REGULAR PAPER

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Evolutionary origin of a plant mitochondrial group II intron from a reverse transcriptase/maturase-encoding ancestor

Abstract Group II introns are widespread in plant cell organelles. In vivo, most if not all plant group II introns do not self-splice but require the assistance of proteinaceous splicing factors. In some cases, a splicing factor (also referred to as maturase) is encoded within the intronic sequence and produced by translation of the (excised) intron RNA. However, most present-day group II introns in plant organellar genomes do not contain open reading frames (ORFs) for splicing factors, and their excision may depend on proteins encoded by other organellar introns or splicing factors encoded in the nuclear genome. Whether or not the ancestors of all of these noncoding organellar introns originally contained ORFs for maturases is currently unknown. Here we show that a noncoding intron in the mitochondrial *cox2* gene of seed plants is likely to be derived from an ancestral reverse transcriptase/maturase-encoding form. We detected remnants of maturase and reverse transcriptase sequences in the 2.7 kb *cox2* intron of *Ginkgo biloba*, the only living species of an ancient gymnosperm lineage, suggesting that the intron originally harbored a splicing factor. This finding supports the earlier proposed hypothesis that the ancient group II introns that invaded organellar genomes were autonomous genetic entities in that they encoded the factor(s) required for their own excision and mobility.

Key words *cox2* · Group II intron · Maturase · Splicing · Evolution · Mitochondrion

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Introduction

Expression of a number of genes in plant cell organelles is dependent upon removal of intervening sequences (introns) that interrupt reading frames and have to be removed by splicing prior to translation of the mRNA. Most introns in higher plant organellar genomes belong to the group II introns (Michel et al. 1989; Sugita and Sugiura 1996; Bonen and Vogel 2001; Michel and Ferat 1995) although a few group I introns have also been described (Vaughn et al. 1995; Cho et al. 1998; Sugiura 1992; Kuhsel et al. 1990). Both group I and group II introns are catalytically active RNA molecules (Pyle and Cech 1991; Belfort 1991; Dürrenberger and Rochaix 1991; Chowira et al. 1994; Michel and Ferat 1995), and group II introns are thought to be the evolutionary ancestors of spliceosomal introns present in the nuclear genomes of eukaryotes (Copertino and Hallick 1993; Hetzer et al. 1997). Group I and group II introns are often referred to as self-splicing introns and, in fact, at least some group I (Pyle and Cech 1991; Dürrenberger and Rochaix 1991) and group II (Winkler and Kück 1991; Padgett et al. 1994) introns can undergo self-splicing in vitro in the absence of any protein factors. However, in vivo, most if not all introns in plant cell organelles seem to require the assistance of proteinaceous splicing factors to be excised (Deshpande et al. 1995; Li et al. 2002; Hess et al. 1994; Jenkins et al. 1997; Vogel et al. 1999). While many splicing factors for plant organellar group II introns appear to be nuclear-encoded (Jenkins et al. 1997; Vogel et al. 1999; Perron et al. 1999; Jenkins and Barkan 2001; Rivier et al. 2001; Till et al. 2001; Ostheimer et al. 2003; Perron et al. 2004), some are encoded by the plastid or mitochondrial genomes (Neuhaus and Link 1987; Hess et al. 1994; Liere and Link 1995; Schuster and Brennicke 1987; Wissinger et al. 1991; Thomson et al. 1994). These organelle genome-encoded splicing factors are referred to as maturases, and homologous reading frames have also been found in mitochondrial genomes of fungi (Anziano and Butow 1991; Szczepanek and Lazowska 1996; Mohr et al. 1993). Often, these maturase open reading frames (ORFs) are encoded

within intronic sequences, suggesting that the splicing factor is produced by translation of the excised group II intron. Maturase proteins encoded by organellar introns are comprised of several distinct domains, some of which can be attributed to defined catalytic activities: a reverse transcriptase domain with seven highly conserved motifs is usually followed by the maturase domain (also referred to as domain X) and sometimes by a DNA-binding motif and an endonuclease domain at the C-terminus (Mohr et al. 1993; Mohr and Lambowitz 2003).

For many maturases, intron substrates are not known. In higher plant chloroplasts, the presence of only a single maturase gene (*matK*) on the plastid genome and the availability of plastid translation-deficient mutants has facilitated a prediction about which introns require the MatK protein for excision: introns in which splicing is defective in the mutants are thought to depend on MatK protein action whereas introns in which splicing is retained in the mutants are believed to be independent of the MatK maturase and, instead, may require nucleus-encoded splicing factors (Hess et al. 1994; Vogel et al. 1997, 1999).

Reading frames homologous to intron-encoded reverse transcriptase/maturase genes have been identified in the fully sequenced genomes of *Arabidopsis* and rice (Mohr and Lambowitz 2003), indicating that some formerly organellar reverse transcriptase/maturase genes have been transferred to the nuclear genome in higher plants. Whether or not these genes are actively expressed and their gene products retargeted to plastids or mitochondria is currently unknown.

The evolutionary history of present-day introns in plant organellar genomes is surrounded by many open questions. While some group I introns could be evolutionarily old in that they stem from homologous introns in homologous bacterial genes (Kuhsel et al. 1990), many group II introns in higher plants appear to have been acquired relatively recently. For example, two introns in the mitochondrial *cox2* gene and an intron in the *nad1* gene first appear in mosses (Qiu et al. 1998), possibly indicating their acquisition with or shortly after the plants' conquest of terrestrial habitats (for review, see e.g., Knoop 2004). Horizontal gene transfer has been suggested as one mechanism leading to organellar gene invasion by intronic sequences (Vaughn et al. 1995; Cho et al. 1998; Sheveleva and Hallick 2004), and fungal introns have been discussed as one possible source of recently acquired introns in plant mitochondria (Vaughn et al. 1995). Origin and evolution of splicing factors for group II introns represent yet another evolutionary puzzle. One possible scenario is that all introns that invaded plant organellar genomes contained coding sequences for the splicing factor(s) required for their excision. However, whether or not the ancestors of noncoding organellar introns originally contained ORFs for maturases and/or reverse transcriptases is currently unknown. We have searched for traces of reverse transcriptase/maturase genes in ancient groups of seed plants, and we report here on the identification of remnants of a reverse transcriptase/maturase reading frame in a large intron within the *cox2* locus of the gymnosperm *Ginkgo biloba*, suggesting that this intron originally harbored a reverse transcriptase/maturase gene that was lost during seed plant evolution.

