832127; ***Plantago albicans*** L. (Plantaginaceae), NHN-W, WAG829909 X111111; ***P. alpina*** L. (syn: *alpinum*), NHN-W, WAG204508 & WAG204477, WAG82952 X111111; cf. ***P. coronopus*** L., NHN-W, WAG82906 X111111; ***P. crassifolia*** Forssk., NHN-W, WAG82908 X111111; ***P. lagopus*** L., NHN-W, WAG82907 X111111; ***P. lanceolata*** L., NHN-W, WAG82905 X111111; ***P. major*** L., NHN-W, WAG201934 X111111; ***P. maritima*** L., NHN-W, WAG135207 X111111; ***P. media*** L., NHN-W, WAG82924 X111111; ***P. monosperma*** Pourr., NHN-W, WAG82937 X111111; ***P. ovata*** Forssk., NHN-W, WAG170986 X111111; ***P. recurvata*** L., NHN-W, WAG82955 X111111; ***P. sempervirens*** Crantz, NHN-W, WAG82956 X111111; ***Plantago spec***., NHN-W, WAG82919, WAG82922 X111111; ***Platanus occidentalis*** L. (Platanaceae), AY832123; ***Sarcocaulon vanderietiae*** L. Bol. (Geraniaceae), AF167133; ***Suregada boiviniana*** Baill. (Euphorbiaceae), AY788284; ***Tacca integrifolia*** Ker-Gawl. (Dioscoreaceae), DQ786153; ***Thismia aseroe*** Becc. (Burmanniaceae), DQ786149; ***T. clavigera*** (Becc.) F. Muell., DQ786150; ***T. rodwayi*** F. Muell., DQ786148; ***T. taiwanensis*** Yang, Saunders and Hsu, DQ786147; ***Thismia sp***., DQ887986; ***Triticum aestivum*** L**.** (Poaceae), X57967; ***Welwitschia mirabilis*** Hook. f. (Welwitschiaceae), AF227478.