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An intron loss in the chloroplast gene *rpo*C1 supports a monophyletic origin for the subfamily Cactoideae of the Cactaceae

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Abstract The deletion of an approximately 700-bp intron in the chloroplast-encoded gene *rpo*C1 was shown in 21 representative species of the subfamily Cactoideae of the angiosperm family Cactaceae. Members of the subfamilies Pereskioideae and Opuntioideae were found to possess the intron, as did members of the related families Aizoaceae, Basellaceae, Didiereaceae, Phytolaccaceae, and Portulacaceae. These results support a monophyletic origin for the most-speciose subfamily of the cactus family, and represent a first report of the loss of this intron in dicots.

Key words Cactaceae · Chloroplast · DNA · Ribosomal polymerase · *rpo*C1 intron

Introduction

The use of structural rearrangements within the chloroplast genome has been applied to infer phylogenetic relatedness among various plant taxa (Palmer 1987; Palmer et al. 1988; Olmstead and Palmer 1994). In a review of the phylogenetic use of cpDNA inversions, deletions, and insertions, Downie and Palmer (1992) cite multiple cases where relatively rare rearrangements in the chloroplast genome provide very robust phylogenetic characters from which evolutionary relationships may be deduced. For example, simple inversions within the chloroplast genome have been observed in certain lineages within the Asteraceae (Jansen and Palmer 1987), Fabaceae (Palmer et al. 1988; Bruneau et al. 1990), and Poaceae (Doyle et al. 1992), which are particularly good indicators of monophyly due to their relatively rare occurrence. Similarly, the loss of chloroplast genes, or their introns, may be used to identify phylogenetically related organisms which share these derived structural variations in their plastid genomes. For example, the loss of the *rpl2* intron (Zurawski et al. 1984; Downie et al. 1991) has provided insights into the evolutionary relationships within and among various families of angiosperms, as well as documenting parallel cpDNA changes in evolutionarily distinct lineages.

Phylogenetic studies using the *rpo*C1–C2 operon have been presented (Liston 1992) where PCR-generated fragments have been restriction-mapped to yield evolutionarily informative site changes in this region. In a survey of several cpDNA structural rearrangements by Katayama and Ogihara (1993), loss of the *rpo*C1 intron was confirmed in a number of grasses [in addition to the reports from maize (Igloi et al. 1990) and from rice (Shimada et al. 1990)]; however, the intron was found to be present in other monocots. Furthermore, research on the plastid gene *rpo*C2 by Cummings et al. (1994) has provided evidence that slipped-strand mispairing most likely gave rise to the additional repetitive coding sequence detected in this gene within certain grass lineages. From these studies it is clear that the *rpo*C1/C2 region within the chloroplast genome may be subject to changes both at the nucleotide level, and those of a larger scale which involve deletions and insertions.

The present study was conducted as part of a broader investigation which assessed molecular variation in cpDNA within the cactus family (Cactaceae). By examining the phylogenetic distribution of cpDNA structural rearrangements, and by comparatively investigating gene-sequence variation, the inferred patterns of evolutionary divergence (phylogeny) within the cactus family may be determined from a morphologically independent source of data. The cacti are a family of approximately 1500–1600 species of dicotyledonous angiosperms, found in xeric to tropical habitats throughout North and South America. Three major

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lineages have been identified within the family (Gibson and Nobel 1986; Barthlott and Hunt 1993) based primarily upon vegetative and floral morphology. These are recognized taxonomically at the subfamilial rank: the subfamily Pereskioideae (18 spp.) includes leafy, nearly succulent shrubs, vines, or small trees; the subfamily Opuntioideae (approximately 260 spp.), glochid-bearing stem succulents, which include the prickly pears, chollas, and related genera, are low or scandent leafless (or seasonally leafy) shrubs, geophytes, and small trees; and the subfamily Cactoideae (approximately 1250 spp.), a stem-succulent group, has extremely diverse morphologies including small and large barrel forms, pendant epiphytes, climbing lianas, geophytes, shrubs, and large trees.

The diversity of morphological structures, together with a propensity for parallel evolution within the cactus family, has confounded its phylogenetic interpretation. Hypotheses regarding the monophyletic origins of each of the subfamilial groups have been proposed based primarily upon morphological interpretation, but the monophyly of these infrafamilial lineages has not been adequately confirmed. In particular, the extensive morphological diversity of the subfamily Cactoideae has necessitated its division into smaller groups at the tribal level (Buxbaum 1958), but the issue of the monophyly of this subfamily has yet to be adequately addressed or substantiated with morphological or other evidence (Gibson and Nobel 1986; Barthlott and Hunt 1993).

