

The complete nucleotide sequence of the cassava (*Manihot esculenta*) chloroplast genome and the evolution of *atpF* in Malpighiales: RNA editing and multiple losses of a group II intron

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Abstract The complete sequence of the chloroplast genome of cassava (*Manihot esculenta*, Euphorbiaceae) has been determined. The genome is 161,453 bp in length and includes a pair of inverted repeats (IR) of 26,954 bp. The genome includes 128 genes; 96 are single copy and 16 are duplicated in the IR. There are four rRNA genes and 30 distinct tRNAs, seven of which are duplicated in the IR. The *infA* gene is absent; expansion of IRb has duplicated 62 amino acids at the 3' end of *rps19* and a number of coding regions have large insertions or deletions, including insertions within the 23S rRNA gene. There are 17 intron-containing genes in cassava, 15 of which have a single intron while two (*clpP*, *ycf3*) have two introns. The usually conserved *atpF* group II intron is absent and this is the first report of its loss from land plant chloroplast genomes. The

phylogenetic distribution of the *atpF* intron loss was determined by a PCR survey of 251 taxa representing 34 families of Malpighiales and 16 taxa from closely related rosids. The *atpF* intron is not only missing in cassava but also from closely related Euphorbiaceae and other Malpighiales, suggesting that there have been at least seven independent losses. In cassava and all other sequenced Malpighiales, *atpF* gene sequences showed a strong association between C-to-T substitutions at nucleotide position 92 and the loss of the intron, suggesting that recombination between an edited mRNA and the *atpF* gene may be a possible mechanism for the intron loss.

Introduction

Cassava, *Manihot esculenta* Crantz subsp. *esculenta* (Euphorbiaceae) is an ancient crop species and starch grains or radiocarbon-dated macroscopic remains are in the archeological record from 1800–7500 BP (Ugent et al. 1986; Dickau et al. 2007). Molecular evidence based on the haplotypes of the single-copy nuclear gene glyceraldehyde 3-phosphate dehydrogenase and genetic variation in five microsatellite loci strongly support the view that cultivated cassava is most likely derived from wild populations referred to *M. esculenta* subsp. *flabellifolia*, particularly from the populations occurring along the southern border of the Amazon basin (Olsen and Schaal 1999, 2001). Cassava, also called manioc or tapioca, is a perennial woody shrub, which is cultivated for its starchy storage roots throughout tropical and subtropical regions of the world, particularly in South America, Africa, and Asia, where it is the major source of dietary energy for more than 500 million people. Africa now produces more cassava than the rest of the world and it is one of that continent's staple food crops

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(Okezie and Kosikowski 1982; Hillocks 2002). The great need for crop improvement coupled with limitations imposed on traditional methods due to polyploidy have made cassava an ideal candidate for genomic approaches. Nuclear genetic maps collected from restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD) and simple sequence repeats (SSR), are available (Fregene et al. 1997; Fregene 2000; Mba et al. 2001), and recently advocated whole genome sequencing (Raven et al. 2006) has been initiated (<http://www.jgi.doe.gov/sequencing/cspseqplans2007.html>).

Several arthropod pests negatively impact cassava roots, foliage, and/or stems, particularly members of Lepidoptera, Diptera, and Hemiptera. There is little or no genetic resistance to these pests and their management is commonly achieved through biological control (El-Sharkawy 2004). Chloroplast genetic engineering would be ideal to address this problem because of the high-dosage strategy that can kill *Bacillus thuringiensis* susceptible and resistant insects (Kota et al. 1999; DeCosa et al. 2001). In addition, multi-gene engineering (DeCosa et al. 2001; Quesada-Vargas et al. 2005) should facilitate the introduction of genes that code for insecticidal proteins. Several other agronomic traits engineered via the chloroplast genome including disease resistance (DeGray et al. 2001), salt tolerance (Kumar et al. 2004a), drought tolerance (Lee et al. 2003), and herbicide resistance (Daniell et al. 1998; Dufourmantel et al. 2007), are valuable for cassava biotechnology. Chloroplast genetic engineering should offer transgene containment via maternal inheritance of transgenes (Hagemann 2004; Daniell 2002, 2007) or engineering cytoplasmic male sterility (Ruiz and Daniell 2005). Other advantages of chloroplast genetic engineering have been reviewed elsewhere (Maliga 2004; Daniell et al. 2005; Grevich and Daniell 2005; Daniell 2006).

Chloroplast genomes of bryophytes, gymnosperms, angiosperms, and their green algal relatives (Chlorophyta) share a basic set of introns which are believed to have been established prior to divergence of vascular and non-vascular plants. In land plant chloroplast genomes there are 17–20 group II introns within tRNA and protein-coding genes. Grasses have only 17 introns because they lack introns within the *clpP* and *rpoC1* genes (Downie and Palmer 1992; Barkan 2004). A group I intron present within *trnL-UAA* is considered the most ancient because it is present in cyanobacteria (Xu et al. 1990) and in chloroplasts of algal lineages, as well as, land plants (Simon et al. 2003). The *Chlamydomonas reinhardtii* chloroplast genome contains five group I and two group II introns but none of these are conserved in land plants. The *Euglena gracilis* chloroplast genome contains 155 introns (groups II and III), accounting for almost 40% of the genome (Barkan 2004). These examples show that introns have been lost or gained during the

course of chloroplast genome evolution. Group I and II introns have been considered mobile genetic elements exhibiting two modes of transposition: (1) intron homing (transmission to an intronless allele) or (2) transposition to ectopic sites, both involving the participation of maturases encoded within the introns (Barkan 2004). Functional group II intron maturases contain reverse transcriptase activity involved in intron homing, endonuclease activity involved in retrotransposition, and domains involved in RNA binding and splicing (Barkan 2004). The only maturase present in land plant chloroplast genomes, *matK* (an ORF in the *trnK-UUU* intron), lacks the reverse transcriptase domain and is therefore incapable of promoting intron mobility (Barkan 2004). Splicing of chloroplast group II introns, including the one in *atpF*, proceeds via lariat formation and depends on host-encoded splicing factors (Jenkins et al. 1997; Vogel et al. 1999; Vogel and Börner 2002; Barkan 2004). Intron presence/absence can be a useful marker for phylogenetic studies even though convergent losses have been documented in angiosperm chloroplast genomes (e.g., Downie et al. 1991a, 1994; McPherson et al. 2004). Detailed studies on the distribution of such structural changes have been sparse and each new fully sequenced chloroplast genome presents previously unsuspected structural variation that merits wider investigation to understand the evolutionary implications.

We report here on the complete sequence and organization of the chloroplast genome of *M. esculenta*, which is the first published genome sequence of a member of the family Euphorbiaceae. The most surprising structural change we uncovered in the cassava chloroplast genome is the loss of the *atpF* intron and the association of this loss with RNA editing. This is the first report of the absence of the *atpF* intron from a land plant chloroplast genome. We also present a detailed survey for this intron loss among members of Euphorbiaceae and in other families of Malpighiales. The phylogenetic implications of the distribution of multiple losses of the *atpF* intron are also discussed.

Materials and methods

DNA sources

A bacterial artificial chromosome (BAC) library of *M. esculenta* cultivar TME3 was constructed by ligating size fractionated partial *Bst*Y1 digests of total cellular, high molecular weight DNA with the pINDIGOBAC536 vector. The average insert size of the cassava library was about 100 kb. Bacterial artificial chromosome-related resources for this public library (BAC library ME_TBa) can be obtained from the Clemson University Genomics Institute BAC/EST Resource Center (<http://www.genome>.

clemson.edu). Bacterial artificial chromosome clone screening, selection and sequencing followed Daniell et al. (2006). Chloroplast genome inserts were identified with a soybean chloroplast DNA probe and the first 96 positive clones were pulled from the library, arrayed in a 96-well microtitre plate, copied, and archived. Selected clones were then subjected to *Hind* III fingerprinting and *Not* I digests. End-sequences were determined and localized on the chloroplast genome of *Arabidopsis thaliana* to deduce the relative positions of the clones; then a single clone that covered the entire chloroplast genome of cassava was chosen for sequencing.

DNA sequencing and genome assembly and annotation

The nucleotide sequence of the selected BAC clone was determined by the bridging shotgun method. The purified BAC DNA was subjected to hydroshearing, end repair, and then size-fractionated by agarose gel electrophoresis. Fractions of approximately 3.0–5.0 kb were eluted and ligated into the vector pBLUESCRIPT IKS+. The shotgun libraries were plated and then arrayed into forty 96-well microtitre plates for the sequencing reactions. Sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Sequence data from the forward and reverse priming sites of the shotgun clones were accumulated until equivalent to eight times the size of the genome and then assembled using Phred-Phrap programs (Ewing and Green 1998). Annotation of the cassava chloroplast genome was performed using DOGMA (Dual Organellar GenoMe Annotator, Wyman et al. 2004) with a custom database of previously published chloroplast genomes. Both tRNAs and rRNAs were identified by BLASTN searches against the same database of chloroplast genomes.