Materials and methods

Plant material

Fresh leaf material of *Gingko biloba* was obtained from a female plant in the Botanical Garden of the University of Freiburg, Germany.

List of oligonucleotides

The following synthetic oligonucleotides were used in this study for polymerase chain reaction (PCR), cDNA synthesis, and DNA sequencing:

P1401	5′GCAGCGGAACCATGGCAATTAG 3′
P1402	5′GAGGTACATCAGCGGGTGTTAC 3′
P1503f:	5′ GGATAATCCCTCATCCTCG 3′
P1502r:	5′ CTACGGTCCCTCGAGTCT 3′
P1501r:	5′ GATTCGATCCTATAGTCGTC 3′
P1500f:	5′ CTTTGCACTTATCGAACATCAG 3′
P1504f:	5′ AGTAGTTGCGGAACTACCG 3′
P1505f:	5′ GGAAAACGGCATTCTTTGAG 3′
P1506r:	5′ AGGGGCGGCAGCGTACTTC 3′
P1507r:	5′ TCGTCGAAGTGACCCTTGA 3′
P1508r:	5′ CATATCGATATATATATCCATCC 3′
P1509r:	5′ CTGAAATATTCACATCTCCCAA 3′
P1510f:	5′ GAGAAGAACGAGGACAACC 3′

Isolation of nucleic acids and DNA gel blot hybridization

Total plant nucleic acids were isolated from fresh leaf tissue by a cetyltrimethylammonium bromide (CTAB)-based method (Doyle and Doyle 1990). Total cellular RNA was extracted using the TriFast reagent (Peqlab GmbH, Erlangen, Germany). RNA samples for cDNA synthesis were purified by treatment with RNase-free DNase I (Roche, Mannheim, Germany). For detection of *cox2* sequences by Southern blot hybridization, DNA samples were digested with restriction enzymes, separated by gel electrophoresis on 0.8% agarose gels, and transferred onto Hybond nylon membranes (Amersham, Buckinghamshire, UK) by capillary blotting. Hybridizations were performed at 65°C in Rapid-Hyb buffer (Amersham) following the manufacturer's protocol and using radiolabeled restriction fragments derived from the amplified *cox2* gene from *Ginkgo biloba* as hybridization probes.

cDNA synthesis and polymerase chain reactions (PCR)

Reverse transcription of DNA-free RNA samples was primed with a random hexanucleotide mixture. 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 30–35 cycles of 45 s at 94°C, 1.5 min at 55°C, and 2 min at 72°C with a 1-min extension of the first cycle at 94°C and a 5-min final extension at 72°C. Primer pair 1401 (binding to conserved *cox2* sequences within exon I) and 1402 (binding to conserved sequences within exon II) were used to amplify the 2.66 kb intronic sequence.

Cloning and DNA sequencing

Amplified *cox2* cDNAs or genomic fragments were purified by agarose gel electrophoresis, and subsequent recovery of the PCR products form excised gel slices using the QIAEX II kit (QIAGEN, Hilden, Germany) or the GFX kit (Amersham). Direct sequencing of amplification products was performed by cycle sequencing followed by automated analysis in a MegaBACE capillary sequencer (Amersham). Regions difficult to sequence directly were subcloned into pBluescript vectors and sequenced from plasmid clones using the universal M13 and reverse primers. To exclude mutations introduced by PCR amplification, four to five independent clones were sequenced.

Bioinformatic analyses

cox2 DNA and cDNA sequences were compared with sequences available in the databases using the NCBI BLAST program. Sequences homologous to reverse transcriptase/maturase genes were identified by translating the intron sequence in all three reading frames followed by removal of the stop codons and running BLAST searches for short, nearly exact, matches. Amino acid sequence alignments of reverse transcriptase/maturase proteins were produced using the ClustalW software (http://www.ebi.ac.uk/clustalw/).

Results and discussion

A large intron in the *cox2* locus of *Ginkgo biloba*

The plant mitochondrial *cox2* gene encoding cytochrome oxidase subunit II has been intensively used for phylogenetic and evolutionary analyses (e.g., Nugent and Palmer 1991; Covello and Gray 1992; Kudla et al. 2002). In particular, the highly variable intron content of the gene has made the *cox2* locus a preferred object of studies on the dynamics of intron evolution in higher plant mitochondria (Hiesel and Brennicke 1983; Laroche et al. 1997; Qiu et al. 1998; Rabbi and Wilson 1993; Albrizio et al. 1994; Kudla et al. 2002). The picture that has emerged from these studies is that the *cox2* locus gained two introns in the moss lineage (Qiu et al. 1998). Subsequently, the two introns have undergone multiple independent loss events during seed-plant evolution (Hiesel and Brennicke 1983; Rabbi and Wilson

1993; Albrizio et al. 1994; Kudla et al. 2002). We have conducted a systematic analysis of intron evolution in mitochondrial *cox2* genes of seed plants (Albertazzi et al. 1998; Kudla et al. 2002; and our unpublished results). In order to trace back the origin of reverse transcriptase/maturase-encoding introns versus noncoding organellar introns, we also included various representatives of primitive angiosperm lineages and determined the molecular structure of their *cox2* loci (Albertazzi et al. 1998 and unpublished data). In the course of this work, we detected an unusually large intron within the *cox2* gene of the maidenhair tree, *Ginkgo biloba*, the only living species of an ancient gymnosperm lineage, the Ginkgoopsida (Zhou and Zheng 2003).

