Origin and Evolution of the Chloroplast trnK (matK) Intron: A Model for Evolution of Group II Intron RNA Structures

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The trnK intron of plants encodes the matK open reading frame (ORF), which has been used extensively as a phylogenetic marker for classification of plants. Here we examined the evolution of the trnK intron itself as a model for group II intron evolution in plants. Representative trnK intron sequences were compiled from species spanning algae to angiosperms, and four introns were newly sequenced. Phylogenetic analyses showed that the matK ORFs belong to the ML (mitochondrial-like) subclass of group II intron ORFs, indicating that they were derived from a mobile group II intron of the class. RNA structures of the introns were folded and analyzed, which revealed progressive RNA structural deviations and degenerations throughout plant evolution. The data support a model in which plant organellar group II introns were derived from bacterial-like introns that had “standard” RNA structures and were competent for self-splicing and mobility and that subsequently the ribozyme structures degenerated to ultimately become dependent upon host-splicing factors. We propose that the patterns of RNA structure evolution seen for the trnK intron will apply to the other group II introns in plants.

Introduction

In plant chloroplasts, the tRNA-Lys(UUU) gene (trnK) contains a group II intron (trnKI1), which encodes the matK open reading frame (ORF). The trnK intron and its encoded matK ORF have generated substantial interest in the fields of plant evolution and molecular biology. In evolutionary studies, the matK ORF has been used as a marker to construct plant phylogenies because the ORF evolves rapidly yet is ubiquitous in plants (Hilu and Liang 1997; Kelchner 2002). From the perspective of molecular biology, the trnK intron is of interest because it represents an unusual form of a group II intron, and the MatK protein has been suggested to have novel properties as a maturase.

Group II introns are self-splicing RNAs and mobile elements found in eubacteria, archaia, and the organelles of fungi, plants, and algae (Bonen and Vogel 2001; Lambowitz and Zimmerly 2004). Although some group II introns consist of an RNA structure alone, many encode a reverse transcriptase (RT) protein within the intron RNA structure, which gives the intron the ability to invade new sites in a genome. The protein also has a second important function in facilitating intron splicing, which it does by binding to the intron RNA structure and stimulating its intrinsic self-splicing properties. Because such a splicing (maturase) function is generally specific to its host intron, such proteins are called maturases.

The trnK intron differs from typical group II introns because its encoded protein, MatK, is a degenerate version of an RT (fig. 1A). Canonical group II intron ORFs contain three conserved domains; an RT domain containing subdomains 0–7; an X domain associated with maturase activity; and an optional En (endonuclease) domain. A fourth DNA-binding domain (D) is located between X and En and has been characterized functionally but is not conserved in sequence (San Filippo and Lambowitz 2002). In contrast, the matK ORF aligns only for RT subdomains 5–7 (poorly conserved) and X (highly conserved), although it is roughly the same size as other group II intron ORFs (Mohr, Perlman, and Lambowitz 1993). The retention of domain X argues that MatK proteins retain maturase activity; however, mobility functions appear to have been lost because the RT catalytic site residues and other RT motifs are not present (Mohr, Perlman, and Lambowitz 1993). Despite these predictions, the complete biochemical functions of MatK have not been demonstrated definitively (below).

In contrast to the ORF structure, the RNA structures of trnK introns are fairly typical of group II introns and fall into the IIA1 class of introns (Michel, Umesono, and Ozeki 1989). Group II intron RNA structures consist of six domains arranged around a central wheel (fig. 1B) (Qin and Pyle 1998). Domain I is largest and constitutes about half of the intron’s size, while domain V is the most highly conserved in sequence and is considered the active site of the ribozyme. The matK ORF is encoded within an extended loop of about 2 kb within domain IV, as is typical of other group II introns. Although there are no gross defects in the RNA structure of trnKI1, this intron along with other plant organellar introns has not been reported to self-splice in vitro (Michel and Ferat 1995; Barkan 2004). Presumably, splicing in vivo relies on protein cofactors, of which the MatK protein is the most obvious candidate.