Analysis of the *atpF* intron

Genomic DNA extractions were initially screened with 15 μ l PCR reactions using primers *atpF*-100F 5'-CTAAGTG TAGTSCTTGGTGTATTGA-3' and *atpF*-465R 5'-AGTTCCTAGAGCTCCTTGTA-3', that are expected to yield a 367-bp intronless exonic fragment for taxa lacking an intron or a ca 1.1-kb fragment for taxa with an intron. If multiple copies of a gene are present which differ greatly in size (i.e., reflecting differences in intron content), then preferential amplification of the shorter fragments can occur. The intron-containing copy may also co-amplify or be suppressed entirely as a PCR artifact (Wurdack, personal observation). To assess whether shorter intronless fragments might represent undesired paralogs, an intron/exon primer combination was tried on intronless taxa using *atpF*-465R and an internal intron primer, *atpF*-Intron-F

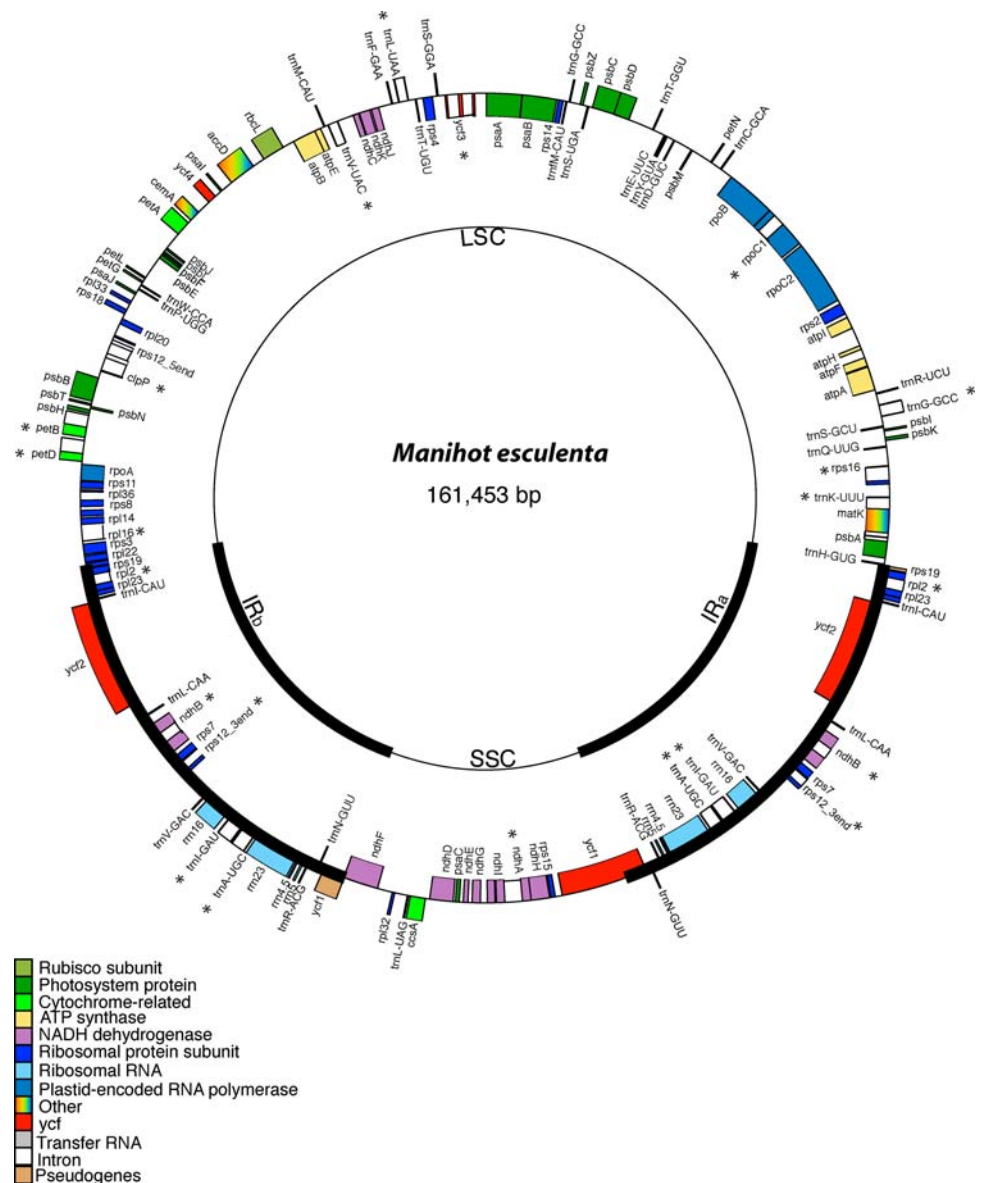
5'-TCGATATARAACACTCATRTCGATA-3'. Primers rooted in the flanking genes (*atpH*-162F 5'-GCT-TAGTCTGGCTTTTATGGAAGC-3' and *atpA*-152R 5'-CCTGCCATTACTTCATCAAGAC-3') were used in combination with the original *atpF* primers or an alternative forward primer (*atpF*-1F 5'-ATGAAAAATRTAACCGATTCTTTC-3') to produce copies from the chloroplast genome for the purposes of sequencing and to establish whether intronless copies had the correct flanking chloroplast genes. Intronless copies could be processed paralogs from RNA intermediates and occur elsewhere in other genome (i.e., nuclear or mitochondrial) but these would not be expected to contain flanking chloroplast sequence. Finally, an intron insertion-site spanning primer *atpF*-ISP-R 5'-CGATTATCTAATAAATCACTTAACAC-3' was used in combination with primers *atpF*-1F or *atpF*-100F to check for intronless copies in select intron-containing taxa. Amplification conditions were 35 cycles of 94°C for 1 min, 52°C for 1 min and 72°C for 2 min. Approximately 7% of the DNAs tested failed to yield bands, mostly due to degraded templates but some could be attributable to primer mismatches.

Phylogenetic analyses

Phylogenetic analyses of the *atpF*-coding region included 76 taxa of which 52 came from previously published, fully sequenced angiosperm chloroplast genomes and the remainder are newly generated, including cassava and 23 additional sequences from a carefully selected subset of the taxa screened here for intron loss. Manual alignment was straightforward with no indels (insertions or deletions) in Malpighiales; among other angiosperms there were six autapomorphic insertions and six deletions (one informative, truncating the terminus of the gene by one codon). Indels were treated as missing data and not excluded or recoded. Analyses were performed with maximum parsimony (MP) using PAUP* ver. 4.0b10 (Swofford 2003), maximum likelihood (ML) using GARLI ver. 0.951 (Zwickl 2006), and Bayesian inference (BI) using MrBayes ver. 3.1.1 (Huelsenbeck and Ronquist 2001). The appropriate model (GTR + I + Γ) was determined with Modeltest ver. 3.7 (Posada and Crandall 1998). For MP, starting trees were generated with 1,000 random taxon addition TBR replicates (MulTrees off) and these were subjected to another round of searching with a limit of 50,000 trees (MulTrees on). Additional MP searches were conducted using the parsimony ratchet as implemented in PAUPRat ver. 1 (Sikes and Lewis 2001) and using five replicates of 200 iterations each under default parameters. For ML, GARLI searches were run under the automated stopping criterion (20,000 generations); likelihood scores and parameter values were optimized in PAUP*. BI used

four Markov chains run for 1.0×10^7 generations and sampled every 100 generations with a discarded burnin of 1.0×10^6 generations. Maximum likelihood and BI searches were done in replicate to determine the concordance between runs. Maximum parsimony non-parametric bootstraps used 1,000 replicates, each with 10 random addition replicates but saving no more than 10 trees per iteration (see Wurdack et al. 2005). Maximum likelihood bootstraps in GARLI used 100 replicates and a 20,000-generation automated stopping criterion. We tested the hypothesis on correlated evolution between *atpF* intron status (present or absent) and RNA-editing (i.e., C or T at nucleotide position 92) using BayesDiscrete (part of BayesTraits; Pagel and Meade 2006). This Bayesian approach looks for correlated evolution between pairs of binary traits and accounts for phylogenetic uncertainty.