Sequencing of the intron and adjacent exon sequences revealed that the intron inserts in an evolutionary conserved position known from *cox2* introns in many angiosperm species, such as *Triticum aestivum* (Bonen et al. 1984), *Petunia hybrida* (Pruitt and Hanson 1989, 1991), *Beta vulgaris* (Mann et al. 1991), and *Acorus calamus* (Albertazzi et al. 1998). The total size of the intron was determined to be 2,660 bp (EMBL database accession number AJ874265), making it one of the biggest plant mitochondrial introns discovered to date. The secondary structural domains conserved in all group II introns are easily identifiable in the sequence (Fig. 1, and data not shown), and strong homology of these domains with *cox2* introns from angiosperm species confirms the singular origin of the intron in seed plants.

Splicing and RNA editing of the *Ginkgo biloba cox2* transcript

A hallmark of mitochondrial gene expression in higher plants is the requirement for an additional RNA processing step, referred to as RNA editing. RNA editing changes the identity of single nucleotides in primary transcripts, predominantly by C-to-U transitions (Gualberto et al. 1989; Covello and Gray 1989; Hiesel et al. 1989; reviewed in Mulligan et al. 1999; Bock 2001). Comparison of exonic sequences of the *cox2* gene from *Ginkgo biloba* with *cox2* gene and protein sequences from other plant species suggested the presence of several potential RNA editing sites in *Ginkgo biloba* (Fig. 1, and data not shown) where conserved amino acid residues could be restored by C-to-U transitions. In order to confirm these potential editing sites experimentally and possibly detect additional sites, we reverse-transcribed DNaseI-treated RNA samples from *Ginkgo biloba* and amplified *cox2* cDNAs. Direct sequencing of the amplified cDNA population confirmed intron excision at the conserved intron-exon borders and revealed numerous sites of C-to-U RNA editing (Fig. 1). The editing sites identified included some that could not be predicted by bioinformatics analyses in that they do not result in changes of the amino acid encoded by the affected triplet. These include a silent CUG-to-UUG transition in exon I (both codons specify leucine) and a CCA-to-UUA transition in exon II that involves two editing events and converts

Fig. 1. Sequence of the *cox2* intron from *Ginkgo biloba* with surrounding exon sequence. Conserved intron domains are shown in color: domain VI in *blue*, domain V in *green* and the two complementary sequences forming the stem-loop structure of domain IV in magenta. Scrambled pieces from reverse transcriptase/maturase sequences are shown in *red bold letters* (see Figs. 2 and 3). Sites of C-to-U mRNA editing are indicated by the *lowercase letter c*. The affected codon is *underlined*, the amino acid specified prior to editing is shown in *lowercase* and followed by the *uppercase letter* for the amino acid specified by the edited codon

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    P  V  M  Q  G  I  I  D  L  H  H  D  I  F  F  F  pL  I  L  I  L  V  lF V
...CCTGTGATGCAAGGAATAATTGACTTGCATCATGATATCTTTTTCTTCcCAATTCTTATTTTGGTCcTCGTA

sL rW M  L  V  R  A  sL W  H  F  H  Y  R  I  N  P  I  P  Q  R  I  V  H  G
TcAcGGATGTTGGTTCGCGCTTcATGGCATTTTCACTATAGAATCAATCCGATCCCGCAAAGGATTGTTCATGGA

T  T  I  E  I  I  rW T  I  sF P  S  I  I  pL M  F  I  A  I  P  S  F  A  L
ACTACTATAGAGATTATTcGGACCATATcTCCTAGTATTATTCcGATGTTCATTGCTATACCATCATTTGCTcTG