There has been considerable speculation about a function of MatK beyond its presumed host intron-specific maturase activity. Over 10 years ago, it was noticed that Epifagus virginiana, a nonphotosynthetic plant, possesses a reduced chloroplast genome that has shed large portions of DNA, including the trnK gene and its intron, yet matK is retained as a free-standing ORF (Wolfe et al. 1992). MatK was therefore suggested to have an important function in chloroplasts beyond splicing of its resident intron. This function was suggested to be splicing of other group II introns still present in the reduced chloroplast genome. If true,
MatK would be the only known “generalized” maturase for group II introns, with a role beyond splicing of its host intron (Emms et al. 1995). Additional indirect evidence for this idea came from the observation that in barley several mutations (Ems et al. 1995). Additional indirect evidence for this idea came from the observation that in barley several mutations associated with an intron structure; these ORFs are not, although it remains formally possible that an uncharacterized chloroplast-encoded protein is involved. Another re-lation notable speculation is that a generalized maturase may be encoded in the nucleus. Angiosperm nuclear ge-nomes contain four group II intron maturase-related genes (nMat-1a, nMat-1b, nMat-2a, and nMat-2b), which are not associated with an intron structure; these ORFs are not closely related to matK (Mohr and Lambowitz 2003).

Previously, we proposed a model for the evolution of group II introns, termed the retroelement ancestor hypothesis, which predicts that the ancestor of all known group II introns was a retroelement in bacteria (Toor, Hausner, and Börner 1999). Because MatK is the only chloroplast-encoded ORF that can be used to link the introns to other ORF-containing introns. The combined RNA structural data support the existence of an ancestor with standard group II intron RNA features, which subsequently accumu-lated deviations and degenerations throughout the molecule during plant evolution. We suggest that the patterns of RNA evolution seen here will apply to other group II introns in plants.

Materials and Methods
Sequence Acquisition

Genomic DNA was prepared from Equisetum arvense as described (Hausner et al. 1999). The trnK11 sequence was polymerase chain reaction (PCR) amplified using standard methods with the primers 5′-GGTGTGCTAACT-CAACGGTAG-3′ and 5′-GGTTGCGGGACTGCAA-CCCGGAACCGTGCG-3′, followed by cloning and sequencing (GenBank accession number AY348551). Charophyte sequences were obtained from isolates F140C (Chara connivens; GenBank accession number AY170442), F146 (Nitella opaca; GenBank accession number AY170449), and F138 (Toypella nidifica; GenBank accession number AY170450) as previously described (Sanders, Karol, and McCourt 2003). Other trnK11 sequences were downloaded directly from GenBank with the following accession numbers: Amborella trichopoda NC_005086; Anthoceros formosae AB086179; Arabidopsis thaliana NC_000932; Atractylodes koreana AB008760; Chaetosphaeridium globosum AF494278; Cycas panzhihuaensis AF143440; Lotus japonicus NC_002694; Marchantia polymorpha X04465; Nicotiana tabacum NC_001879; Nymphaea alba NC_006050; Orzya sativa AF148650; Pellia borealis AF238498; Physcomitrella patens NC_005087; Pinus thunbergii D11467; Plagiocnium insignae AY522573; Podocarpus macrophyllus AF228111; Porella baueri AY168653; Psilotum nudum AP004638; Sphagnum inudatum AY342156; Torreya grandis AF228108; Zea mays ZMA86363.
Phylogenetic Analysis

Sequences were aligned initially with the ClustalX program (Thompson et al. 1997) and manually refined. For figure 2, the final data set after ambiguously aligned positions were removed was 424 amino acids, 376 of which were parsimony informative (PI). ProtTest was used in part to identify the best-fit model for subsequent analyses (Abascal, Zardoya, and Posada 2005) [Jones-Taylor-Thornton {JTT} model (\( \alpha = 2.35; I = 0.04 \)] (Jones, Taylor, and Thornton 1992)]. Metropolis-coupled Markov chain Monte Carlo Bayesian analysis was performed using the program MrBayes V3.0b4 (Ronquist and Huelsenbeck 2003). This analysis was initiated from a random starting tree, and four chains were run simultaneously for 2,000,000 generations, with trees sampled every 100 generations. After discarding the first 100,000 trees (“burn-in”), posterior probabilities were computed from the remaining trees. Bootstrap replicates (2,000 each) were generated using three different phylogenetic methods. Minimum evolution values were obtained using the programs SEQBOOT, PROTDIST, and FITCH within the PHYLIP 3.63 package (Felsenstein 2004). Maximum likelihood values were generated using PHYML with four rate categories and optimization of tree topology, branch lengths, and rate parameters (Guindon and Gascuel 2003). Finally, un-weighted maximum parsimony values were obtained using PAUP* V4.0b10 (Swofford 2003), using a heuristic search method with random sequence addition starting trees (10 rounds) and Tree Bisection-Reconnection branch rearrangements. Gaps were treated as missing data.

