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Loss of the mitochondrial *cox2* intron 1 in a family of monocotyledonous plants and utilization of mitochondrial intron sequences for the construction of a nuclear intron

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Abstract The intron content of plant organellar genes is a useful marker in molecular systematics and evolution. We have tested representatives of a wide range of monocotyledonous plant families for the presence of an intron (*cox2* intron 1) in one of the most conservative mitochondrial genes, the *cox2* locus. Almost all species analyzed were found to harbor a group II intron at a phylogenetically conserved position. The only exceptions were members of a single monocot family, the Ruscaceae: representatives of all genera in this family were found to lack *cox2* intron 1, but instead harbor an intron in the 3′ portion of the *cox2* coding region (*cox2* intron 2). The presence of *cox2* intron 1 in families of monocotyledonous plants that are closely related to the Ruscaceae suggests that loss of the intron is specific to this family and may have accompanied the evolutionary appearance of the Ruscaceae. Interestingly, sequences that are highly homologous to *cox2* intron 2 are found in a nuclear intron in a lineage of monocotyledonous plants, suggesting that the originally mitochondrial group II intron sequence was transferred to the nuclear genome and reused there to build a spliceosomal intron.

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Introduction

Mitochondria are DNA-containing organelles that serve as the power stations of eukaryotic cells and also harbor a number of biochemical pathways. Mitochondrial genomes differ greatly in size, structural organization, coding capacity and gene expression, both within and between the kingdoms of eukaryotic organisms. The mitochondrial genomes of higher plants are much larger (200–2400 kb) and more complex than those of protists (5.7–76 kb), animals (14–42 kb) and fungi (18–176 kb) (Backert et al. 1997; Gray et al. 1998). The great size difference between the highly compact mitochondrial genomes of animals and those of higher plants is mainly due to the presence of large intergenic spacers, introns and duplicated sequences in plant mitochondrial genomes (Backert et al. 1997; Unseld et al. 1997).

A characteristic feature of plant mitochondrial genomes is their remarkably slow evolution rate (Wolfe et al. 1987). The nucleotide substitution rate in plant mitochondria has been calculated to be less than one-third of that in chloroplast DNA (Wolfe et al. 1987). Consequently, mitochondrial sequences are significantly less informative for molecular phylogenetic analyses of relatively closely related species than chloroplast sequences. However, structural characters, such as gene arrangement, intron content and sequence duplications, are useful mitochondrial markers in molecular phylogeny.

Cytochrome oxidase is a highly conserved multisubunit protein complex of the inner mitochondrial membrane in all respiring eukaryotes. In plants, typically three of its subunits are encoded by the mitochondrial genes *cox1*, *cox2* and *cox3*. Of these, the *cox2* gene encoding subunit II of cytochrome oxidase has received particular attention because (1) structural rearrangements in the mitochondrial genome involving the *cox2* locus have been

implicated in cytoplasmic male sterility (cms) in certain plant species (Young and Hanson 1987; Mann et al. 1989); (2) deletions in the *cox2* locus have been shown to result in cytoplasmically inherited striped-leaf phenotypes in maize (Gu et al. 1993, 1994); and (3) instances of mitochondrial gene transfer to the nucleus in certain dicotyledonous plants make the *cox2* gene a useful genetic marker for the study of molecular evolution (Nugent and Palmer 1991, 1993; Covello and Gray 1992). Moreover, during the evolution of dicotyledonous plants, the *cox2* locus has undergone multiple changes in its intron content. In most angiosperm species, the *cox2* locus contains a single group II intron at a well conserved insertion site. This intron, however, is absent from at least two lineages of dicotyledonous plants (Wolfe et al. 1987; De Benedetto et al. 1992), suggesting that it has been lost at least twice in dicot evolution (Hiesel and Brennicke 1983; De Benedetto et al. 1992; Qiu et al. 1998). Some angiosperm species harbor a second group II intron in the 3′ portion of the *cox2* reading frame (Lippok et al. 1992; Unseld et al. 1997; Qiu et al. 1998). This intron 2 is not homologous to intron 1 and its presence in the *cox2* gene appears to be independent of the presence of intron 1. Whereas the gene in carrot carries both introns (Lippok et al. 1992), the Arabidopsis *cox2* lacks intron 1 but possesses intron 2 (Unseld et al. 1997). Both *cox2* introns were acquired early in bryophyte evolution, possibly shortly after plants became established in land habitats (Qiu et al. 1998).