Fig. 1 Map of the *M. esculenta* plastid genome represented in its circular monomeric form. Large and small single copy regions (*LSC*, *SSC*) are separated by the inverted repeats (*IRa* and *IRb*, 26,954 bp, respectively). Genes illustrated inside the circle are transcribed in the clockwise direction and genes illustrated outside the circle are transcribed in the counter-clockwise direction. Split genes or genes with introns are marked with asterisks



Results

Size, gene content, order, and organization of the cassava chloroplast genome

The complete nucleotide sequence of the chloroplast genome of *M. esculenta* has been determined (Fig. 1, Genbank accession number EU117376). This genome is 161,453 bp in length and includes a pair of inverted repeats (IR) of 26,954 bp separated by small and large single copy (SSC, LSC) regions of 18,250 and 89,295 bp, respectively. The genome has 128 genes; 96 are single copy and 16 are duplicated in the IR. There are four rRNA genes and 30 distinct tRNAs, seven of which are duplicated in the IR. The genome consists of 49.82% protein-coding, 1.7% tRNA, and 5.6% rRNA genes, and 42.87% non-coding sequence.

The G + C and A + T contents in the cassava chloroplast genome are 35.87 and 64.13%, respectively. The overall A + T content is similar to poplar (63.26%), tobacco (62.2%), citrus (61.52%), maize (61.5%), and rice (61.1%). The A + T content of LSC and SSC regions are 66.73 and 70.36%, respectively, whereas the IR has 57.70% A + T due to the presence of the rRNA gene cluster.

The *infA* gene, coding for translation initiation factor 1, is absent in the cassava chloroplast genome. The expansion of IRb has duplicated 62 amino acids of the 3' end of *rps19*. There are indels within certain coding sequences. For example, between coordinates 540–560 of the 23S rRNA gaps are required to align the cassava sequences with sequences from other species. Moreover, the 23S rRNA (2,822 bp) is 11–12 bp longer than other core eudicots including members of the Solanaceae, *Atropa* (2,811 bp, Schmitz-Linneweber et al. 2002), *Lycopersicon* (2,810 bp, Kahlau et al. 2006), *Nicotiana* (2,810 bp, Shinozaki et al. 1986), *Solanum* (2,810 bp, Daniell et al. 2006), and species from other families, including *Arabidopsis* (2,810 bp, Sato et al. 1999), *Citrus* (2,809 bp, Bausher et al. 2006), *Coffea* (2,810 bp, Samson et al. 2007), *Cucumis* (2,810 bp, Plader et al. 2007), *Daucus* (2,806 bp, Ruhlman et al. 2006), *Glycine* (2,811 bp, Saski et al. 2005), *Gossypium* (2,810 bp, Lee et al. 2006), *Populus* (2,809 bp, Okumura et al. 2006), and *Spinacia* (2,810 bp, Schmitz-Linneweber et al. 2001).

Evolution of *atpF* and loss of its intron

Seventeen genes in cassava have introns, 15 of which contain a single intron including six in tRNAs, while two (*clpP*, *ycf3*) have two introns. The alignment of the *atpF* sequences of cassava and *Populus* (i.e., the closest relative with a completely sequenced chloroplast genome) indicates that the intron is missing from the former (Fig. 2). The presence/absence of the *atpF* intron in 251 taxa representing 34 of the ~38 families of Malpighiales and 16 taxa from the closely related sister groups, Celastrales and Oxalidales (including Huaceae), was determined by a PCR survey (Appendix, Fig. 3). This screening determined the intron was missing not only in cassava but also from closely related members of Euphorbiaceae, some Phyllanthaceae, and Picrodendraceae, and all sampled members of Elatinaceae, Lophopyxidaceae, Malesherbiaceae, Passifloraceae, and Turneraceae. PCR product size variation probably due to small intron indels was evident on agarose gels for some taxa but large size differences (>ca 50 bp) indicating that larger indels were not detected. Secondary PCR screening on intronless taxa with the intron/exon primer pair did not detect intron-containing copies. Screening using the exon/intron-insertion-site spanning primers did not detect intronless copies in intron-containing taxa.

Amplification using primers in the flanking genes *atpH* and *atpA* and sequencing of representative intronless taxa (see Appendix) verified intron absence at the same splice site. The length of the *atpF* protein-coding sequence was found to be 555 bp in all fully sequenced Malpighiales (some additional taxa sampled here have truncated sequences due to the use of internal *atpF* primers). Phylogenetic analyses (MP, ML, BI) were performed using *atpF* sequences from 76 taxa including 23 Malpighiales representing intron-containing and closely related intronless taxa. The data set had 576 aligned coding positions of which 380 were variable, and 267 were parsimony informative. Maximum parsimony searches reached the set tree limit of 50,000 (actual number of optimal trees is greater) most parsimonious trees (length 1,626 steps; retention index of 0.589; consistency index of 0.385/0.325 including/excluding uninformative characters, respectively) that in strict consensus are moderately resolved, except among rosids, Solanaceae, and basal angiosperms (results not shown). The parsimony ratchet found no shorter trees. The ML analyses resulted in an optimal tree with $-\ln L = 8741.21059$ (Fig. 4). The topologies and relative levels of support were similar among trees from the three methods of analysis. Fifteen nodes (four in Malpighiales) had strong support (≥ 95 posterior probability, PP; $\geq 85\%$ bootstrap) across all analyses. Malpighiales were resolved as monophyletic with ML and BI analyses (PP = 98) but not with MP (i.e., unresolved mostly with other rosids). Euphorbiaceae were monophyletic with high to moderate support. Phyllanthaceae were paraphyletic due to the inclusion of Picrodendraceae but this has weak support. In cassava and all other sequenced Malpighiales, the *atpF* gene sequences showed a strong association between C-to-T substitutions at nucleotide position 92 and losses of the intron (mapped in Fig. 4).

Discussion

Implications for homologous recombination of transgenes in chloroplast genomes

Complete chloroplast genome sequences provide information on intergenic spacer regions for homologous recombination of transgenes in chloroplast vectors. Transformations of the *Arabidopsis*, potato, and tomato chloroplast genomes were achieved using the tobacco chloroplast genome flanking sequences but efficiencies were much lower than in tobacco because of less than 100% sequence identities (Sikdar et al. 1998; Sidorov et al. 1999; Ruf et al. 2001). In another study, *Petunia* flanking sequences were used to transform the tobacco plastid genome and transformation efficiency was lower than was observed with endogenous flanking sequences (DeGray et al. 2001). Furthermore,

Fig. 2 Alignment of *atpF* genes from cassava and *Populus alba* showing that there is 94.1% sequence identity in the exon regions and the precise loss of the intron in cassava. The nucleotide position 92 is highlighted in cassava, where C-U editing occurs. Primers used for DNA sequencing are also highlighted

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Manihot ATGAAAAATATAACCGATTCTTTCGTTTCCTTGGGTCCTGCGCCATCCGCCGGGAGTTTCGGGTTTAATA
Populus ATGAAAAATATAACCGATTCTTTCGTTTCCTTGGGTCCTGCGCCATCCGCCGGGAGTTTCGGGTTTAATA

Manihot CCGATATTTTAGCAACAACCTTAATAAATCTAAGTGTAGTCCTTGGTGTATTGATTTTTTTGGAAAGGG
Populus CCGATATTTTAGCAACAACCTCAATAAATCTAAGTGTAGTCCTTGGTGTATTGATTTTTTTGGAAAGGG
                                     atpF-100 forward

Manihot GGTGT-----
Populus AGTGTGTGCGAGTTGTTTATTTCAGAATAGGCTGGATTGAACCAGCTGCACCTTTTTGTTTCGTTTAA

Manihot -----
Populus CTAGGAAATTTAACTAGAAAAGAAAGGTGCATGATCCTGCGAATTACTCCTGAATAAATTAAGAAATCA

Manihot -----
Populus TCTTTAAGAACCATAGCATTTCGTGATTCATTGGTAAATAGATTTTGATTCTCTATCAACCAATAATGTG

Manihot -----
Populus GGACCATTAACATGGTTAAAGCTAAACTGTTTGAAGTCCAGACAGAGCAGGTTACTCTTCTACTAGTAT

Manihot -----
Populus GTTAATACATACATAAAAGGATTCAAGATGAATAGTTGGAAATGTTTTCCGGTATAGAACACTCATGTCC

Manihot -----
Populus AAAAAGGATTTGAACCCTTTTTTTTTTCAAAAAAATGGGTATTTACCCATTCTATCCAATGCTGAATCG

Manihot -----
Populus ATAACCTACACATAAAGTAAAGTCTTTGGATTTGAAGAAAAAAGAAACAACCTTACTGACAATTACTTG

Manihot -----
Populus TTTGGTCAGAAGAGTCTCCGAATATTCGGTCTTGGATTAGTGATTAGTTTAGTTTGGAAATCCGAAT

Manihot -----
Populus AATGAACCAGAAAAAGAGGATAGGCTCATTCCAGTCAAAAAAGAGATGGGAATTTCCCATAGTAATTGA

Manihot -----
Populus ACTAATTGAGCGTGAGAGCCAAATGAATCGAAAGACTCATGTTTGGTTCGGGAAGGATCATGGAAGTTT

Manihot -----
Populus TGCAATGAATGAAAAATAATCTACTTTTCATTAAGTGATTTATTAGATAATCGAAAAACAGAGAATTTTGA

Manihot ATACTATTTCGAAATTCAGAAAACTACGCGAGGGGGCTATTGAACAGCTGAAAAAGCCCGGGCCCGCTT
Populus ATACTATTTCGAAATTCAGAAAGACTACGCGGAGGAGCCATTGAACAGTTGAAAAAGCCCGGGCCCGCTT