L  Y  S  M  D  E  V  V  V  N  P  A  I  T  I  K  A  I  G  H  Q  rW Y  rW T
TTATACTCAATGGACGAGGTAGTAGTGAATCCAGCCATTACTATAAAAGCTATTGGACATCAAcGGTATcGGA
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intron
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GTGCGCCCCTTAACGAGGGTGATGGAAGTGCAACAAAATGCACCGGACTACTTCTTATATGGTTCGCAAAGCATC
TGGCTTACCGAATGATAGGGGATAATCCCTCATCCTCGTCGACCTCGAGAGACTCGAGGGACCGTAGCATGCCA
GAAACGGAGAGTTGAAGTGGTTAGACCTAGTACCCCTATCGGATAAGAGAAATGCTCCCCTAGCCGAGGAGCCTA
TGGTTCCATTCTTGTTGTTGTTGGAGGTACACATCCCTCTCCTCGGTGTGGGCAAGCAGTGATATACGAGAAATA
GATGCTCAGCCTGCAATGTCCGATAACAGCGCTGAAGTGGTGAATCTATCGGCACCGCAGCAGTGGCATACAACT
TCCGGACCTAACGGGCCGGCCTGGCCCCGTAACCTTCCGGAATGGGGGATCCCCGTTGGTAGCAGCAACCACGGTA
GTAGTTGCGGAACTACCGGGCGGGAGAAGAACGAGGACAACCATTGACTGTTGTTCCTGCTCCTCATTCGGGGAC
GGAGGTCCTACGGTAGGTAACAGTAGGCACAAGCACTTTACCGAAGGGGACCAGCGCTTATACTCCTCCACCGTA
TATCGGAGTCTCGGCTATTCGCAGCGAGAAGCAAGGGATGTCGTGAACGGTAGTGGAACGGGGTCGCGGGGAATT
TACCTATTCATGGATAGATAGATACATCCATCTATCTATAGATGAATAGATCCCTTCGTATTGATGCGTATATAT
AGATATAGATATATACCCATCTATATAGGTACCTATCCATCTGTATATAGAGATGGATAGATATATATATATCCT
ATATATACGTGAGATATAGATATAGATATCTTGTAGATATAGATTCGATATCTATCTAGATCTATAGATATATAC
ATATCTATGTCAGGGATGGATATATATATGATATGTATATATAAGGATGAATCTCTTTATTCATTGGGAGATGT
GAATATTTCAGTCCATCCGACCATTCAATGGGGATCTACCTACGAAACCGTTGCATATGACGGGGCAAGATCCA
ACCGTCGACAATTCCACTCTCGACGGCCGGCGGGCGTGATCGGTCTTATCATCGATCAAATGAGGAAAAAATCCT
TCCAGTTAGGACTGACCCGAATAACAATTTGCGGGGAGATGCGATGCGTCGTCCCCTTGTACTATATTCTGTCAG
GGACGAGAGAATAGTACGAGAAGCGATCAGAATGCCTCCCTTGAAGAAATGCGAGTGTTCGAACCGAAATGGTT
TGTG**GGGTTCAGACCT**GGAATAAGAAACA**CTCGGTACTGCCCGCAAATGGAGCGGTATCACCTGGCTCCTTTGGG
GGGATATCAAGGGTCACTTCGACGATCTGGACCATACTTTTTCGGCATCACTTCCCAGTAAGAAGGGAAAG**TGAA
AGACGAGAATCTGATAGACCCGTACCGAAAACTGGCGGATCTGTGAACAACAGAGAACGAGAACCCAAAACGGTC
CCCCCGAACCTCCCGAAGAAAGAAAGTGGGGGTGAAGGAATTGGAACTGGGACCAAGCGGAGATAGAACCAGAA
TCACCCACCTAGCTGGTAGGTTCATTGGAACATTCATTCTGGGTACCGGGAAAAAGAAGTACGCTGCCGCCCCTT
ATCTCTTATTTCTTCATTCAGGGGTTATGGAATGGGCAAGCACTTTCTCTTCTCTTATTTCTTCATTCAAGGGTG
GGCAAGCACTTGCTCTAGTCCGACCCATTCAATAATGGGTAGTAGGATCTTCTTGTTGGAAGCTCCTATCAACGA
GCTCACGTCCAAACTCGCGAGGAAACGCATCTTGAGTGCAACGAAAGGAAGGGTTTGCTAGCGATGCGAAAGGAC
CCATTCGACTCAATCAATCAATTAATAGGGGGACAGGATTGATCGATGACCACAGGGGGAATGAT**TTACGCTA
TGACGTGGTAATTCGAGGTCTCATGAACTACTACTCGTTCATGGACAAC**GGGAACCCTCTGATTCCTCCATTCAG
GGGCAGGCCGAGGGAATCCTATGGATTCTCCCAAGATTCCTGGCCGTCCCCACCATTACTAGATAGAGGAAGGGG
TAGGAAATGGAGAATCTCTACTCGAGTGTTCATAGAATTGGGCCCCTGACAATTGGAGATGTGGGGAATCACTCT
TGATCTATTCATTTGGGGGCCCGACCGTTCCTACAACTTTGCCGACTAGCATCCCTTTCAACTTTGCACTTATCG
AACATCAGAAGAAGACGACTATAGGATCGAATCAGCTTTTCGTGGTGAACTAGGTCGTCCCATACCTTTTCATAC
GCGTGTCTGCTGTGTGTGTGGAACCACTGAGAATGTGGAACCACATGTAATAGTAATAAATAGAAGTCCTGGG
GGTTTCACCTTGTTTCTCGGTTTCACTATGGCTGGCTCGATCGAATGGTAAAAAGGTCCCCCCAGTTGTCCGTAG
GGCCACAGTTCGATCCACCGGAGTATTACGGTCTATACCTGAGAGGGGGGAGAACCACCCGACTAGATAATAGTA
TACTGCTCGAGTGTCA**ATGGTAGG**CGTGGA**GAGCTTCTTGCGGGGAAACTTGCAAGTACAGTTT**GGG**GGGGAGGCG
GATGTACCTGAACAAGGACTAGGCGTCGACCC**AAC
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exon
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  hY E  C  S  D  Y  N  S  S  D  G  Q  S  pL T  F  D  S  Y  tM I  P  E  D  D
CTcATGAGTGTTCAGACTATAACAGTTCCGATGGACAGTCACcAACTTTTGACAGTTATAcGATTCCAGAAGATG

  pL E  L  G  Q  sL R  L  sL E  V  D  N  R  V  V  V  P  A  K  T  R  pL R  M
ATccAGAATTGGGTCAATcACGTTTATcAGAAGTGGACAATCGAGTGGTTGTACCAGCCAAAACTCGTCcACGTA