Here we report loss of the intron of the chloroplast gene *rpo*C1 within the subfamily Cactoideae, the most speciose subfamily of the dicotyledonous family Cactaceae. Prior to this report, the loss of this intron has only been reported in the grass family (Poaceae) in the monocots.

Materials and methods

Plant materials and DNA isolation. Genomic DNA samples were isolated from various Cactaceae and outgroup taxa as included in Table 1; these taxa were chosen to be representative of the major lineages within the Cactaceae as currently recognized (Gibson and Nobel 1986; Barthlott and Hunt 1993). Outgroup taxa were included to assess the phylogenetic distribution of the intron loss in related Caryophyllalean families. DNA samples were isolated using a modified organelle-pellet isolation method (Wallace, unpublished) where mucilaginous tissues are ground in a blender with 30–50 volumes of 0.35 M sorbitol buffer (0.35 M sorbitol; 50 mM TRIS-HCl, pH 8; 5 mM EDTA; 5 mM 2-mercaptoethanol; 0.1% w/v bovine serum albumin), filtered (Miracloth, Calbiochem), and the organelles pelleted at 2500 rpm in a Sorval GSA rotor at 4 °C for 45 min; the pellets remaining after the supernatant is decanted are resuspended in 2 x CTAB buffer (Doyle and Doyle 1987) at 60 °C for about 1 h. Isopropanol-precipitated DNAs were then further purified in CsCl/ethidium bromide ultracentrifuge gradients, followed by dialysis against TE (10 mM TRIS, pH 8, 1 mM EDTA).

DNA characterization – PCR amplification of rpoC1 and rpoC2 Genes. For each of the taxa examined, 5 µl (< 10 ng) of genomic DNA were used in 100 µl polymerase chain reactions, employing the reaction buffer and *Taq* polymerase (Amplitaq, Perkin-Elmer/Cetus), following the manufacturer's protocols. The primers for the *rpo*C genes were generously supplied by Aaron Liston, Oregon State University. Primers *rpo*C1 195 [forward; 5′-AAGCGGAATTTGTGCTTGTG-3′, (Liston 1992)] and *rpo*C1 2505 [reverse, 5′-TATGACCAACAGTGGTTCG-3′, (Asmussen and Liston, unpublished)] were used. Thermal cycling for amplification was: 1-min initial melting at 94 °C; 35 cycles of 94 °C for 1 min; 50 °C for 2 min, and 72 °C for 3 min, followed by a 7-min extension step at 72 °C. PCR products for all taxa were compared in 1% agarose gels for amplification quality/quantity and product size; the PCR products separated in the gels were then Southern (1975) transferred to nylon filters (Zetabind; AMF CUNO) after depurination in 0.25 M HCl (11 min) and denaturation in 0.4 M NaOH.

Preparation of rpoC1 exon- and intron-specific hybridization probes. A 395-bp *Hin*dIII-*Xba*I DNA fragment was isolated from a cloned cpDNA library from tobacco (Shinozaki et al. 1986) fragment *Bam*12a, corresponding to coordinates 21 947 to 25 128 (obtained from J. Palmer, Indiana University). This *Hin*dIII-*Xba*I fragment has endpoints of 23 607 and 23 212, respectively, and represents an internal (395-bp) region of the 740-bp intron of *rpo*C1 in tobacco. The fragment was isolated in low-melting-point agarose (SeaPlaque, FMC Corporation), and directly nick-translated (BRL NT system) for use as an intron-specific DNA probe. From the same chloroplast DNA fragment (*Bam*12a), the 964-bp *Bam*HI-*Xba*I fragment (corresponding to cpDNA coordinates 21 947 and 22 911, respectively) was used as an exon-specific probe (*rpo*C1 exon 2). Hybridization for both probes was conducted for 16–20 h at 63 °C, in 4 x SSC, 0.5% SDS, 2.5 x Denhardt's solution, 10 mM EDTA, and 25 µg/ml of carrier DNA (Sambrook et al. 1989).