Monocotyledonous plants diverged from dicotyledonous plants approximately 200 million years ago (Wolfe et al. 1989). In order to gain further insights into the evolutionary dynamics of the intron content of plant mitochondrial genes, we have undertaken a systematic survey of monocotyledonous plants with respect to the presence of *cox2* intron 1. We report here that species from a wide range of monocot families all carry this intron. Interestingly, we found the intron to be absent from all representatives of a single monocot family, the Ruscaceae. This suggests that this intron was lost simultaneously with, or shortly before, the diversification of the Ruscaceae. Surprisingly, molecular analysis of the *cox2* locus in *Ruscus aculeatus* revealed the presence of intron 2 in the same genomic location as, and homologous to, the *cox2* intron 2 sequences previously discovered in dicotyledonous species. Most surprisingly, analysis of the intron 2 sequences revealed striking homology with a nuclear intron in an alcohol dehydrogenase gene of a monocotyledonous plant, suggesting that the mitochondrial intron sequence was transferred to the nuclear genome, where it was reused to build a spliceosomal intron.

Materials and methods

Plant material

Fresh leaf material of species of the family Ruscaceae was obtained from plants in the Botanical Gardens of the Universities of Freiburg and Munich, Germany. Material from *Semele androgyna* was kindly provided by Dr. R. M. Maier (Munich). *Haemanthus albiflos* and *Dracaena marginata* plants were kindly made available by Dr. S. Ruf (Freiburg). All other plant species were either grown under greenhouse conditions, or fresh leaf samples were harvested from plants in the Botanical Gardens in Freiburg and Halle/Saale, Germany.

Oligonucleotides

The synthetic oligonucleotides used in this study for RNA ligation, reverse transcription, PCR and/or DNA sequencing are listed in Table 1:

Isolation of nucleic acids

Total plant nucleic acids were isolated from 1–5 g of leaf tissue by a cetyltrimethylammonium bromide (CTAB)-based method (Doyle and Doyle 1990). For restriction enzyme digestion, DNA samples were further purified by CsCl gradient centrifugation. Total cellular RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, Calif.). Samples for DNA and RNA analyses were purified by treatment with DNase-free RNase A or RNase-free DNase I (Roche Diagnostics, Mannheim, Germany), respectively.

Blotting and hybridization techniques

Total cellular DNA from *Ruscus aculeatus* was digested with restriction enzymes, fractionated by gel electrophoresis on 0.8% agarose gels and transferred onto Hybond N nylon membranes (Amersham, Little Chalfont, Bucks., UK) by capillary blotting. Blots were probed with a radiolabeled product generated by PCR amplification using total *R. aculeatus* DNA with the primer pair P1401/P1402 (Table 1). Labeling of the PCR product with [^{32}P]dATP was carried out by random priming (Amersham). To test for the presence of intron 1 in various monocotyledonous plant species, Southern blots or dot blots were probed with a *cox2* intron 1-specific probe (prepared by PCR amplification of the intron from *Zea mays*; Fox and Leaver 1981). A radiolabeled *Xho*I-*Bam*HI restriction fragment of the intron from *Ruscus* was used as a probe for the detection of *cox2* intron 2 sequences.

Table 1 Oligonucleotides used in this study

Primer	Sequence (5′→3′)
P5′cox1	AGACTCCCATGCCTTTCT
P5′cox2	CCGATCGCCTACTCCAACAAT
P3′Ra	CTTCAGCTTCCCCGGTTTA
P1401	GCAGCGGAACCATGGCAATTAG
P1402	GAGGTACATCAGCGGGTGTTAC
P1405	CCAGGTACAGCATCACATTTG
P1406	CATCTTTCCTAGTATCATCC
P1407	GGAACTACTATCGAGATTC
P1408	CCGTAGAGAAAGTCTCTCTC
P1409	GCTTCCCCGGTTTAGTTGGT
P1410	TCAAAGAATGAAGCAAAGC
P1411	CTCATCTACGTATGATTGTAAC
P924	CAATGGACGAGGTAGTAGTA
P925	CTATTATAGTCCGAATACTCAT
Pcox8	CTTTTGACAGTTATATGATTC
Pcox11	TCACTGCACTGACCATAGTAAA
P5′Rus	GGACCAAGCAATTTCCGTAGAG
P3′lig	rCrCTGCAGCTACTGGCCGTCGTTTTACTCrAox[a]
PM13	GTAAAACGACGGCCAGT
PNM13	CGACGGCCAGTAGCTGCA