For figure 3, a second data set was assembled with the same \textit{matK} sequences plus representatives from major classes of group II introns. These sequences were aligned across RT subdomains 5–7 and domain X, and after unalignable positions were removed, 143 amino acids remained (139 PI). Phylogenetic reconstruction was as described above, with the exception of the evolutionary model. Due to the highly divergent data set and small number of characters, a relatively simple model of evolution was chosen (JTT). It has previously been shown that complicated models (e.g., identified by likelihood ratio tests) used with highly divergent data sets can result in incorrect topologies (Posada and Crandall 2001\textsuperscript{a}; Pontikivska 2004). In addition, the ability of model selection tests to choose the correct model is compromised when, as is the case here, the number of characters is small (Posada and Crandall 2001\textsuperscript{b}). Bayesian and maximum parsimony analyses were performed as described above, and the NEIGHBOR program (PHYLIP3.63 package) was used to obtain a second set of bootstrap values. Bacterial class C was chosen as an
outgroup because it has the most divergent RNA structure. Rooting the tree with other classes of ORFs did not affect the placement of *matK* ORFs within the ML lineage.

**RNA Secondary Structure Folding**

Intron RNAs were folded using MFOLD (Zuker 2003). Initial secondary structure calculations were progressively refined by the addition of secondary structure constraints to produce agreement with consensus structures (Michel, Umesono, and Ozeki 1989; Toor, Hausner, and Zimmerly 2001). Structures were also refined by detailed comparisons with relatives, with the requirement that alignable sequence in two introns be folded identically. The *Arabidopsis* structure proved problematic in the ID(iii) region and was resolved by comparison with four related angiosperm sequences not otherwise used in this article (not shown). Remaining uncertainties after extensive comparisons are listed in the legend of Supplementary Data Figure 1 (Supplementary Material online).

**Results and Discussion**

Initially, we collected *trnK* intron and *matK* ORF sequences from representative plants to sample the diversity of *trnK* sequence. Thousands of *matK* sequences are present in the databases, but many entries contain only ORF sequence and lack the flanking intron RNA sequence. Among full-length *trnK* intron sequences, there was good representation among higher plants; however, the only early-branching plant representatives were the charophyte alga *C. globosum* and the liverwort *M. polymorpha*. To increase data for primitive representatives, we PCR amplified and sequenced *trnK*I1 from *E. arvense* (horsetail fern; GenBank accession number AY348551) and also from three additional charophytes (*C. connivens, N. opaca,* and *T. nidifica*; GenBank accession numbers AY170442, AY170449, and AY170450, respectively). Subsequently, *trnK* sequences for two other liverworts, three mosses, and a hornwort were reported to GenBank. The final data set of *trnK* sequences represents the spectrum of plant diversity and comprises 25 introns, including four charophyte algae (*C. globosum, C. connivens, N. opaca,* and *T. nidifica*), three liverworts (*M. polymorpha, P. baueri,* and *P. borealis*), a hornwort (*A. formosae*), three mosses (*S. inundatum, P. patens,* and *P. insignes*), two fern allies (*P. nudum* and *E. arvense*), four conifers (*P. thunbergii, P. macrophyllus,* *T. grandis,* and *C. panzhihuaensis*), two early-branching angiosperms (*A. trichopoda* and *N. alba*), two monocots (*O. sativa* and *Z. mays*), and four eudicots (*A. thaliana,* *N. tabacum, Lotus japonica,* and *A. koreana*). All the sequences were complete except for three of the conifers and *Tolyptella*, which were missing some or all of the RNA domain VI.

It is of interest to examine which organisms lack *trnK* or its intron, as this gives information about intron gain and loss. As previously noted, the earliest branching plants to contain *trnK*I1 are charophyte algae (Turmel, Otis, and Lemieux 2002; Sanders, Karol, and McCourt 2003), while earlier branching algae (red algae, glaucophytes, and green algae) contain only *trnK* but no intron (*Chlorella* GenBank accession number NC_005353; *Chlamydomonas* GenBank accession number NC_001865; and *Nephroselmis* GenBank accession number NC_000927). Even the early-branching streptophyte *Mesostigma* lacks the intron (GenBank accession number NC_002186), suggesting that a group II intron inserted into the *trnK* gene of a primitive streptophyte after divergence of chlorophytes and *Mesostigma*. In all instances, the intron-encoded ORF has the degenerate features of *matK* ORFs diagrammed in figure 1(A).