Sequencing the exon1-exon2 junction of the rpoC1 gene The approximately 1.8-kb PCR product from *Ferocactus flavovirens* was used as a double-stranded template from which a single stranded sequencing template was made, using an asymmetric amplification (Gyllensten 1989) employing the *rpo*C1 2505 reverse primer only. The *rpo*C1 195 forward primer was used in sequencing reactions. The sequence determined for portions of *rpo*C1 exon 1 and *rpo*C1 exon 2 were compared to published sequences of tobacco (Shinozaki et al. 1986), maize (Igloi et al. 1990), and rice (Shimada et al. 1990).

Results

Comparison of PCR product sizes

The difference of approximately 700 bp in the observed (1.8-kb) versus expected (2.5-kb) PCR amplicon size for members of the subfamily Cactoideae (Fig. 1 A) was hypothesized to have been due to the loss of the *rpo*C1 intron, as has been reported in grasses (Igloi et al. 1990; Shimada et al. 1990). PCR products for members of the subfamilies Pereskioideae and Opuntioideae were found to produce the expected amplicon size of about 2.5-kb, as did the outgroup taxa from the Aizoaceae, Didiereaceae, Phytolaccaceae, and Portulacaceae (Fig. 2). Small amounts of amplification products corresponding to the 2.5-kb amplicon in four cactoid taxa (*Arrojadoa*, *Lepismium*, *Rhipsalis*, and *Espostoa*) were detected (Fig. 1 A). The possibility of hetroplasmy is extremely unlikely between these cactoid taxa with other members of subfamilies Pereskioideae and Opuntioideae. Since these fragments did hybridize weakly to