  I
TGATT...
```

a proline into a leucine codon (Fig. 1). In the latter case, the editing in first codon position seems unnecessary in that a single editing, even in second codon position, would be sufficient to alter the codon identity.

As group II intron splicing and C-to-U RNA editing are absent from the nucleocytosolic compartment in higher plants, intron excision and high-frequency editing of the *cox2* transcript confirm the mitochondrial localization of the *cox2* gene in *Ginkgo biloba*.

Mitochondrial genes are occasionally found in more than one copy per genome, usually due to gene duplications that often are confined to a small taxonomic group (e.g., Rothenberg und Hanson 1987; Gutierres et al. 1997). In order to exclude this possibility for the *Ginkgo biloba cox2*

locus and to confirm that *cox2* is a single copy mitochondrial gene, we performed a Southern blot analysis using a *cox2* gene-specific probe. A single hybridizing band was detected in all restriction analyses (Fig. 2), ultimately confirming that *cox2* is indeed a single copy mitochondrial gene in *Ginkgo biloba*.

Remnants of an intron-encoded reverse transcriptase/maturase

Presence of an unusually large intron within the *cox2* locus of *Ginkgo biloba* prompted us to search the intron for the presence of potential ORFs. Intron-encoded ORFs poten-
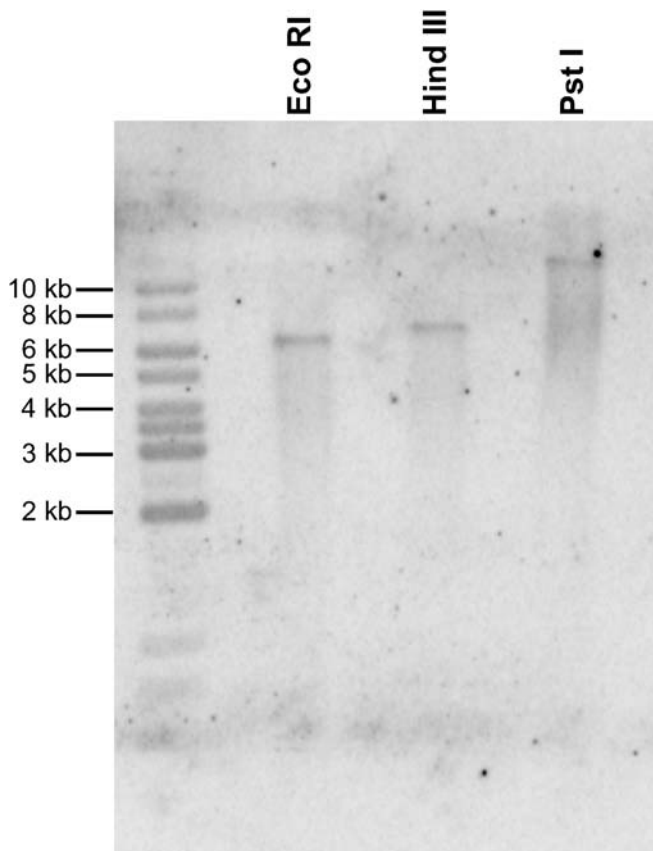
Fig. 2. Detection of *cox2* sequences in *Ginkgo biloba* by DNA gel blot analysis. Total DNA was digested with the restriction enzymes indicated, separated by agarose gel electrophoresis, blotted, and hybridized to a *cox2* gene-specific probe. Detection of a single hybridizing band in all lanes confirms that *cox2* is a single copy mitochondrial gene in *Ginkgo biloba*

tially encoding reverse transcriptase/maturase proteins most often insert in the loop of domain IV (Schuster and Brennicke 1987; Wissinger et al. 1991) and, as domain IV of the *Ginkgo biloba* intron contains a large insertion (loop size: 1,967 bp; Fig. 1), it seemed possible that it harbors a maturase-encoding gene. However, conceptual translation of the intron sequence in all three reading frames failed to detect a sufficiently long ORF (Fig. 3, and data not shown). Moreover, bioinformatics analysis of the longest theoretical ORFs revealed no significant homology to any protein in the databases. Also, detailed analysis of the conceptual translation of the 1,967-bp loop of domain IV (Fig. 1) revealed no potential ORF larger than 100 amino acids (Fig. 3, and data not shown). In order to trace back the evolutionary history of this long, presumably noncoding, insertion in intron domain IV, we refined the search criteria by removing stop codons and searching for short, nearly exact, matches at the level of the amino acid sequence. Interestingly, these searches identified altogether three regions with significant homology to intron-encoded reverse transcriptase/maturase proteins in fungal and bryophyte mitochondrial genomes (Figs. 1, 2, and 3). The first two stretches are homologous to conserved regions in the

reverse transcriptase domains RT IIa and RT III of intron-encoded reverse transcriptases/maturases (Fig. 4; Mohr and Lambowitz 2003). The third stretch displays homology to the conserved domain X, the presumed intron maturase domain, which is located downstream of the reverse transcriptase domain in most intron-encoded reverse transcriptase/maturase proteins (Fig. 4; Mohr et al. 1993; Mohr and Lambowitz 2003).

For several reasons, it seems highly unlikely that a functional reverse transcriptase/maturase protein is encoded in the *Ginkgo biloba cox2* intron: (1) The sequences surrounding the reverse transcriptase/maturase-related stretches are full of stop codons (Fig. 3, and data not shown); (2) the short sequence stretches homologous to reverse transcriptase/maturase ORFs are in different reading frames (Fig. 3); (3) no regions of homology with the other highly conserved reverse transcriptase/maturase domains could be detected (Fig. 3, and data not shown). Instead, these reverse transcriptase/maturase-related sequences are likely to represent pseudogenic remnants of a functional reverse transcriptase/maturase gene formerly present in this intron.