A second observation is that no RNA structure is associated with the *matK* sequence of the fern *Adiantum*, based on the lack of a domain 5 and other diagnostic features, nor is there a *trnK* gene in the genome (GenBank accession number NC_004766). The retention of *matK* without the *trnK* gene resembles the situation of *Epifagus* and strengthens the argument for a second function for the MatK protein, which is most likely facilitation of splicing other group II introns retained in the genome.

To reconstruct the evolutionary history of the *trnK* intron, we first analyzed the *matK* ORFs phylogenetically to establish a framework for later comparison with RNA structures. Amino acid sequences were aligned, with ambiguously aligned positions excluded, for a data set of 424 amino acids. The resulting phylogeny (fig. 2) is in agreement with known plant phylogenetic relationships and indicates strict vertical inheritance as expected (Savolainen and Chase 2003).

A more informative analysis examined the relationship between *matK* ORFs and other group II intron ORFs, as it has implications for the origin of the *trnK* intron. The ORF alignment included representatives from all major intron subgroups and included sequence from RT domains 5–7 and X, again with unalignable positions excluded, for a final data set of 143 amino acids. Phylogenetic analyses indicated that *matK* ORFs fall within the ML (mitochondrial-like) class of introns (fig. 3A). (It should be noted that the ML class is not only a phylogenetic clade of intron ORFs that are predominant in fungal mitochondria but also contains bacterial members; *matK* is the only known chloroplast member in this class.) The support placing *matK* within the ML class is moderate in figure 3(A) because of few characters and high sequence divergence. However, the conclusion is further supported by sequence identity within domain X shared by ML class and *matK* ORFs but not other classes (fig. 3B). This sequence conservation was previously observed between *matK* and fungal mitochondrial introns (Mohr, Perlman, and Lambowitz 1993), while domain X of the chloroplast-like (CL) clade has been noted to be divergent from the ML clade (Zimmerly, Hausner, and Wu 2001). Additional support placing *matK* in the ML clade comes from the shared A1 RNA secondary structures of ML and *trnK* introns, while other classes have B or C class RNA structures (Michel, Umesono, and Ozeki 1989; Toor, Hausner, and Zimmerly 2001). Together, one can firmly conclude that the *trnK* family of introns descended from an RT-encoding intron of the ML class with an A1 RNA structure. However, the phylogenetic resolution in figure 3(A) and in other analyses (not shown) were not sufficient to suggest close relatives, and so one cannot distinguish if the *trnK*I1 ancestor came from a bacterial, mitochondrial, or unknown chloroplast source.
FIG. 3.—Phylogenetic relationship of matK ORFs with respect to other group II intron ORFs. (A) RT subdomains 5–7 and domain X were aligned for matK and ORFs of representative introns of Classes B, C, D, CL1, and CL2 (bacterial classes B, C, and D and CL classes 1 and 2; see Zimmerly, Hausner, and Wu [2001] for class definitions). The phylogeny shown is the most likely tree retrieved from Bayesian analysis (JTT model). Thick lines denote Bayesian posterior probabilities/C21 95%. Bootstrap values for 2,000 replicates are shown for neighbor-joining (JTT model; top) and unweighted maximum parsimony (bottom). The tree was outgroup rooted with ORFs from class C. Species abbreviations and GenBank accession numbers are as follows: O.b.: Oenothera berteriana, M63034; Z.m.: Zea mays, U09987; T.a.: Triticum aestivum, X57965; S.t.: Solanum tuberosum, AJ003130; A.t.: Arabidopsis thaliana, X98300; V.f.: Vicia faba, M30176; P.a.: Podospora anserina, X55026; M.p.: Marchantia polymorpha, M68929; K.l.: Kluyveromyces lactis, X57546; S.c.: Saccharomyces cerevisiae, V00694; P.: Podospora anserina, X55026.