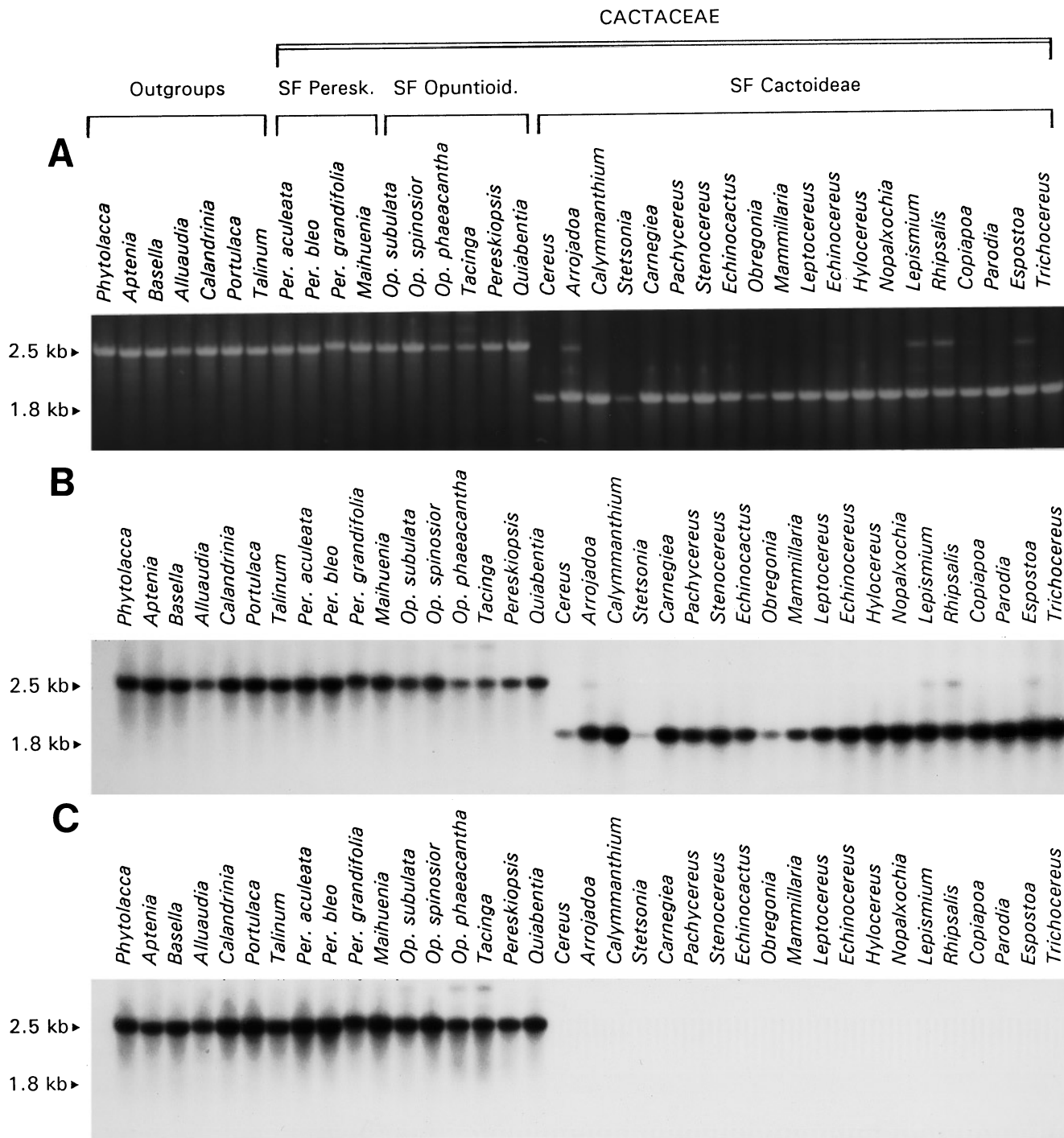

Fig. 1 **A** PCR fragments generated using primers 195F and 2505R for the chloroplast gene *rpo*C1. Fragments obtained from amplifications of members of the Cactaceae subfamily Cactoideae are approximately 700 bp smaller than similar amplifications from members of the subfamilies Pereskioideae and Opuntioideae, and outgroup taxa. **B** autoradiogram of PCR products hybridized to a 964-bp exon-specific probe for *rpo*C1 exon 2, showing sequence homology in both amplicon size classes (2.5 and 1.8 kb). **C** autoradiogram of same filter as above, hybridized to a 395-bp intron-specific probe. Lack of hybridization to amplicons from the subfamily Cactoideae confirms the loss of the *rpo*C1 intron

the exon probe (Fig. 1 B), but were not detected with the the intron-specific probe (Fig. 1 C), we conclude that these spurious PCR products are due to small amounts of DNA contaminants in these four samples.

Hybridization to exon- and intron-specific probes

The PCR fragments transferred to nylon membranes were examined for homology to the *rpo*C1 exon and

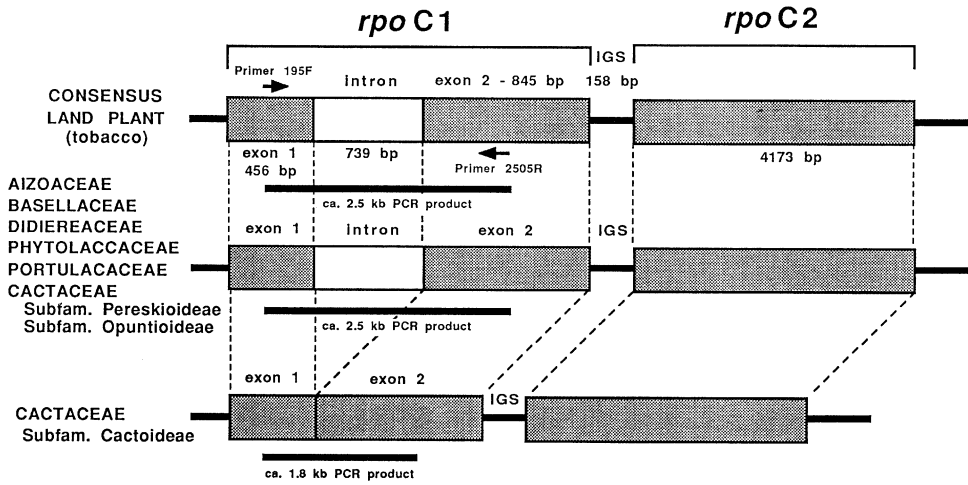

Fig. 2 Diagram of the *rpo*C1/C2 operon, summarizing the taxonomic distribution of the *rpo*C1 intron in all taxa investigated relative to the consensus land plant (tobacco) gene organization. Twenty representative species from throughout the subfamily Cactoideae share the loss of this intron; the remaining cactus subfamilies and related outgroup taxa have the intron in their cpDNA genomes

intron regions using specific probes as outlined above. All PCR products hybridized with the exon-specific probe for *rpo*C1 exon 2 (Fig. 1 B) which confirmed that *rpo*C1 was amplified for both size classes of amplicons. Using the same filter with the 395-bp intron-specific probe, hybridization was observed only in PCR fragments of approximately 2.5 kb, which included all related outgroup taxa, and members of subfamilies Pereskioideae and Opuntioideae of the Cactaceae. No hybridization was observed in any of the 1.8-kb PCR fragments obtained from amplifications of *rpo*C1 in members of the Cactaceae subfamily Cactoideae (Fig. 1 C), providing further evidence that the intron was lost in these species.