[a] rN, ribonucleotide; rN_{ox}, oxidized ribonucleotide (dialdehyde)

Cloning and DNA sequencing

Amplified *cox2* cDNAs or genomic fragments were purified by agarose gel electrophoresis and subsequent recovery of the PCR products from excised gel slices using the QIAEX II kit (Qiagen, Hilden, Germany). PCR products were directly sequenced by a modified chain-termination method (Bachmann et al. 1990) or by cycle sequencing techniques, using a Perkin Elmer thermocycler and fluorescence-labeled oligonucleotide primers or chain terminators. PCR products cloned into pBluescript vectors were used to determine the intron sequences. To exclude sequencing errors, both strands of several independent clones were sequenced.

Amplification of mRNA 3′ ends (RLM-RACE)

RNA ligation-mediated PCR (RLM-RACE; Liu and Gorovsky 1993) was used to determine the sequence of the 3′ end of the *R. aculeatus cox2* transcript. The procedure was essentially performed as described earlier (Albertazzi et al. 1998). Briefly, the 3′ ends of the total cellular RNA population were ligated to the synthetic oligonucleotide P3′lig (Table 1) using T4 RNA ligase (Amersham). This oligonucleotide carries two ribonucleotides at its 5′ end, as well as a ribonucleotide with an oxidized ribose moiety (dialdehyde) at its 3′ end to prevent self-ligation of the oligonucleotides. The ligation products were reverse transcribed with the primer PM13 (Table 1) and the *cox2* transcript population was subsequently amplified with a 5′ *cox2*-specific primer (P1401) and the 3′ primer PM13 (complementary to the 5′ sequence of oligonucleotide P3′lig). Reamplification reactions were carried out with the primer pair Pcox8/PNM13 (Table 1).

cDNA synthesis and PCR

Reverse transcription of DNA-free RNA samples was primed with a random hexanucleotide mixture or (for 3′ end mapping) with specific primers. Elongation reactions were performed with SuperScriptTMII RNase H-free reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Total cellular DNA or first-strand cDNAs were amplified by 30 to 40 cycles of 40 s at 93°C, 1.5 min at 55°C, and 2 min at 72°C with a 3-min extension of the first cycle at 93°C and a final 7-min extension at 72°C. The 5′ primer 924 or 1401 (which bind to conserved *cox2* sequences within exon I) and the 3′ primer 925, 1402 or Pcox11 (binding to conserved sequences within exon II) were used in standard PCR tests for the presence of intron 1 in the *cox2* genes from various monocotyledonous species.

Computer analyses

DNA and cDNA sequences determined in the course of this work were compared with *cox2* sequences available in the databases using the NCBI BLAST program. A phylogenetic tree for mitochondrial *cox2* sequences was constructed using the TREECON software package (Van de Peer and De Wachter 1993).

Results and discussion

Lack of *cox2* intron 1 in representatives of the family Ruscaceae

Monocotyledonous plants are estimated to have diverged from dicotyledonous plants approximately 200 million years ago (Wolfe et al. 1989). Most of the molecular studies on organellar genes and their intron-exon structure have been limited to representatives of a single family of monocotyledonous plants, the Poaceae. In order to gain further insight into the evolutionary dynamics of the intron content of plant mitochondrial genes, we have undertaken a systematic survey of monocotyledonous plants with respect to the presence of *cox2* intron 1. We tested a wide range of species (Table 2) using PCR assays with primer pairs derived from conserved regions of exons I and II of the *cox2* gene (see Materials and methods). The results were subsequently confirmed for most of the species by DNA hybridization analyses and/or partial DNA sequencing. An overview of the distribution of intron 1 in *cox2* genes from monocotyledonous plants is provided in Table 2. In the initial screen, *Ruscus aculeatus* was included as a

Table 2 Monocotyledonous species tested for presence of intron 1 in the mitochondrial *cox2* gene