Evolutionary implications

Removal of group II introns relies on an RNA-catalyzed splicing mechanism but requires protein factors in vivo, presumably to help fold the intronic RNA into the catalytically active secondary and tertiary structures (Michel et al. 1989; Copertino and Hallick 1993; Scott and Klug 1996; Michel and Ferat 1995). In addition to their splicing activity, group II introns can behave as mobile genetic elements, for example, by inserting themselves into intronless alleles, a process known as intron homing (Belfort and Perlman 1995; Curcio and Belfort 1996; Bonen and Vogel 2001). In several cases, bifunctional proteins referred to as reverse transcriptases/maturases mediate both splicing and homing of the intron: while the reverse transcriptase activity plays a role in intron mobility, the maturase domain functions in intron removal (Mohr et al. 1993; Mohr and Lambowitz 2003).

The detection of remnants of a reverse transcriptases/maturase gene in the *cox2* intron of *Ginkgo biloba* has interesting implications. A systematic survey of land plants for the presence of this intron led to the conclusion that the intron was acquired once during early land-plant evolution in a common ancestor of all land plants, exclusive of liverworts (Qiu et al. 1998). Our data suggests that the intron, when it was acquired, possessed a reverse transcriptases/maturase gene and hence was a mobile group II intron. Subsequently, degeneration of the reverse transcriptase/maturase gene has occurred, with the intron in *Ginkgo biloba* reflecting an intermediate on the way to complete loss of the reverse transcriptase/maturase sequences. Consequently, the intron in present-day angiosperms no longer shows any evidence of the mobile past of the intron.

What could have been the source of the intron when it was acquired in early land-plant evolution? Our bioinformatics analyses show that the closest relatives of the reverse

Fig. 3. Identification of remnants of reverse transcriptase/maturase sequences within the sequenced *cox2* intron from *Ginkgo biloba*. Translation of the relevant parts of the intron in all three reading frames is shown. Nucleotide numbers of the intronic sequence are indicated above the DNA sequence. Amino acid sequence stretches homologous to reverse transcriptase/maturase genes in mitochondrial introns from other organisms are underlined and in bold (cp. Fig. 4), the corresponding DNA sequences are marked in *red* and *bold*. Note that all three frames contain numerous stop codons. This, together with the reverse transcriptase/maturase-homologous sequence stretches being in different reading frames, strongly suggests that there is no functional reverse transcriptase/maturase protein encoded in the intron. Instead, these sequences may represent pseudogenic remnants of an intact reverse transcriptase/maturase gene that is likely to have been present in the ancestral intron
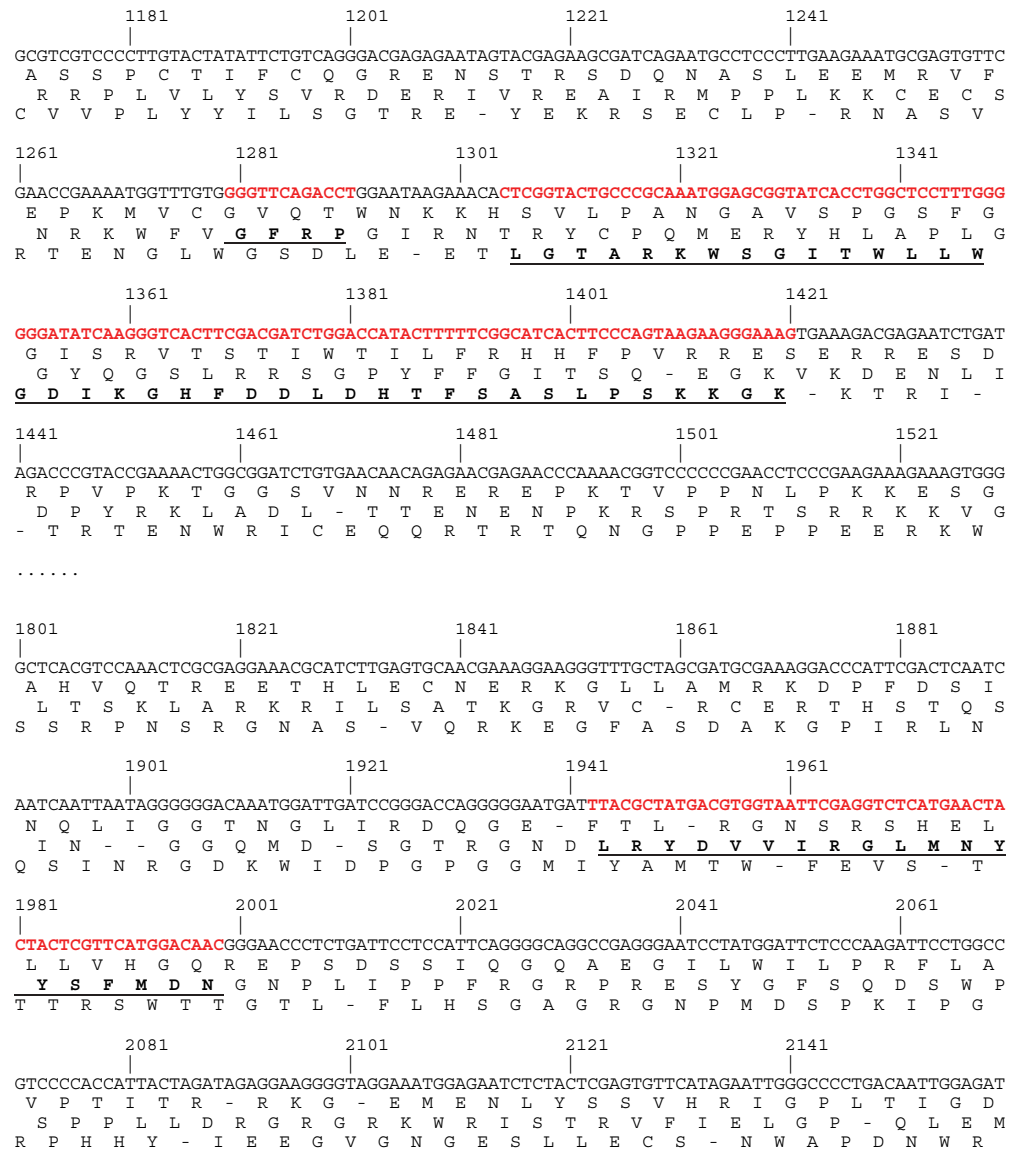
transcriptase/maturase sequences in *Ginkgo biloba* come from fungal homologues and a reverse transcriptase/maturase gene within an intron in the mitochondrial 18S rRNA gene from the liverwort *Marchantia polymorpha* (Fig. 4). Hence, two scenarios seem feasible: (1) transposition of the intron from a mitochondrial gene with a mobile intron (e.g., the 18S rRNA) into the *cox2* locus; (2) acquisition from a fungus via horizontal gene transfer. Recent studies have provided evidence for horizontal gene transfer of mitochondrial genes and introns both from fungi to higher plants and between higher plant species (Vaughn et al. 1995; Marienfeld et al. 1997; Cho et al. 1998; Bergthorsson et al. 2003; Won and Renner 2003; Davis and Wurdack 2004; Mower et al. 2004). At present, we cannot distinguish between the two above possibilities of *cox2* intron acquisition, but molecular phylogenetic analysis of the homologous introns in mosses, hornworts, and ferns may help to resolve this issue.