Sequence of *rpo*C1 exon 1-exon 2 junction

To confirm the intron loss inferred from the PCR-product size differences and lack of hybridization with the intron-specific probe, the junction between exon 1 and exon 2 was sequenced (Fig. 3) in a representative species from the subfamily Cactoideae. The *rpo*C1 intron (740 bp) reported in tobacco (Shinozaki et al. 1986) was found to be completely lost in *F. flavovirens*, similar to the intron loss reported in maize (Igloi et al. 1990) and rice (Shimada et al. 1990). Since all other samples from the subfamily Cactoideae show the same PCR fragment size and do not hybridize to the intron-specific probe, we conclude that the intron has been lost in all examined samples from the subfamily Cactoideae.

Discussion

Our survey of the Cactaceae for variation within the *rpo*C1/C2 operon revealed a significant structural change in one subfamily of the cacti, the Cactoideae.

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Nicotiana 5'...†AACAATGCGGGGTCGAATTTGTTGATTCTCGGATACGAAGGTATCAAATGGGCTA
Ferocactus  ... AATAATGCGGAGTCGAATTTGTTAATTCTCGAGTACGAAGGTATCAAATGGGTTA

Nicotiana  TATCAAACTCGCATGCCCAGTAACCCATGTGTGGTATTTAAAACGTCTTCCTAGTTATAT
Ferocactus TATAAAATTGGCATGCCCAGTAACTCACGTGTGGTATTTGAAACGTCTTCCTAGTTATAT

                                                          exon 1 end ↓
Nicotiana  TGCG-AATCTTTTAGATAAACCTCTTAAAGAATTAGAAGGCCTAGTATACTGCGATGTGT
Ferocactus CGCGAATTTTTTGGAATAAACCTCTCAAAGAATTATAGGGCCTAGTATACTGCGAT----

Nicotiana  GATTTGATCGAAATTCTGATTTTACAGACTCGGAAGGAGAAACTGTCATCCCAGTCAATC
Ferocactus ------------------------------------------------------------

Nicotiana  CAATTGGGATGCCCTTGTAGTCAATACTCCCAAATAAAAAGGGAATTGATCTATGGTCGA
Ferocactus ------------------------------------------------------------

Nicotiana  TTTCGTAACAAATCAAATAAAGAGGAATTTCTATTTGTACCTCGTAAAAAAAGCTTTTTA
Ferocactus ------------------------------------------------------------

Nicotiana  TTTTGTAGAATGAAACATTTCCTTTCTTTTCGTGGAAAGAAATTATGTTCAAGTAAGCAA
Ferocactus ------------------------------------------------------------

Nicotiana  ATATGTCATGGTTCTAGGAGTCTATCTATCGCGTATAGACTTTAAGGGCGTGGTGGCCTA
Ferocactus ------------------------------------------------------------

Nicotiana  ACCATCGAGGTGAAGTCGGGACCTAAAAGATCGAATCGAACAATATATAGACAAGTAAAT
Ferocactus ------------------------------------------------------------

Nicotiana  CCCTTATGGGTTCCAAGGTATTCCTTTAATTATTAAGAGTTAAGGGATTTATCATTCAAA
Ferocactus ------------------------------------------------------------

Nicotiana  GGGAAGTAGACTACTCAAGAATTTTCTATTTTATTTGTTGGAATTGAATTTGAATAAAGA
Ferocactus ------------------------------------------------------------

Nicotiana  ATTAATAGAAGACGGAATCACTGAAATCTTGTTTCGTCTTCACTGGGAACTTGAGTAAGG
Ferocactus ------------------------------------------------------------

Nicotiana  AGTAGACCTTTTTATTTTGGGATTTTCTAGAGTTTGAAAACGAGAACTCCTTTCCTTATT
Ferocactus ------------------------------------------------------------