Manihot ACGGAAAGTGGAAATAGAAGCAGATCAGTTTCGAACGAATGGATATTCTGAGATAGAACGAGAAAAATTG
Populus ACGGAAAGTGGAAATAGAAGCGGATCAATTTCCAGTGAACGGATACCTCTGAGATAGAACGAGAAAAATTG

Manihot AATTTGATTAATTCAACTTATAAGACTTTTGAACAATTAGAAAATTACAAAAATGAAACCATTCAATTTG
Populus AATTTGATTAATTCAACTTATAAGACTTTTGAACAATTAGAAAATTACAAAAACGAAACCATTCAATTTG

                                     atpF-465 reverse
Manihot AACAAACGAACGATTAATCAAGTCCGACAACGGGTTTTCCAACAAGCCTTACAAGGAGCTCTAGGAAC
Populus AACAAACGAAGCGATTAATCAAGTCCGACAACGGGTTTTCCAACAAGCCTTACAAGGAGCTCTAGGGAC

Manihot TCTGAATAGTTGTTTGACCAACGAGTTGCATTTACGTACCATCAATGCTAATCTTGGCATGTTGGCGCG
Populus TTTGAATAGTTGTTTGACCAACGAGTTACATTTACGTACTATTAGTGCCAATATTGGCATGTTGGGGCG

Manihot ATAAAAGAAATAACTGATTAG
Populus ATGAAAGAAATAACTAATTAG

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when *Solanum nigrum* flanking sequence was used in plastid transformation of tobacco, the efficiency of homologous recombination was very low even though flanking sequence identity was 98% (Zubkot et al. 2004). These examples emphasize the advantages of species-specific vectors for efficient homologous recombination within the intergenic spacer regions of chloroplast genomes. Species-specific chloroplast vectors were successfully employed in

developing plastid transformants in carrot, cotton, and lettuce (Kumar 2007, 2004a, b; Kanamoto et al. 2006; Ruhlman et al. 2007). In addition to intergenic spacer regions, regulatory elements like promoters, 5'-UTRs and 3'-UTRs are important for expression of transgenes in plastids. Recently, high-level GFP expression was achieved in lettuce using endogenous regulatory elements (Kanamoto et al. 2006). Therefore, it is important to obtain complete

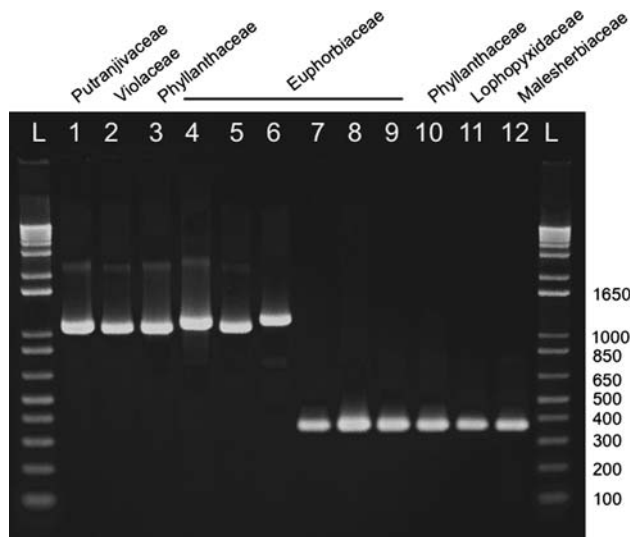


Fig. 3 Ethidium bromide stained 1.5% agarose gel showing amplification products for selected Malpighiales taxa screened for the presence/absence of the *atpF* intron. Intron present, lanes 1 *Putranjiva roxburghii*, 2 *Hybanthus concolor*, 3 *Bischofia javanica*, 4 *Ricinus communis*, 5 *Suregada glomerulata*, 6 *Tetrorchidium cf. macrophyllum*. Intron absent, 7 *Elateriospermum tapos*, 8 *Manihot grahamii*, 9 *Micrandra inundata*, 10 *Savia bahamensis*, 11 *Lophopyxis maingayi*, 12 *Malesherbia weberbaueri*. L Invitrogen 1 Kb Plus DNA Ladder. Underlined taxa have been sequenced from these PCR products. See Appendix for sample details

genome sequences of crop plants for various biotechnology applications (Daniell et al. 2006; http://www.ncbi.nlm.nih.gov/genomes/ORGANELLES/plastids_tax.html).

Genome organization

The plastid genome of *M. esculenta* has the ancestral angiosperm genome organization (Raubeson et al. 2007) and in particular is co-linear with *Populus*, its closest fully sequenced relative. Major structural differences include the presence of *rps16* and *rpl32* (both absent in *Populus*) and the loss of *infA* (present in *Populus*) in cassava. The last is an unusually unstable gene, having been lost from the chloroplast genome of angiosperms on many separate occasions and transferred multiple times to the nuclear genome. Using two *infA* gene probes from *Antirrhinum* and a Southern hybridization approach accompanied by selective sequencing, Millen et al. (2001) ascertained that *infA* was lost or is a pseudogene in at least 24 lineages of angiosperms, including most rosids and five Euphorbiaceae sampled. *Hevea*, a close relative of cassava in Euphorbiaceae, was definitively determined in that study to possess a pseudogene based on sequencing; poor hybridization suggests other Malpighiales contain pseudogenes (Millen et al. 2001). In *Populus* and 17 other sequenced chloroplast genomes sequenced, *infA* is also pseudogene.

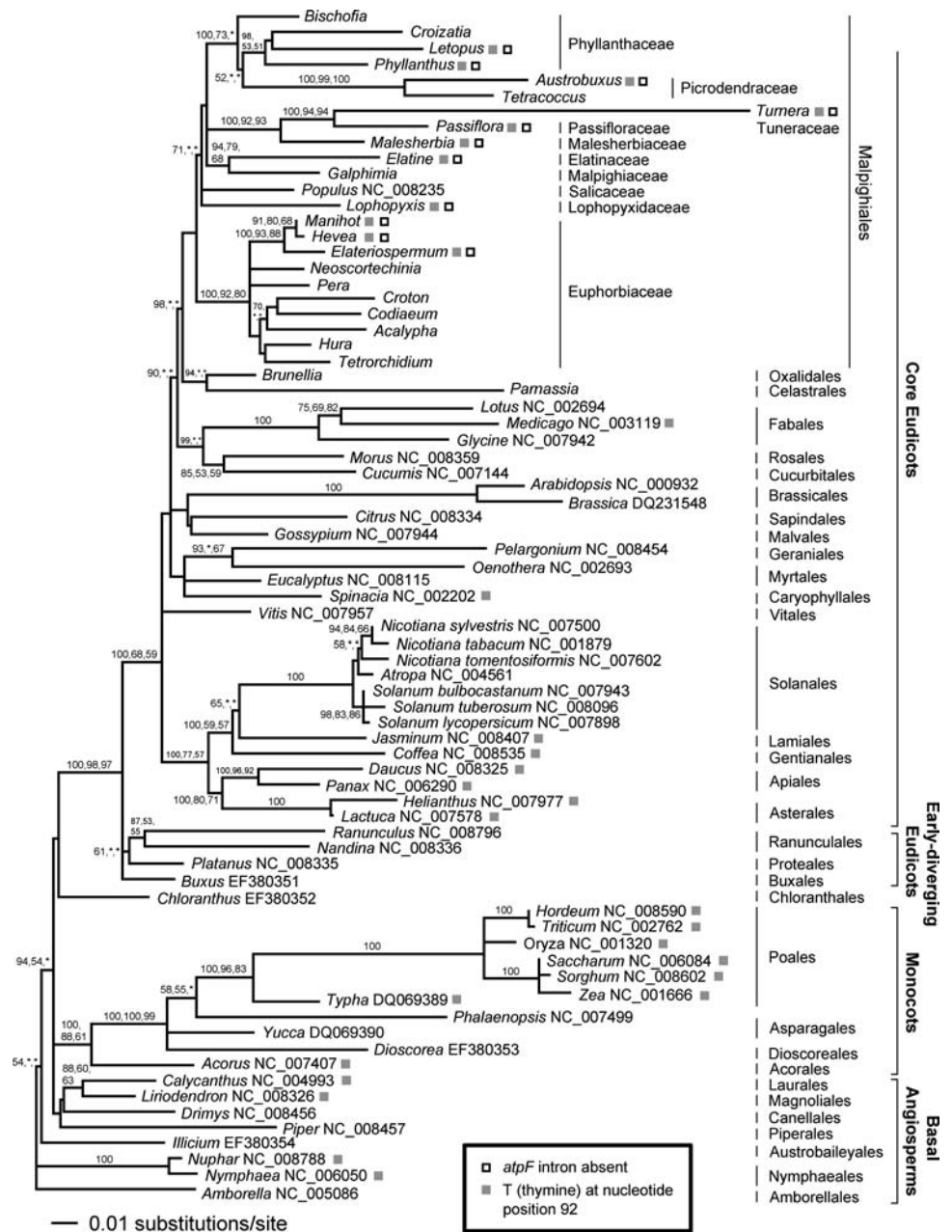
Evolution of *atpF* and loss of its intron