A recent analysis of mitochondrial introns has provided evidence for ORF remnants in several introns of the liverwort *M. polymorpha* and a few other bryophytes but failed to detect such sequences in the higher plant *Arabidopsis thaliana* (Toor et al. 2001). Our finding that remnants of a reverse transcriptase/maturase gene are present in the *cox2* intron of *Ginkgo biloba* represents the first case of traces of reverse transcriptase/maturase sequences in an intron of a higher plant lineage. Due to their high degree of evolutionary degeneration (Figs. 1, 3, and 4), ORF remnants were detectable only by refined bioinformatics searches, and the presence of rather short sequence traces in the gymnosperm lineage (as represented by *Ginkgo biloba*) provides an explanation as to why they are undetectable in the angiosperm lineage (Toor et al. 2001). It thus seems conceivable that the last recognizable traces of the reverse transcriptase/maturase ORF in the *cox2* intron were lost during seed-plant evolution. This underscores the impor-

Fig. 4. Amino acid sequence alignment of reverse transcriptases/maturases with partial homology to the translated intron of the *cox2* gene from *Ginkgo biloba*. N- and C-termini of the proteins display no significant conservation and no homology to the *cox2* intron from *Ginkgo biloba* and, therefore, were omitted from this alignment. *Asterisks* mark residues that are identical in all sequences in the reverse transcriptase/maturase alignment, *colon* indicates conserved substitutions, and *dot* denotes semiconserved substitutions (according to ClustalW; http://www.ebi.ac.uk/clustalw/). Regions exhibiting homology with short sequence stretches in the translated *cox2* intron from *Ginkgo biloba* are aligned with the respective *Ginkgo biloba* sequences (*bold, underlined*). Species and accession numbers are as follows: *P.a. Podospora anserina* (CAA38781 and CAA38778), *M.p. Marchantia polymorpha* (P38478), *A.m. Allomyces macrogynus* (S63652), *S.o. Schizosaccharomyces octosporus* (AF275271.2)

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P.a. cox1 i1   KSEARKGQGSLGEMLMYNERGQCINAYEVICKLEALYTAYMNIKSEPGNMTPRVDSETLD 219
M.p. rrn18 i1  --------------MTKCN--------YEQLLDPEIFRLAYELKKSKSGNMKPGADKETLD 39
P.a. cox1 i4   FIAAAGNGTANEMKLVLKDK-KFVNLYQLICSKDLLIQAYRNVRSNPGGMTPSIDNITYD 231
A.m. cox1 i3   DTDAVKIGTGGYSKLVKLANGKFTGLYDTIMSIEFLLLAYRNIKSKAGNMTPGPDDQTLD 237
S.o. cox2 i1   SIKASLKNTKNVDKTENDLNKFYELNYRQLYNLDLIKTAYLDLKSNSDNFEKNNNNNSLD 235
                                        *   :  .: :  **   :*:...:    :. : *

P.a. cox1 i1   GISKEWFEKISEQLKSEQFRFRPTRRVYIPKANGKMRPLGIASPRDKIVQEVFRAILEQV 279
M.p. rrn18 i1  GFSQAYVEKVVRQLKDESFQFRPSRREFIPKADGKLRSLGIPSPRDKIVQEVMRRILEPV 99
P.a. cox1 i4   GINDEFLEKLILELKSERFKFTSVKRVYIPKANGKTRPLGIPTSKDKIVQEAMKILLELI 291
A.m. cox1 i3   GITPGFFEDLIKSLKNESFRFKSVKRTFKKKTNGKMRPLGIPAPRDKIVQEAMRILLEFI 297
S.o. cox2 i1   KISNEWAEKTCKSLKDRSFIFKPSRKIMVTCRNNQKREISIANGKDRVIQQAFKMILQSA 295
               :.   : *.   .**.. * *  .::       ::: * :.*.  .*::*:::. :*:

                       GFRPG        LGTARKWSGITWLLWGDIKGHFDDLDHTFSASLPSKKGK
P.a. cox1 i1   LEPRFHSSSHGFRPGRGCHSALATIRYWNGIKWFIEGDIKGFFDNIDHHILEKLLVKHFQ 339
M.p. rrn18 i1  FEPRFLDSSHGFRPHRSPHTALRQIRRWTGTSWMIEGDIKGYFDNIDHHLLAGFIAELVK 159
P.a. cox1 i4   YEPIFLDVSHGFRPKRSCHTALHQISKWNGTTWMLEGDIKGFFNEVDHQVLIKILEKKIK 351
A.m. cox1 i3   FEPIFSDFSHGFRPQRSCHTALKEVSKWNGMTWCIEGDIKSFFDNVDHKILENFLREKIE 357
S.o. cox2 i1   YEPIFLNYSHGFRPGRSPHSAIFEVRKWTRITWMIKGDIKGFFDNVDHHILANLLSKKIK 355