Nicotiana  TGGTGTACCTACTTGAGCCGGATGAAAGGAAACTTTCACGTCCGATTTTGAAGGGGGGGA
Ferocactus ------------------------------------------------------------

                                  Exon 2→
Nicotiana  GATCCTATAGAATCCTATCCCAATTTTTCTTTTGCTAGGCCCATAACTAAAAAGCCCACT
Ferocactus ------------------------TTTTCATTTGCTAGGCCCATAGTGAAAAAAGCCAGC

Nicotiana  TTCTTACGATTACGAGGTTTATTCGAATATGAAATCCAATCTTGGAAATACAGCATCCCA
Ferocactus TTTTTACGATTACGAGGTTTATTCGAATATAAAATCCAATCCTGGAAATACAGCATCCGA

Nicotiana  CTTTTTTTTACTACCCAGGGTTTCGATACATTTCGCAATCGAGAAATC......3'
Ferocactus TCTTTTTTTACTACCCAAGGTTTTGATACATTTCGAAATAGGGAAATC......
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Fig. 3 Nucleotide sequence determined for the *rpo*C1 3′ exon 1 and 5′ *rpo*C1 exon 2 junction in *F. flavovirens*, a member of the subfamily Cactoideae, aligned to a homologous sequence in tobacco, confirming loss of the intron. The sequence from tobacco was obtained from Shinozaki et al. (1986); the nucleotide position marked by † indicates position 24 004 of the tobacco genome; * indicates position 22 951. Gaps (deleted nucleotides) are represented with a dash (-)

Loss of the *rpo*C1 intron was confirmed by differences in PCR amplicon size, non-hybridization to an intron-specific cpDNA probe, and by demonstrating a conserved nucleotide sequence for relevant portions of exons

1 and 2 of *rpo*C1 and the loss of the intron sequence (Fig. 3) at their junction, as has been previously reported in maize (Igloi et al. 1990) and rice (Shimada et al. 1990).The sequence determined for this junction of *rpo*C1 3′ exon 1 and 5′ exon 2 from *F. flavovirens* showed that there were no remaining nucleotides from the intron (complete deletion), and that exon 2 continues to read in-frame with exon 1, which is the same condition reported in rice and maize. This represents a parallel loss of the *rpo*C1 intron in grasses (Katayama and Ogihara 1993) and in one subfamily of the cacti within the dicots. Further surveys of various cpDNA genomes may disclose additional parallel losses of this and other cpDNA introns; such a survey is presently being conducted (J. Palmer, personal communication).

Table 1 Plant specimens used to examine intron loss in *rpo*C1. Absence of the *rpo*C1 intron confirmed by PCR product size, hybridization to an intron-specific cpDNA probe, and sequencing through *rpo*C1 3′ exon 1 into 5′ exon 2 of *Ferocactus flavovirens*. + = intron present; − = intron absent