Species[a]	Family	Order	Presence of *cox2* intron 1
Acorus calamus	Acoraceae	Acorales	+
Asparagus sprengeri	Asparagaceae	Asparagales	+
Danaë racemosa	Ruscaceae	Asparagales	−
Ruscus aculeatus	Ruscaceae	Asparagales	−
Ruscus hypoglossum	Ruscaceae	Asparagales	−
Semele androgyna	Ruscaceae	Asparagales	−
Dracaena marginata	Dracaenaceae	Asparagales	+
Hosta sieboldiana	Aphyllanthaceae	Asparagales	+
Allium ramosum	Alliaceae	Asparagales	+
Allium sativum	Alliaceae	Asparagales	+
Galanthus nivalis	Amaryllidaceae	Asparagales	+
Haemanthus albiflos	Amaryllidaceae	Asparagales	+
Narcissus pseudonarcissus	Amaryllidaceae	Asparagales	+
Fritillaria imperialis	Liliaceae	Liliales	+
Iris germanica	Iridaceae	Liliales	+
Neottia nidus-avis	Orchidaceae	Liliales	+
Anthurium hookeri	Araceae	Arales	+
Anubias barteri	Araceae	Arales	+
Arum maculatum	Araceae	Arales	+
Calla palustris	Araceae	Arales	+
Colocasia eodorata	Araceae	Arales	+
Cryptocoryne ciliata	Araceae	Arales	+
Dieffenbachia maculata	Araceae	Arales	+
Orontium aquaticum	Araceae	Arales	+
Peltandra virginica	Araceae	Arales	+
Sauromatum venosum	Araceae	Arales	+
Lemna minor	Lemnaceae	Arales	+
Pontederia cordata	Pontederiaceae	Pontederiales	+
Sparganium erectum	Sparganiaceae	Typhales	+
Typha angustifolia	Typhaceae	Typhales	+
Ananas comosus	Bromeliaceae	Bromeliales	+
Tradescantia virginiana	Commelinaceae	Commelinales	+
Oryza sativa	Poaceae	Poales	+
Triticum aestivum	Poaceae	Poales	+
Stipa capillata	Poaceae	Poales	+
Zea mays	Poaceae	Poales	+

[a]With the exception of *Acorus calamus*, all species are classified according to Dahlgren et al. (1985)

representative of the family Ruscaceae. Among all the species tested, *R. aculeatus* was the only one that yielded a PCR product corresponding in size to an intron-free *cox2* gene (Fig. 1, Table 2). This result was confirmed by the lack of hybridization of isolated *R. aculeatus* DNA to a *cox2* intron 1-specific probe (data not shown), providing further evidence that the gene from *Ruscus* does not harbor this intron. We then set out to determine whether lack of the intron was specific to *R. aculeatus* or was a general characteristic of the family Ruscaceae. We obtained plant material from members of all three genera belonging to the Ruscaceae, *Danaë*, *Ruscus* and *Semele* (Dahlgren et al. 1985). PCR analysis, as well as DNA sequencing, revealed that all four species examined lack the intron 1 in their *cox2* gene, suggesting that the absence of the intron is a shared feature of all Ruscaceae and that loss of intron occurred in the common ancestor of all three genera of Ruscaceae (Fig. 1, Table 2).

Both classical (Dahlgren et al. 1985) and molecular (Chase et al. 1993) systematics place the Ruscaceae in the order Asparagales. The presence of the *cox2* intron 1 in all other Asparagales families analyzed here (Table 2) indicates that the intron was still present when the Asparagales diversified into different families. Although we could not test all families of the Asparagales due to the limited availability of plant material, the presence of the *cox2* intron 1 in the Dracaenaceae (considered to be closely related to the Ruscaceae; Dahlgren et al. 1985; Chase et al. 1993) suggests that the loss of this intron may have occurred shortly before the diversification of the Ruscaceae and thus might be specific to this family.