With the exception of the loss in cassava reported here, a group II intron in *atpF* at nucleotide position 148 (*atpFi148*) has been found in all previously sequenced land plant chloroplast genomes. Most green algae (Chlorophyta and charophycean green algae which are early-branching members of the streptophytes and sister to land plants) also appear to lack this intron. Among charophytes, however, *Staurastrum* does possess *atpFi148*, but its sister group *Zygnema* does not (Turmel et al. 2005, 2006). A single gain within the streptophytes before the origin of land plants followed by losses in charophytes has been suggested (Turmel et al. 2006). Details on exactly where the *atpFi148* was gained and where losses have occurred must await detailed sampling for this intron and a more robust phylogeny of the early-branching lineages of the streptophytes. Another charophyte, *Chara*, has a non-homologous *atpF* intron at a different insertion site (*atpFi1380*) (Turmel et al. 1999, 2006). Splicing of the *atpF* intron is well studied and specific host-encoded (nuclear) protein cofactors are required. Among assayed chloroplast introns in *Arabidopsis* and maize, *crs1* is a required specific cofactor for the *atpF* intron where it promotes intron folding (Jenkins et al. 1997; Vogel et al. 1999; Ostersetzer et al. 2005; Asakura and Barkan 2006). Intron recognition by *crs1* has been mapped to elements of specific intron domains in maize (Ostersetzer et al. 2005), although these are not highly conserved either across angiosperms or in the Malpighiales sequenced here. Lack of splicing of the spinach *atpF* intron in transgenic *Chlamydomonas* suggests host factors are also taxon specific (Deshpande et al. 1995). Mutations causing the loss of specific splicing factors such as *crs1* could drive the concomitant evolutionary loss of the *atpF* intron since splicing defects are lethal by preventing biogenesis of the ATP synthase complex (Jenkins et al. 1997).

RNA editing and loss of *atpF* intron

Copies (processed paralogs sensu Bowe and dePamphilis 1996) of organellar genes lacking otherwise canonical introns can be the products of reverse-transcription of RNA intermediates. These may be integrated elsewhere (i.e., mitochondrial or nuclear genomes) or replace the native intron-containing copy via recombination (Bonen and Vogel 2001; Itchoda et al. 2002). RNA editing has been documented for *atpF* (Corneille et al. 2000; Tillich et al. 2006) and in particular for codon 31, where C-U editing occurs to correct a non-synonymous second position substitution (nucleotide 92) and conserve a leucine residue. Processed paralogs from RNA intermediates would be expected to show an editing signature. Cassava and all other intronless taxa sequenced for *atpF* appear by bioin-

Fig. 4 Phylogenetic tree of the 76-taxon *atpF* dataset. Tree shown is from a ML analysis with $-\ln L = 8741.21059$. *Number series* above or below branches indicate support from BI, ML, and MP analyses, respectively; *single numbers* indicate all 3 values are identical. *Asterisk* indicates <50% support in that analysis. Intron absence is indicated with an *open square*; all other taxa possess the *atpF* intron. A *filled gray square* indicates the presence of a thymine at nucleotide 92; all other taxa possess a cytosine at that site, which is potentially RNA edited as a C-U conversion. *GenBank numbers* are indicated for published data, all other taxa are newly generated (see [Appendix](#))



formatic prediction to have lost this RNA-editing in codon number 31 (i.e., the conversion of CCA \rightarrow CTA and the resulting amino acid change of P \rightarrow L), which may be evidence of replacement via an RNA intermediate. The association of intron absence and this substitution is absolute among sequenced Malpighiales but not elsewhere in the tree where editing occurs but the intron is present (mapped in Fig. 4). This observation suggests recombination between an edited mRNA and the cognate chloroplast genome allele may be a possible mechanism for the loss of the *atpF* intron. Although this mechanism has been previously suggested for mitochondrial genes (e.g., *nad4*, Itchoda et al. 2002), it has not yet been reported for chloroplast

genes. However, further studies are needed to confirm this hypothesis. In particular, *atpF* is part of an ATP synthase transcriptional unit consisting of *atpI/H/F/A* and full polycistronic and partial length transcripts due to post-transcriptional processing have been detected (Stahl et al. 1993; Miyagi et al. 1998; Knauf and Hachtel 2002). Editing also occurs in *atpA* (Tillich et al. 2006) and two sites (codons 264 and 383) do not appear to be edited in cassava. A third site, codon 265, is ambiguous and would need experimental verification as although editing is possible (i.e., a cytosine is present in cassava), it appears dispensible and the third-codon position change is synonymous (Corneille et al. 2000). If a transcriptional unit was involved in recombination,

then such additional editing changes beyond *atpF* might be expected. Other possible mechanisms involved in the loss of introns include maturases that have reverse transcriptase and endonuclease activity for intron homing, and retrotransposition. However, the only maturase (*matK*) identified in land plant chloroplast genomes lacks reverse transcriptase activity (Barkan 2004) and therefore may not play any significant role in the loss of introns.

Phylogenetic distribution of *atpF* intron losses

To determine the distribution and phylogenetic utility of the *atpF* intron loss, we screened a broad diversity of Euphorbiaceae and other Malpighiales DNAs mostly used in prior phylogenetic studies (i.e., Davis and Wurdack 2004; Wurdack et al. 2004, 2005; Berry et al. 2005; Davis et al. 2005). Our results, when considered in the context of these studies, suggest that the intron has been lost at least seven times in Malpighiales (losses each from Elatinaceae, Euphorbiaceae, Picrodendraceae, Lophopyxidaceae, and Passifloraceae s.l., and twice from Phyllanthaceae). The minimum number of losses estimated here can be established with confidence even though there is uncertainty in the relationships of some groups (i.e., between groups of Malpighiales families and at the subfamily level in Euphorbiaceae), as prior studies indicate the seven clades with losses all have strongly supported sister-groups that possess the intron. Optimizations with fewer losses would imply the unlikely secondary gain of an intron at this site.

The intron loss in Euphorbiaceae is confined to the articulated crotonoids (sensu Wurdack et al. 2005), a clade of the subfamily Crotonoideae with many members possessing articulated laticifers that are the source of rubber production. This strongly supported (100% bootstrap; Wurdack et al. 2005; Tokuoka 2007) group is nested within the family and comprises approximately eight genera and ca 175 species (six genera sampled here, *Cnidoscolus*, *Elateriospermum*, *Glycydendron*, *Hevea*, *Manihot*, and *Micrandra*; not sampled, *Micrandropsis* and *Cunuria*). The affinities of *Elateriospermum* (traditionally treated as a monotypic tribe), the first-branching lineage of the articulated crotonoids, had been disputed prior to molecular phylogenetic analyses (Wurdack et al. 2005). The absence of the intron in *Elateriospermum* confirms its affinities. Although our sampling in this clade is incomplete, we believe this represents a single loss before the divergence of *Elateriospermum* and all nested members are expected to lack the intron. The intron absences in Phyllanthaceae are confined to two nested clades: (1) all Phyllanthaceae and (2) part of Poranthereae (clades F1 and F3, respectively; sensu Wurdack et al. 2004; Kathriarachchi et al. 2005; Hoffmann et al. 2006) and probably represent two separate losses. Additional sampling is needed for Poranthereae to see if the loss is confined to