Taxon	Group[a]	Source[b]	*rpo*C1 intron present
Related Families			
Phytolacca americana	Phytolaccaceae	Wallace 1989-CT-011	+
Aptenia coridfolia	Aizoaceae	Kirstenbosch Bot. Garden s.n.	+
Basella alba	Basellaceae	ISU Botany Dept. GH s.n.	+
Alluaudia procera	Didiereaceae	Univ. Mass. Greenhouse s.n.	+
Calandrinia grandiflora	Portulacaceae	Bot. Garten Jena 3307	+
Talinum paniculatum	Portulacaceae	Smith College BG 746	+
Portulaca oleracea	Portulacaceae	Wallace 1989-CT-012	+
Cactaceae			
Subfamily Pereskioideae			
Pereskia aculeata	Pereskia	BERL 249–02–85–30	+
Pereskia bleo	Pereskia	BERL 277–01–80–80	+
Pereskia grandifolia	Pereskia	BERL 166–62–83–10	+
Maihuenia poeppigi	Maihuenia	F. Kattermann FK-278	+
Subfamily Opuntioideae			
Opuntia subulata	Austrocylindropuntia	Univ. Connecticut GH	+
Opuntia spinosior	Cylindropuntia	D. Pinkava 14308	+
Opuntia phaeacantha	Platyopuntia	C. Christy 516	+
Tacinga funalis	Tacinga	KEW 100–74–01111	+
Pereskiopsis porteri	Pereskiopsis	BERL 169–03–84–30	+
Quiabentia verticillata	Quiabentia	BERL 236–10–85–20	+
Subfamily Cactoideae			
Cereus aethiops	Tribe Cereeae	HNT 41779	−
Arrojadoa rhodacantha	Tribe Cereeae	Univ. Connecticut GH s.n.	−
Calymmanthium substerile	Tribe Browningieae	HNT 46555	−
Stetsonia coryne	Tribe Browningieae	HNT 9620	−
Carnegiea gigantea	Tribe Pachycereeae	DES s.n.	−
Pachycereus marginatus	Tribe Pachycereeae	HNT 'Bed 2N'	−
Stenocereus griseus	Tribe Pachycereeae	DES 1953–4041–0101	−
Echinocactus grusonii	Tribe Cacteae	Univ. Connecticut GH s.n.	−
Ferocactus flavovirens	Tribe Cacteae	H. Cota 8051	−
Obregonia denegrii	Tribe Cacteae	Arid Lands Greenhouses s.n.	−
Mammillaria voburnensis	Tribe Cacteae	L. Lippold s.n.	−
Leptocereus quadricostatus	Tribe Echinocereeae	R. Ross s.n.	−
Echinocereus viridiflorus	Tribe Echinocereeae	Wallace 1992-CO-004	−
Hylocereus undatus	Tribe Hylocereeae	HNT 39872	−
Nopalxochia horrichii	Tribe Hylocereeae	HNT 36756	−
Lepismium warmingianum	Tribe Rhipsalideae	BONN 04643	−
Rhipsalis heteroclada	Tribe Rhipsalideae	BONN 04551	−
Copiapoa coquimbana	Tribe Notocacteae	F. Kattermann FK-83	−
Parodia mairanana	Tribe Notocacteae	HNT s.n.	−
Espostoa blossfeldiorum	Tribe Trichocereeae	DES 1967–8766–0101	−
Trichocereus pasacana	Tribe Trichocereeae	DES 1987–0477–2119	−

[a] For related familes, sensu Cronquist (1981); for Cactaceae, sensu Barthlott and Hunt (1993)
[b] Source – abbreviations: BERL, Botanisches Garten, Berlin-Dahlem, Germany; BONN, Univ. Bonn Botanisches Garten, Germany; DES, Desert Botanical Garden, Phoenix, Arizona; HNT, Huntington Botanical Garden, San Marino, Calif.; KEW, Royal Bot. Gardens, Richmond, England; s.n. = no accession number

This synapomorphic deletion of a chloroplast sequence identifies the most-speciose subfamily of the cacti (Gibson and Nobel 1986; Barthlott and Hunt 1993) as a monophyletic lineage. Prior to these results, there existed no anatomical or other morphological character which clearly supported the monophyly of the subfamily Cactoideae. The evolution of this subfamily has previously been assumed to be monophyletic since it lacked the specialized areolar spines (glochids), characteristic pollen structure, and funicularly arillate seed found in the subfamily Opuntioideae, and also was significantly more specialized than the small subfamily Pereskioideae. This condition of "monophyly by exclusion" is phylogenetically unacceptable, and data has yet to be presented which could rule out polyphyly for subfamily Cactoideae. The loss of the *rpo*C1 intron in members of subfamily Cactoideae provides a very significant molecular character which confirms a monophyletic origin for this diverse group of cacti, and refutes the possibility of polyphyly for this subfamily.

These *rpo*C1 intron-loss data are consistent with other molecular data derived from comparative gene-sequencing studies of the same taxa included in this investigation (Table 1) plus additional taxa from throughout the Cactaceae. Phylogenetic analyses of plastid gene-sequence variation for *rbc*L (Wallace, Cota and Hills, in preparation) and for *ndh*F (Cota and Wallace, unpublished data) also place members of the subfamily Cactoideae within a monophyletic lineage.

The loss of the *rpo*C1 intron represents a relatively rare evolutionary event (Downie and Palmer 1992), and provides a robust molecular character to confirm the monophyletic origin of the subfamily Cactoideae, while additionally placing this subfamily as the most derived within the Cactaceae. While no sister-group relationships at the subfamilial level have yet to be shown, it is clear that the Cactoideae is a unique, highly specialized lineage. Further studies of cpDNA variation in the cacti will provide additional molecular characters from which other evolutionary relationships may be determined.

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