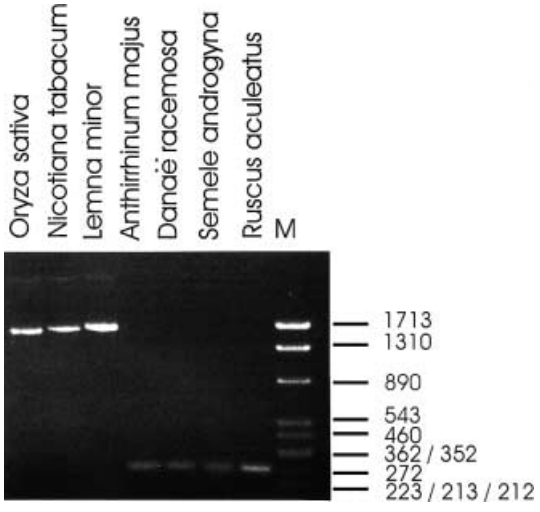

Fig. 1 PCR test for the presence of *cox2* intron 1 in representatives of the Ruscaceae. For comparison, two other monocotyledonous and two dicotyledonous species were also included. Amplification with the primer pair Pcox10/P1402 (Table 1) yields a ~1.6-kb fragment for the intron-containing *cox2* genes from *Oryza sativa* (Kao et al. 1984), *Nicotiana tabacum* and *Lemna minor*. The intron-free *cox2* from the dicot *Anthirrhinum majus* (Albertazzi et al. 1998) produces a PCR product of 284 bp. The three genera of the family Ruscaceae, represented by the species *Danaë racemosa*, *Semele androgyna* and *Ruscus aculeatus*, yield PCR products of virtually identical size (~284 bp) suggesting that their *cox2* genes also lack the intron 1. M, molecular weight marker (sizes in bp are indicated on the *right*)

cox2 is a single-copy mitochondrial gene in *R. aculeatus*

The presence of an intron-free *cox2* gene in the Ruscaceae can be explained by two alternative hypotheses: (1) the *cox2* gene in the mitochondrial genome has lost its intron; or (2) the *cox2* gene was transferred to the nucleus during the evolution of this genus. Such evolutionary gene transfers to the nuclear genome are known to have occurred via an RNA or cDNA intermediate (Nugent and Palmer 1991; Covello and Gray 1992; Adams et al. 1999; Selosse et al. 2001). Consequently, nuclear *cox2* genes usually do not contain introns (except between the acquired transit peptide sequence and the sequence of the mature protein; Nugent and Palmer 1991). In some cases, the mitochondrial genome retains a copy of the transferred gene which then becomes an inactive pseudogene and is no longer expressed (Covello and Gray 1992; Adams et al. 1999). In view of these considerations, we set out to determine (1) whether *cox2* is present as a single, intron-free gene copy in Ruscaceae, and (2) whether *cox2* is a genuine mitochondrial gene in this monocot family.

Total DNA from *R. aculeatus* was digested with various restriction enzymes and hybridized to a *cox2*-specific probe. A single, strong, hybridization signal was obtained with each digest, suggesting that the gene is present as a single locus (Fig. 2). Even upon re-hybridization of the blot at reduced stringency, no evidence for additional specific hybridization signals could be found (not shown), strongly suggesting that only a single copy of *cox2* is present in *Ruscus*. The mitochondrial localization of the gene was ultimately proven in our subsequent analyses by the finding that the *Ruscus cox2* mRNA is subject to numerous modifications by C-to-U RNA editing, an mRNA processing step that is characteristic of mitochondrial transcripts, and is not found in the nucleo-cytoplasmic compartment (see below).

Ruscus has an intron in the 3′ portion of the *cox2* coding region

We next wanted to determine the molecular structure of the *cox2* locus in *Ruscus*. The complete sequence of the gene was determined by a PCR-based approach employing sets of conserved primers. The sequence of the 3′ end was identified by a modified RLM-RACE strategy (Liu and Gorovsky 1993). As expected, the reading frame was not interrupted by an intron 1-homologous sequence (Fig. 3; EMBL Database Accession No. AJ427967). Instead, we detected an intron in the 3′ portion of the *cox2* reading frame. This intron (subsequently referred to as *cox2* intron 2) has previously been found in the dicot *Daucus carota* (Lippok et al. 1992) and several other angiosperm species (Unseld et al. 1997;

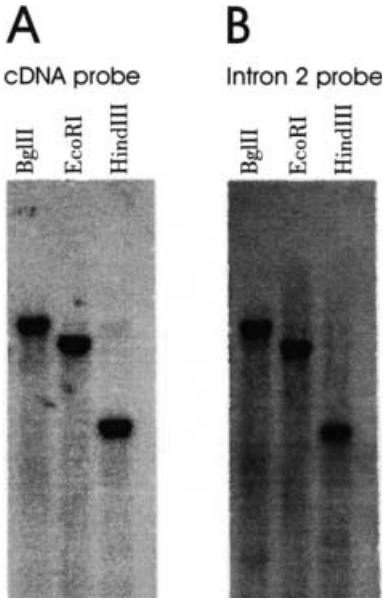

Fig. 2A, B Southern hybridization assay to detect *cox2* sequences in *Ruscus aculeatus*. **A** Total cellular DNA was digested with the restriction enzymes *Bgl*II, *Eco*RI and *Hin*dIII, transferred to a nylon membrane, and hybridized to a radiolabelled *cox2*-specific probe. The presence of a single strongly hybridizing band in all lanes indicates that *cox2* is a single-copy mitochondrial gene in *R. aculeatus*. **B** The blot shown in **A** was stripped and re-hybridized to an intron 2-specific probe, confirming the presence of intron 2 in the 3′ portion of the *Ruscus cox2* locus