Leptopus or extends to other genera. Monophyly of this clade is strongly supported and although internal intergeneric relationships are poorly resolved (Wurdack et al. 2004; Kathriarachchi et al. 2005), the presence of both intron and intronless taxa confirms this loss is separate from that in Phyllanthaceae. The loss in Picrodendraceae is confined to nested closely related Australasian members of the family and probably represents a single loss. *Podocalyx*, sister to the rest of the family (Davis and Wurdack 2004; Davis et al. 2005), possesses the intron. Malesherbiaceae, Passifloraceae, and Turneraceae are closely related and have been proposed to be united in a single family (i.e., Passifloraceae sensu lato; APG 2003). The absence of the intron may reflect a single loss and represent an additional synapomorphy uniting the smaller segregate families. These families are nested within a large clade of Malpighiales with mostly parietal placentation whose other sampled members (including *Populus*) contain the intron. Passifloraceae are known to have other chloroplast gene and intron losses (Downie et al. 1994a, b; Hansen et al. 2006; Jansen et al. 2007). The loss of the intron in Lophopyxidaceae appears isolated as the family is monotypic, and because its strongly supported sister group (Davis et al. 2005), Putranjivaceae, possesses the intron. Only one member of digeneric Elatinaceae was sampled and it is unclear if this is an isolated loss or characterizes the entire small family (samples of *Bergia*, the second genus in the family failed to amplify); members of the strongly supported sister family, Malpighiaceae (Davis and Chase 2004), possess the intron. Given the isolated taxonomic positions of these losses and our uneven sampling outside of Euphorbiaceae, additional intron losses are likely not only in Malpighiales but also throughout angiosperms. We expect densely sampled future studies among other groups of angiosperms to uncover similar patterns of sporadic loss.

Phylogenetic analyses of the *atpF* coding sequence recovered trees that are largely congruent with established angiosperm relationships (Fig. 4) and with surprisingly high levels of support. This indicates that the molecular evolution of intronless alleles has not dramatically changed with the loss of the intron. Such changes might occur if some of the data represented paralogs in a new genetic environment (i.e., transferred to the nucleus or mitochondrion) and can also negatively impact phylogenetic reconstruction (Bowe and dePamphilis 1996), which does not appear to be the case here. Higher-level relationships among families of Malpighiales are poorly resolved even with four or more genes (e.g., Davis et al. 2005) and supported relationships would not be expected with a single short gene such as the coding region of *atpF*. The unsupported paraphyletic relationship of Phyllanthaceae and with its sister group Picrodendraceae is probably an artifact of short branches and sparse taxon sampling.

In conclusion, this is the first published chloroplast genome sequence of a member of the family Euphorbiaceae. In order to achieve efficient homologous recombination in chloroplast transformation it is important for vectors to have 100% sequence identity of flanking sequences. In addition, having endogenous regulatory elements in the vector is highly desirable to hyper-express foreign genes in the chloroplast. The chloroplast genome sequence provides the necessary information for the design and construction of an efficient chloroplast vector for this important crop species. The loss of *atpF* intron among members of Euphorbiaceae and other Malpighiales provides a new structural change to define clades in this large angiosperm group. Furthermore, a strong association between C-to-T changes at nucleotide position 92 and the loss of the intron suggests that recombination between an edited mRNA and the *atpF* gene may be a possible mechanism for the intron loss.

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Appendix

Taxa and voucher information for 250 accessions (251 taxa) of Malpighiales and related families examined for *atpF* intron status. Taxa sequenced for *atpF* are followed by the GenBank accession number. Herbarium acronyms follow Index Herbariorum (<http://sciweb.nybg.org/science2/IndexHerbariorum.asp>).

Intron present

(i) Malpighiales

Achariaceae: *Acharia tragodes* Thunb., Cloete s.n. (BOL); *Erythrospermum phytolaccoides* Gardn., Chase 1277 (K); *Hydnocarpus* sp., Chase 1301 (K); *Kiggelaria africana* L., Chase 5607 (K). **Balanopaceae:** *Balanops vieillardii* Baill., Chase 1816 (K). **Bonnetiaceae:** *Bonnetia sessilis* Benth., Berry s.n. 25.7.98 (MO). **Caryocaraceae:** *Anthodiscus amazonicus* Gleason & A. C. Sm., van der Werff 13889 (MO); *Caryocar glabrum* Pers., Mori 22997 (NY). **Clusiaceae s.l.:** *Mammea siamensis* T. Anders., Chase 1216 (K); “*Mesua*” sp. (probably *Kayea* sp.), Coode 7884 (K); *Montrouzieria cauliflora* Planch. & Triana, Lowry 5601 (MO); *Pentapthalangium latissimum* Lauterb., Chase 2100 (K); *Rhedia macrophylla* Planch. & Triana, Chase 1219 (K). **Ctenolophonaceae:** *Ctenolophon englerianus* Mildbr., McPherson 16911 (MO). **Dichapetalaceae:** *Dichapetalum* sp., Chase 624 (K?). **Euphorbiaceae s.s.:**

Acalypha californica Benth., Levin 2192 (SD) GenBank EU293932; *Adriana tomentosa* (Thunb.) Gaudich. var. *tomentosa*, Cameron s.n. (US); *Aleurites moluccana* (L.) Willd., Wurdack s.n. (US); *Amperea xiphoclada* (Sieber ex Spreng.) Druce, Chase 1936 (K); *Anthostema madagascariense* Baill., Hoffmann 291 (K); *Aparisthium cordatum* (A. Juss.) Baill., Bell et al. 93-60 (US); *Astraea lobatum* Klotzsch, Steinmann 2024 (RSA); *Baloghia inophylla* (G. Forst.) P. S. Green, Chase 3062 (K); *Bernardia myricifolia* (Scheele) S. Watson, McGill & Sundell 5459 (K); *Bertya rosmarinifolia* Planch., Chase 1938 (K); *Beyeria leschenaultii* (DC.) Baill., Chase 1939 (K); *Blumeodendron* sp., Chase 1252 (K); *Calycopeplus casuarinoides* L.S. Sm., Steinmann 1407 (RSA); *Caperonia palustris* (L.) A. St.-Hil., Wurdack D073 (US); *Cavacoa aurea* (Cavaco) J. Léonard, Luke & Robertson 1922 (US); *Cephalomappa malloticarpa* J.J. Sm., M. Chase 1256 (K); *Chaetocarpus africanus* Pax, Gabon, White 1319 (MO); *Cladogynos orientalis* Zipp., Chamchanroon 2010 (L); *Claoxylon australe* Baill. ex Müll. Arg., Chase 1937 (K); *Clutia pulchella* L., Chase 5876 (K); *Cnesmone javanica* Blume, Huq 10189 (US); *Codiaeum variegatum* (L.) Blume, Wurdack D033 (US), GenBank EU293933; *Colliguaja integerrima* Gillies & Hook., Landrum 8234 (MO); *Conceveiba martiana* Baill., Bell 93-176 (US); *Croton alabamensis* E.A. Sm. ex Chapm. var. *alabamensis*, Wurdack D008 (US), GenBank EU293934 *Croton alabamensis* E.A. Smith ex Chapman var. *texensis* Ginzburg, Nesom 7850 (NY); *Croton betulinus* Vahl, Steinmann 2026 (RSA); *Croton chilensis* Müll. Arg., Wurdack D646 (US); *Croton conduplicatus* Kunth, Berry 5542 (MO); *Croton cuneatus* Klotzsch, Berry 7589 (PORT); *Croton elliotii* Chapm., Wurdack D455 (US); *Croton flavens* L., Steinmann 2015 (RSA); *Croton guianensis* Aubl., Berry 5535 (MO); *Croton martinianus* V.W. Steinm., Steinmann 606 (RSA); *Croton nanus* Gagnep., Poona 4256 (BKF); *Croton repens* Schldtl., Steinmann 1062 (RSA); *Croton setigerus* Hook., Hughey s.n. (US); *Croton socotranus* Balf. f., Wurdack D644 (US); *Croton speciosus* Müll. Arg., Berry 7590 (MO); *Croton suaveolens* Torr., Devender 96-257 (RSA); *Croton trinitatis* Millsp., Berry 7586 (MO); *Crotonogynopsis usambarica* Pax, Tanzania, Polhill et al. 5129 (K); *Dalechampia spathulata* (Scheidw.) Baill., Wurdack D010 (US); *Dichostemma glaucescens* Pierre, Reitsma 1285 (NY); *Ditaxis argothamnoides* (Bertol. ex Spreng.) Radcl.-Sm. & Govaerts, Wurdack D105 (US); *Ditrysinia fruticosa* (Bartram) Govaerts & Frodin, Wurdack D149 (US); *Ditta myricoides* Griseb., Cedeño s.n. (US); *Dodecastigma amazonicum* Ducke, Mori 22811 (US); *Endospermum moluccanum* (Teijsm. & Binn.) Kurz, Chase 1258 (K); *Enriquebeltrania crenatifolia* (Miranda) Rzed., Cabrera 10768 (NY); *Erythrococca* sp., Cameroon, Cheek s.n. (K); *Euphorbia epithymoides* L., Chase 102 (NCU); *Euphorbia mesembryanthemifolia* Jacq.,