               **  * .  ****** *. *:*:   :  *.  .* ::****.:*:::**::*  :: : .:

P.a. cox1 i1   DQRFIDLYWKMVKAGYVEFDKDK-SSIIGVPQGGIASPILSNLVLNELDEFVQNIVDEFN 398
M.p. rrn18 i1  DQRLLALYWKLVRAGYVNQGKAEPHLLTGVPQGRILSPLLSNIYLHQFDLFMEEIKVKY- 218
P.a. cox1 i4   DQRFFDLLWKLFRAGYIDDGVKY-NTYTGVPQGGVISPVLSNIYLHEFDLFVETLIKKYS 410
A.m. cox1 i3   DQRFIDLFWKLVRAGYLEKGVSF-DSPLGVPQGGIILSPLLSNIYLHAFDMFMEKVMSDHS 416
S.o. cox2 i1   DKNLIDFYWKLVRAGYVNNGNYK-VSNLGIPQGGIILSPLLANIYLHELDVYMEQLIQKYT 414
               *:.:: : **::***:: .       *:*** : **:*:*: *: :* ::: :  ..

P.a. cox1 i1   EKLKGGKHTSKNPAYVVIDSRIGKITRLERKLKSK--GQELDSGRKLERMKLIKVRATMP 456
M.p. rrn18 i1  --TTTGALSKNNPIYLKARN---KYYKLVKSLKAS--SAEIIRAR----------RDMLK 261
P.a. cox1 i4   --SEKDFISKVNPVIVKYSSKLSRLNDEYQTTKDKEILKEIIKLR--------AERNKLP 460
A.m. cox1 i3   --SDSKSISKVNPKMVSYSNKLSKLNKQYQDTKDPAILKEIRALR--------LERNKIP 466
S.o. cox2 i1   ---------VNKPVSKKNKEHTRLFSEITAKSKKKFPDFELIKRMR-------KELRRIP 458
                        :*         .           *         *:                :

P.a. cox1 i1   SMIPNP-DLAKIYYVRYADDWLIGVAGSSETARAIKERIAAYLKDILKLELSMEKTLITN 515
M.p. rrn18 i1  MTYGIQ-TGSRVRYVRYADDWVIGVTGPKALAVQIKEEVSTFLQEKLKLSLQAEKTRITN 320
P.a. cox1 i4   SRIR---NGIRVRYTRYADDWVIGIIGDQELVAKIKEECKAFLRDILKLELSEEKTKITN 517
A.m. cox1 i3   SRIR---TGTRVRYVRYADDWLVGIIGPRELAVSLKEMIASFLAQELKITLSPEKTKISH 523
S.o. cox2 i1   STIRDSSTGTRVRYYYNRYGDDYVIGVVGPKNLAETIQNLVSDFLKNELLIDLNKEKSQITH 518
                :: * **.**::*: *    .  ::: :    :* : * *. **: *::

P.a. cox1 i1   ASEDKAYFLGTEIQRISSVKGEIKRFKNIKGHPQ-RIPTTSTVMNAPISKLVTKLADKGI 574
M.p. rrn18 i1  LSRSEALFLGTLIS-ITTRK--YVQSQKVGGGHR-RASLGRIRLCIPIDILIGKLSQMG- 375
P.a. cox1 i4   ITEKEVRFLGVDIKRKDSGESKIIQRQVKGRLIKSRINNNRLYFYVPVRDIINKLEKAGF 577
A.m. cox1 i3   FGKDLISFLGVNLRIPKPAESKVVLRKGAKGHTLARVNHTRVFFYIPVDKILAKLAAQGF 583
S.o. cox2 i1   LTSKSLKYLGYEIFRRNRKYSESQLTYNSKTNTYRKGSNHRVILYAPINDLINKLVDHKF 578
                     .   :** :         .         :::       :* : :: **

                                   LRYDVVIRGLMNYYSFMDN
P.a. cox1 i1   VIWKSKALNEDNLIPQPILKWVNLPIRDIILRYKMIWNGYINYYSFADNKPRLVLIY-WI 633
M.p. rrn18 i1  -------ACDEKGTPKAVTKWIFLNVGEIINKYMAVFRGYYNYYSFADDIHHLLQII-YI 427
P.a. cox1 i4   IKTYTSANGREKLAPNAITKWIFLDHRSILLRYNAIRGLLNYYSFVDNKIAFHSIVNFL 637
A.m. cox1 i3   LKDYVPGG---KIVTNAITKWIFLDHRSIISKYNSIINGFLSYYSFVDNLNAFHLIINYI 640
S.o. cox2 i1   AILNG-----TQAKPKAITKWIYLEPREIISRYNGILNGIYYYYKGVDNLNQLSYTN-WI 632
                :  .:.: **: *   .*: :*  : .*    **. .*:  :    ::

P.a. cox1 i1   LRKSLAKTLATKLKLGTVRKVYLKFGVNLRFEILGTDNKSIEFTKGNLLPTPK-NFK--G 690
M.p. rrn18 i1  LRYSAINTVARKLGLNTA-KVIKRFGVDLIFRDHTNEIK-------HKLNFPR-SLP--N 476
P.a. cox1 i4   IHHSCAKTIARKLNLPNRAQAFNKFGRYLTAPGEG-KLKPMELFTLDSFKKTT-SLLKSY 695
A.m. cox1 i3   LVHSCAKTLARKFRLGSRAGAFKKFGGDLAAPAPSPQDKPLGLNLKKSYAKTN-KFN--I 697
S.o. cox2 i1   LRFSAVFTLSRKWNISPK-KVFKKLGKNLTVKTVNSKGKETSISLALPKTLSRNRLFKLD 691
                :  *   *:: *  :       . ::* *    . *      .              :
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tance of including early branching seed-plant lineages in reconstruction of the evolutionary history of plant group II introns.

Another interesting question is how the maturase function lost by degeneration of the reverse transcriptase/maturase gene in the *cox2* intron was compensated for. The two most plausible possibilities may be: (1) that the gene was transferred to the nucleus; (2) that the splicing function was taken over by another intron-encoded maturase in the mitochondrial genome. The single chloroplast-genome-encoded maturase gene *matK* provides precedence for the latter scenario in that it appears to be involved not only in the splicing of the group II intron it resides in (*trnK*) but also in the splicing of several other plastid introns (Hess et al. 1994; Jenkins et al. 1997; Vogel et al. 1999).

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