Qiu et al. 1998). The intron in *Ruscus* is located at the same conserved position and shares strong sequence homology with *cox2* intron 2 sequences from *Daucus* and *Arabidopsis* (data not shown). Hybridization with an intron 2-specific probe detected fragments of the same size as hybridization with a probe derived from the *cox2* coding region (Fig. 2A, B), confirming the presence of intron 2 in the *Ruscus cox2* locus. The entire sequence of the intron from *Ruscus* was determined (EMBL Database Accession No. AJ427967). The intron is 2199 bp long, and exhibits all the structural features expected in a group II intron.

The processed *R. aculeatus cox2* transcript potentially specifies a protein of 257 amino acids (Fig. 3) and is highly homologous to *cox2* sequences isolated earlier from other plant mitochondrial genomes. cDNA analysis confirmed correct splicing of intron 2 in vivo (data not shown).

RNA editing of the *R. aculeatus cox2* transcript

A characteristic feature of plant mitochondrial mRNAs is their post-transcriptional processing by pyrimidine-to-pyrimidine conversions, commonly referred to as RNA editing (Covello and Gray 1989; Gualberto et al. 1989; Hiesel et al. 1989; for review see e.g. Hanson et al. 1996; Maier et al. 1996). In most instances, RNA editing events restore codons for conserved (and hence potentially functionally important) amino acid residues. Having determined the complete nucleotide sequence of the *cox2* locus from *R. aculeatus*, we were interested in comparing the DNA sequence with that of the mRNA to identify possible sites of mRNA editing and reliably deduce the amino acid sequence of the *Ruscus* Cox2 protein. For this purpose, cDNAs were synthesized and the amplified cDNA population was directly sequenced. Upon comparison of the cDNA sequence with the genomic sequence, we identified a total of 19 sites of C-to-U RNA editing, which change the coding properties of 18 codons at the mRNA level (Fig. 3). One CCA codon harbors two editing sites, with the second C being edited in virtually every RNA molecule and the first C being only partially edited. This partial editing does not affect the protein sequence, since both CUA and UUA codons specify leucine. Partial editing at the first codon position may reflect either some inaccuracy in editing site selection (with the second C being targeted but the first being erroneously recognized with reduced efficiency) or the silent editing reaction at the first codon position is tolerated due to the lack of selective pressure. Processing of the *Ruscus cox2* transcript by editing unambiguously demonstrates that the *cox2* gene is located in the mitochondrion.

Evolutionary implications

Compelling evidence argues in favor of a specific loss of the *cox2* intron 1 in the Ruscaceae rather than an acquisition of the intron in other plant families. First, a homologous intron is present in *Acorus calamus*, which is believed to be the most ancient surviving representative of the ancestral monocotyledonous plants (Duvall et al. 1993; Albertazzi et al. 1998). Moreover, the presence of a homologous intron at a conserved insertion site in all other monocotyledonous, as well as in most dicotyledonous, plants strongly suggests that the intron was already present in the common ancestor of angiosperm plants (Qiu et al. 1998). Hence, its absence from the Ruscaceae is most likely to be due to loss of the intron at the time of, or shortly before, the evolutionary appearance of this family.

The absence of *cox2* intron 1 from all four species that are assigned to the Ruscaceae by classical systematics (Fig. 1) is a good indication that they are indeed closely related and share a common ancestor that lacks the intron. In order to confirm this close association by additional molecular characters, we sequenced large portions of the *cox2* genes from the three other representatives of the family Ruscaceae, *Danaë racemosa*, *Ruscus hypoglossum* and *Semele androgyna*. We also determined the corresponding sequences from *Haemanthus albiflos*, a member of the Amaryllidaceae, since the Amaryllidaceae is one of the monocot families most closely related to the Ruscaceae (Dahlgren et al. 1985). As expected, all species grouped as Ruscaceae by classical systematics cluster together in the phylogenetic tree derived from plant *cox2* sequences (not shown) and