- Wurdack D102 (US); *Euphorbia pulcherrima* Willd. ex Klotzsch cv. 'Brilliant Diamonds', Wurdack D084 (US); *Excoecaria agallocha* L., Motley & Cameron 2053 (NY); *Excoecaria cochinchinensis* Lour., Chase 1260 (K); *Garcia nutans* Vahl ex Rohr, Wurdack D051 (US); *Gitara venezolana* Pax & K. Hoffm., Bell et al. 94-312 (US); *Gymnanthes lucida* Sw., Wurdack D055 (US); *Hippomane mancinella* L., Wurdack D053 (US); *Homalanthus populneus* (Geiseler) Pax, Chase 1266 (K); *Hura crepitans* L., Wurdack D089 (US) GenBankEU293935; *Jatropha gossypifolia* L., Taylor 11763 (MO); *Jatropha integerrima* Jacq., Wurdack D047 (US); *Joannesia princeps* Vell., Chase 1262 (K); *Klaineanthus gaboniae* Pierre, White (ser. 2) 334 (MO); *Koiloceras bantamense* Hassk., Chase 1263 (K); *Lasiocroton bahamensis* Pax & K. Hoffm., Wurdack D058 (US); *Leucocroton microphyllus* (A. Rich.) Pax & K. Hoffm., HAJB 81915 (HAJB); *Mabea* sp., Bell et al. 94-30 (US); *Macaranga grandifolia* (Blanco) Merr., Wurdack D046 (US); *Mallotus japonicus* (L. f.) Müll. Arg., Wurdack D004 (US); *Mallotus philippensis* (Lam.) Müll. Arg., Wurdack D822 (US); *Manniophyton africanum* Müll. Arg., White 3366 (MO); *Maprounea guianensis* Aubl., Wurdack D332 (US); *Melanolepis multiglandulosa* Reichb. & Zoll., Motley 2493 (NY); *Micrococca capensis* (Baill.) Prain, Kurzweil 463/89 (K). *Monadenium guentheri* Pax, Chase 1007 (K); *Monotaxis grandiflora* Endl., Horn 2386 (DUKE); *Moultonianthus leembruggianus* (Boerl. & Koord.) Steenis, Challen et al. 3 (K); *Nealchornea yapurensis* Huber, Fine s.n. (US); *Neoboutonia mannii* Benth. & Hook. f., Fay 6701 (MO); *Neoscortechinia kingii* (Hook. f.) Pax & K. Hoffm., Chase 1265 (K) GenBank EU293936; *Neoshirakia japonica* (Siebold & Zucc.) Esser, Wurdack D005 (US); *Omphalea diandra* L., Chase 570 (K); *Ostodes paniculata* Blume, Chase 1267 (K); *Pachystroma longifolium* (Nees) I. M. Johnst., Prince s.n. (US); *Pausandra martinii* Baill., Berry 7466 (MO); *Pedilanthus tithymaloides* (L.) Poit., Wurdack D034 (US); *Pera bicolor* (Klotzsch) Müll. Arg., Gillespie 4300 (US), GenBank EU293937 *Pimelodendron zoanthogyne* J.J. Sm., Chase 1268 (K); *Plukenetia volubilis* L., Armbruster 38 (?) *Pogonophora schomburgkiana* Miers ex Benth., Larpin 1022 (US); *Pseudosenefeldera inclinata* (Müll. Arg.) Esser, Wurdack D385 (US); *Ricinocarpos tuberculatus* Müll. Arg., Chase 2164 (K); *Ricinodendron heudelotii* (Baill.) Heckel, Chase 1269 (K); *Ricinus communis* L., Wurdack D009 (US); *Seidelia triandra* (E. Mey.) Pax, Giess 13381 (MO); *Senefelderopsis croizatii* Steyerem., Berry s.n. (MO); *Spathiostemon javensis* Blume, Chase 1261 (K); *Stillingia sylvatica* L. subsp. *tenuis* (Small) D. J. Rogers, Wurdack D117 (US); *Strophoblachia fimbriicalyx* Boerl., Chase 1270 (K); *Sumbaviopsis albicans* (Blume) J.J. Sm., Chase 1271 (K); *Suregada glomerulata* (Blume) Baill., Chase 1272 (K); *Synadenium grantii* Hook. f., Wurdack D259 (US); *Syndyophyllum occidentale* (Airy Shaw) Welzen, Aik et al. SAN-111913 (L); *Tannodia cordifolia* (Baill.) Baill., Ralimanana 293 (K); *Tetrorchidium cf. macrophyllum* Müll. Arg., Bell et al. 93-204 (US), GenBank EU293938; *Tragia fallax* Müll. Arg., Bell et al. 94-378 (US); *Tragia urticifolia* Michx., Wurdack D074 (US); *Tragiella anomala* (Prain) Pax & K. Hoffm., Mwasumbi et al. 16202 (MO); *Trewia nudiflora* L., Chase 1273 (K); *Triadica sebifera* (L.) Small, Wurdack D059 (US); *Trigonostemon verrucosus* J. J. Sm., Chase 1274 (K); *Vernicia montana* Lour., Chase 2105 (K). **Euphroniaceae:** *Euphronia guianensis* (R. H. Schomb.) H. Hallier, Mori 23699 (NY). **Goupiaceae:** *Goupia glabra* Aubl., Prevost 3031 (CAY). **Humiriaceae:** *Humiria wurdackii* Cuatrec., Wurdack s.n. (US); *Sacoglottis gabonensis* Urb., Stone 3283 (MO); *Vantanea guianensis* Aubl., Pennington 13855 (K). **Hypericaceae:** *Cratoxylum formosum* (Jack) Benth & Hook. f. ex Dyer, Chase 1218 (K). **Irvingiaceae:** *Irvingia malayana* Oliv., Simpson 2638 (K?); *Klainedoxa gabonensis* Pierre, Bradley et al. 1092 (MO). **Ixonanthaceae:** *Cyrillopsis paraensis* Kuhlm., Hentrich 68 (NY); *Ochthocosmus longipedicellatus* Steyerem. & Luteyn, Berry 6561 (MO). **Lacistemataceae:** *Lacistema aggregatum* Rusby, Pennington et al. 583 (K); *Lozania pittieri* (Blake) L. B. Sm., Pennington et al. 584 (K). **Linaceae:** *Durandea pentagyna* K. Schum., Takeuchi 7103 (MO); *Linum perenne* L., Chase 111 (NCU); *Reinwardtia indica* Dumort., Chase 230 (NCU). **Malpighiaceae:** *Dicella nucifera* Chodat, Anderson 13607 (MICH); *Galphimia gracilis* Bartl., Adelson s.n. (MICH), GenBank EU293939. **Medusagynaceae:** *Medusagyne oppositifolia* Baker, Fay s.n. (K) [Kew 1981-2059]. **Ochnaceae s.s.:** *Elvasia calophyllea* DC., Amaral s.n. (K?); *Lophira lanceolata* Tiegh., Schmidt 1902 (MO); *Ochna multiflora* DC., Chase 229 (NCU). **Pandaceae:** *Galearia filiformis* (Blume) Boerl., Chase 1334 (K); *Microdesmis pierlotiana* J. Léonard, Gereau et al. 5654 (MO); *Panda oleosa* Pierre., Schmidt et al. 2048 (MO). **Phyllanthaceae:** *Amanoa strobilacea* Müll. Arg., McPherson 16826 (MO); *Apodiscus chevalieri* Hutch., Schmidt et al. 2094 (MO); *Aporusa frutescens* Blume, Chase 1251 (K); *Astrocasia neurocarpa* (Müll. Arg.) I. M. Johnst. ex Standl., Wurdack D743 (US); *Bischofia javanica* Blume, Levin 2200 (SD), GenBank EU293940; *Cleistanthus oblongifolius* (Roxb.) Müll. Arg., Chase 1257 (K); *Croizatia brevipetiolata* (Secco) Dorr, Dorr et al. 8555 (US), GenBank EU293941; *Didymocistus chrysadenius* Kuhlm., Gillespie et al. 4805 (US); *Discocarpus essequeboensis* Klotzsch, Hoffman 996 (US); *Gonatogyne brasiliensis* (Baill.) Müll. Arg., Cordeiro & Esteves 1384 (K); *Heywoodia lucens* Sim, Saufferer & Muchai SS-1544 (US); *Hieronyma oblonga* (Tul.) Müll. Arg., Bell 94-252 (US); *Hymenocardia acida* Tul., Walters et al. 897 (MO); *Lachnostylis bilocularis* R. A. Dyer, Kurzweil 83/88 (K); *Leptonema glabrum* (Leandri) Leandri, McPherson &