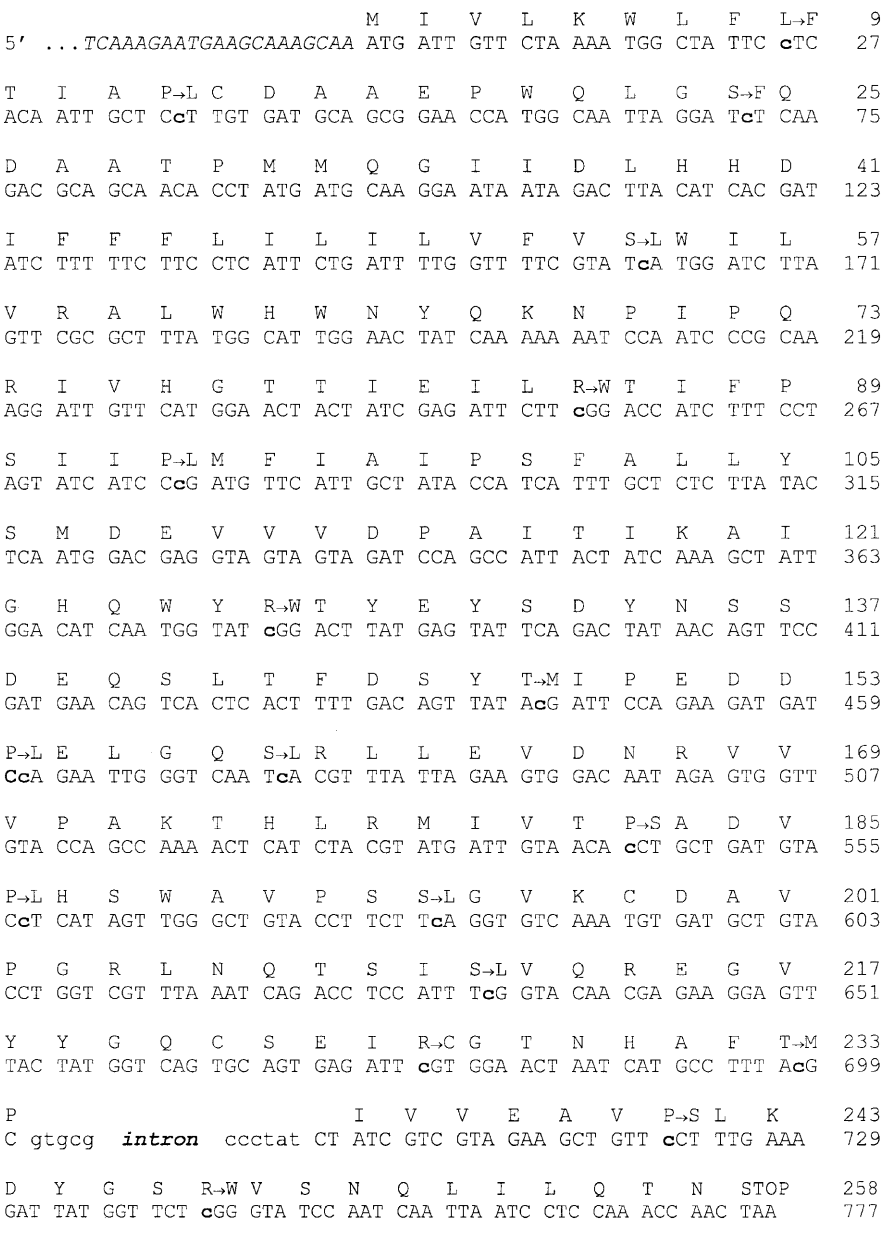

Fig. 3 Nucleotide and deduced amino acid sequences of the *cox2* locus from *Ruscus aculeatus*. Deviations of the mRNA sequence from the sequence of the DNA template due to C-to-U RNA editing are indicated in *bold lower case letters*, and the resulting amino acid changes caused by RNA editing are indicated *above* the codons. The C shown in *bold upper case* (codon 154) is edited only in some mRNA molecules. Part of the 5′ untranslated sequence is shown in *italics*, and parts of the intron are denoted by *lower case letters*

form a branch separate from *H. albiflos* and all other monocotyledonous plants.

In summary, our data provide evidence that an evolutionary loss of *cox2* intron 1 has occurred in at least one lineage of monocotyledonous plants. Furthermore, they underline the utility of the intron content of mitochondrial genes (which can be inexpensively tested by rapid PCR assays with sets of conserved primers) as a source of molecular markers in plant phylogeny and systematics.

The presence of intron 2 in the *R. aculeatus cox2* locus is intriguing and, to the best of our knowledge, is the first *cox2* intron 2 sequence to be found in a monocotyldeonous plant. When we searched the databases for homologous sequences in other species, we unexpectedly found a strong homology with a nuclear intron in an alcohol dehydrogenase gene (*adhB*) from the monocotyledonous plant *Washingtonia robusta* (Fig. 4). The palm *W. robusta* has three nuclear *adh* loci, one of which has been completely sequenced (*adhB*; Morton et al. 1996). The sequence homologous to the *cox2* intron 2 from *R. aculeatus* is part of intron 1 of *adhB* in *Washingtonia*. Although it is unlikely to be critical for spliceosomal removal of intron 1, the presence of this sequence suggests an interesting evolutionary scenario in which *cox2* intron 2 sequences were transferred from the mitochondrial to the nuclear genome and part of the originally mitochondrial group II intron was used to build a spliceosomal intron in the nuclear *adhB* gene.

Massive gene transfer from the mitochondrial to the nuclear genome has occurred since the acquisition of the α-proteobacterial endosymbiont by the pre-eukaryotic archaebacterium-like organism (see Gray 1993 and Martin and Herrmann 1998, for review). Gene transfer to the nucleus is an ongoing process, in that a number of

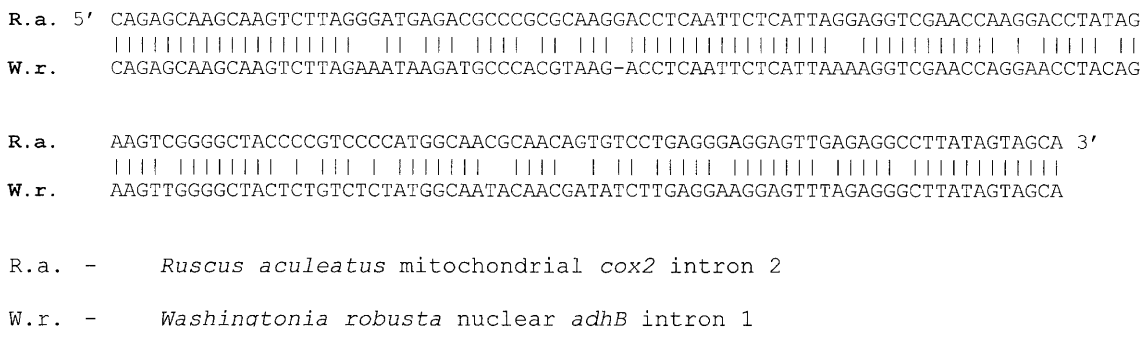

Fig. 4 Homology between intron 2 of the *Ruscus aculeatus cox2* gene and intron 1 of the nuclear alcohol dehydrogenase gene B (*adhB*) of the monocotyledonous species *Washingtonia robusta* (Morton et al. 1996). A stretch of 154 nucleotides from the mitochondrial intron is highly homologous (83%) to the nuclear intron, suggesting that a DNA transfer event accounts for one of the two sequences

recent gene transfer events have been detected that have resulted in some variability in the coding capacities of mitochondrial genomes even among relatively closely related organisms (Nugent and Palmer 1991; Aubert et al. 1992; Grohmann et al. 1992; Knoop et al. 1995; Adams et al. 2000, 2001). In addition, plant nuclear genomes can harbor large pieces of mitochondrial DNA which serve no apparent function and hence are commonly referred to as "promiscuous DNA" (Sun and Callis 1993; reviewed by Thorsness and Weber 1996). Occasionally, DNA transfer in the reverse direction can also occur, as revealed by the detection of *copia*-, *gypsy*- and LINE-like retrotransposon fragments in the mitochondrial genome of *Arabidopsis thaliana* (Knoop et al. 1996; reviewed in Marienfeld et al. 1999). However, in the case of the *cox2*/*adhB* sequence, we strongly favor the idea of a mitochondrion-to-nucleus transfer event because the high homology between the two sequences (Fig. 4) points to a relatively recent transfer event whereas the *cox2* intron 2 sequence is evolutionarily old and clearly precedes the divergence of monocots and dicots (Qiu et al. 1998). To the best of our knowledge, this is the first time that a mitochondrial group II intron sequence has been found to be part of a spliceosomal intron in a nuclear gene.

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