Rabenantoandro 18389 (MO); *Maesobotrya vermeulenii* (de Wild.) J. Léonard, Bradley et al. 1032 (MO); *Poranthera huegelii* Klotzsch, Spjut 7369 (US); *Richeria grandis* Vahl, Merello et al. 1714 (MO); *Sauropus racemosus* Beille, Soejarto et al. 10648 (NY); *Spondianthus preussii* Engl., Merello et al. 1661 (MO); *Zimmermania ovata* E.A. Bruce, Faden s.n. (US). **Picrodendraceae:** *Androstachys johnsonii* Prain, Chase 1904 (K); *Hyaenanche globosa* (Gaertn.) Lamb. & Vahl, Chase 1445 (K); *Picrodendron baccatum* Krug & Urb. ex Urban, Wurdack D050; *Podocalyx loranthoides* Klotzsch, Berry & Aymard 7226 (MO), GenBank EU293955 (partial sequence not used in phylogenetic analyses); *Tetracoccus dioicus* Parry, Levin 2202 (DUKE), GenBank EU293942. **Podostemaceae:** *Marathrum* c.f. *oxycarpum* Tul., Gabriel da Cachoeira 9/1996 (AM); *Podostemum ceratophyllum* Michx., Horn & Wurdack s.n. (DUKE). **Putranjivaceae:** *Drypetes capillipes* Pax & K. Hoffm., Harris 4884 (K); *Drypetes fallax* Pax & K. Hoffm., Harris 4892 (K); *Drypetes macrostigma* J.J. Sm., Chase 1259 (K); *Putranjiva roxburghii* Wall., Wurdack D057 (US), GenBank EU293956 (partial sequence not used in phylogenetic analyses). **Quiinaceae:** *Quiina pteridophylla* (Radlk.) Pires, Pires s.n. (CPATU) *Touroulia guianensis* Aubl., Pires (CPATU). **Rhizophoraceae s.s.:** *Bruguiera gymnorhiza* Lam., Chase 12838 (K); *Carallia brachiata* (Lour.) Merr., Chase 2151 (K); *Crossostylis multiflora* Brongn. & Gris ex Pancher & Sebert, Lowry 5686 (MO); *Paradrypetes subintegriifolia* G. A. Levin, Acevedo-Rdgz. & Cedeño 7560 (US); *Cassipourea lanceolata* Tul., Schatz 3689 (MO). **Salicaceae s.l.:** *Abatia parviflora* Ruiz & Pav., Pennington 676 (K); *Casearia nitida* Jacq., FTG 72496; *Flacourtia jangomas* Steud., Chase 2150 (K); *Ilesia polycarpa* Maxim., Wurdack D22 (US); *Poliathyrsis sinensis* Oliver, Wurdack D029 (US); *Scyphostegia borneensis* Stapf, Beaman 911 (BH). **Trigoniaceae:** *Trigonistrum hypoleucum* Miq., Og B128 (L). **Violaceae:** *Hybanthus concolor* Spreng., Wurdack D148 (US); *Hymenanchera alpina* Oliv., Chase 501 (K); *Rinorea bengalensis* Kuntze, Chase 2148 (K).

(ii) Other related rosids (Celastrales, Oxalidales + Huaceae)

Brunelliaceae: *Brunellia acutangula* Humb. & Bonpl., Stergios 20646 (US), GenBank EU293943. **Celastraceae:** *Brexia madagascariensis* Thouars, Wurdack D764 (US); *Maytenus senegalensis* (Lam.) Exell, Collenette 4/93 (K); *Plagiopteron suaveolens* Griff., Chase 1335 (K); *Siphonodon celastrineus* Griff., Chase 2097 (K); *Stackhousia minima* Hook. f., Molloy s.n. (CHR); *Tripterygium regelii* Sprague & Takeda, Chase 1003 (K). **Cephalotaceae:** *Cephalotus folicularis* Labill., Chase 147 (NCU). **Connaraceae:** *Rourea minor* Leenh., Chase 1221 (K). **Cunoniaceae:** *Eucryphia milliganii* Hook. f., Chase 2528 (K). **Elaeocarpaceae:**

Crinodendron hookerianum Gay, Chase 909 (K); *Sloanea* sp., Chase 343 (NCU). **Huaceae:** *Afrostryax* sp., Cheek 5007 (K). **Lepidobotryaceae:** *Ruptiliocarpon caracolito* Hammel & Zamora, Hammel 19102 (MO). **Oxalidaceae:** *Averrhoa carambola* L., Chase 214 (NCU). **Parnassiaceae:** *Parnassia grandifolia* DC., Wurdack D795 (US), GenBank EU293944.

Intron absent

(i) Malpighiales

Elatinaceae: *Elatine hexandra* DC., Chase 2978 (K), GenBank EU293945. **Euphorbiaceae s.s.:** *Cnidocolus urens* (L.) Arthur var. *stimulosus* (Michx.) Govaerts, Wurdack D002 (US); *Elateriospermum tapos* Blume, Soepadmo & Suhaimi s193 (NY), GenBank EU293946; *Glycydendron amazonicum* Ducke, Gillespie et al. 4546 (US); *Glycydendron* cf. *amazonicum* Ducke, Mori et al. 23265 (US); *Hevea* sp. [cf. *pauciflora* (Spruce ex Benth.) Müll. Arg.], Gillespie 4272 (US), GenBank EU293947; *Manihot esculenta* subsp. *esculenta* Crantz, EU117376; *Manihot foetida* Pohl, Olsen s.n.; *Manihot grahamii* Hook., Wurdack s.n. (US); *Manihot pauciflora* Brandegee, Villasenor 1248 (NY); *Manihot walkerae* Croizat, Olsen s.n.; *Micrandra inundata* P. E. Berry & A. Wiedenhoft, Berry 6350 (MO). **Lophopyxidaceae:** *Lophopyxis maingayi* Hook. f., Adelbai P-10203 (US), GenBank EU293948. **Malesherbiaceae:** *Malesherbia weberbaueri* Gilg, Weigend 2000/365 (NY), GenBank EU293949. **Passifloraceae s.s.:** *Paropsia madagascariensis* (Baill.) H. Perrier, Zyhra 949 (WIS); *Passiflora coccinea* Aubl., Chase 2475 (K), GenBank EU293950. **Phyllanthaceae:** *Breynia disticha* J. R. Forst. & G. Forst. cv. 'Rosea Picta', Wurdack D012 (US); *Flueggea virosa* (Roxb. ex Willd.) Voigt subsp. *virosa*, Wurdack D101 (US); *Glochidion puberum* (L.) Hutch., Wurdack D003 (US); *Leptopus colchicus* (Fisch. & C. A. Mey. ex Boiss.) Pojark., Wurdack D778 (US), GenBank EU293951; *Leptopus esquirolii* (H. Lév.) P. T. Li, 1980 Sino-American Bot. Exped. 1678 (US); *Margaritaria tetracocca* (Baill.) G. L. Webster, Wurdack D044 (US); *Phyllanthus epiphyllanthus* L., Wurdack D056 (US), GenBank EU293952; *Phyllanthus fluitans* Benth. ex Müll. Arg., Wurdack D761 (US); *Phyllanthus juglandifolius* Willd., Wurdack D759 (US); *Phyllanthus nutans* Sw., Wurdack D762 (US); *Reverchonia arenaria* A. Gray, Atwood 17245 (NY); *Savia bahamensis* Britton, Wurdack D048 (US). **Picrodendraceae:** *Austrobuxus megacarpus* P. I. Forster, Forster 21239 (BRI), GenBank EU293953; *Choriceras majus* Airy Shaw, Forster 21230 (BRI); *Dissiliaria muelleri* Baill., Forster 14629 (BRI); *Whyanbeelia terrae-reginae* Airy Shaw & B. Hyland, Forster 21231 (BRI). **Turneraceae:** *Tricliceras longipedunculatum* (Mast.) R. Fern., Kanji et al. 304 (NY); *Turnera ulmifolia* L., Wurdack s.n. (US), GenBank EU293954.

(ii) Other related rosids (Celastrales, Oxalidales + Hua-ceae): none noted.

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