B

Arabidopsis
Pinus
Equisetum
Sphagnum
Marchantia
Chaetosphaeridium
matK

Class ML

Class B
Class D
Class CL1
Class CL2
Class C

Other Classes

--- 0.1 substitutions per site

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Together, the information supports the following model for the origin of the trnK intron and the degeneration of its ORF (fig. 4). The distant ancestor is predicted to be an ML class intron encoding a RT ORF and being competent for mobility, and this ancestor intron might have come from any source (bacterial, mitochondrial, or chloroplast). The ancestor intron likely invaded the trnK gene in streptophytes after the divergence of chlorophytes and Mesostigma, placing the origin of the trnK intron and MatK protein at approximately 1200–800 MYA (Yoon et al. 2004). Sometimes after the initial insertion but before divergence of other derived charophytes, the intron lost its mobility properties by acquiring drastic mutations in RT subdomains 0–4 and En and more modest mutations in RT subdomains 5–7 and X. It is possible that this degeneration coincided with the second function of MatK as a generalized maturase (Ems et al. 1995; Vogel, Börner, and Hess 1999). Consistent with its putative function as a maturase, the entire length of the ORF is under selective pressure (Young and dePamphilis 2000), in agreement with experiments showing that maturase function relies on the entire RT domain in addition to the X domain (Cui et al. 2004). Subsequent to ORF degeneration, the trnK intron was inherited only vertically, because its mobility functions were lost.

In order to examine evolution of the intron RNA structures, the trnK II sequences were folded into secondary structures, based on agreement with consensus structures for IIA1 introns and comparative support (Michel, Umesono, and Ozeki 1989; Toor, Hausner, and Zimmerly 2001) (see Methods). Structure models of individual introns can be found in Supplementary Data Figure 1 (Supplementary Material online). Although one might expect the foldings to be straightforward because the introns are homologous and several structures are published (Michel, Umesono, and Ozeki 1989), it was in fact difficult to arrive at refined structures, due to many sequence changes, numerous deletions and lengthy insertions, and substantial structural variation. Consequently, it was critical to use extensive sequence comparisons among related trnK sequences to arrive at accurate foldings. It was found that with careful comparisons, regions initially appearing irregular could be refolded into fairly conventional structures with minor irregularities. This complication underscores the general difficulty of folding plant organellar introns into accurate secondary structures, in contrast to bacterial group II introns, and shows the necessity of detailed structural comparisons for plant introns. Despite these comparisons, some uncertainties remained, which are listed in the legend of Supplementary Data Figure 1 (Supplementary Material online). In all cases, the uncertainties correspond to deviations for which no folding could be found to agree with the consensus structure or with closely related introns, and we predict that these regions deviate from the standard structure, even if the modeled pairings are not completely accurate.

The final secondary structures were compared with each other to analyze their differences. First, consensus structures were made for each subgroup of trnK introns (e.g., mosses, eudicots), with the consensus structure representing 100% identity of homologous positions in the RNA structures. Further consensus structures were made for combined subgroups until a consensus structure was obtained for all trnK introns. Comparisons among the consensus structures and among individual introns showed many deviations, which are listed in table 1. The changes can be categorized according to how many introns contain them, which suggests the timing of acquisition. One type of deviation is found in isolated examples, and suggests a relatively recent change. For example, there is a deletion of domain ID(ii)1 in Arabidopsis and a deletion of domain IC2 in Porella which can be predicted to have occurred recently because they are found in only one example (table 1).
Table 1: Deviations Within *trnK11* RNA Structures Compared to the Predicted ML Class Ancestor

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<th>Wheel Sequence</th>
<th>5' End</th>
<th>J1/2</th>
<th>J2/3</th>
<th>J5/6</th>
<th>3' End</th>
<th>l(i)</th>
<th>IB</th>
<th>n-α'</th>
<th>IC1</th>
<th>(i)</th>
<th>0 Motif</th>
<th>IC2</th>
<th>Size</th>
<th>ID(iii)</th>
<th>IC3</th>
<th>D2</th>
<th>D2(τ)</th>
<th>D3(Ψ)</th>
<th>D3</th>
<th>D4</th>
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<td>B</td>
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<td>sh</td>
<td>1</td>
<td>Loss***</td>
<td>67</td>
<td>Loss</td>
<td>1'</td>
<td>MP</td>
<td>AU</td>
<td>1'</td>
<td>MP</td>
<td>+</td>
<td>Loss of</td>
<td>ID(iii)2</td>
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<td>2 1st B's</td>
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<td>7</td>
<td>Loss***</td>
<td>1 bMP</td>
<td>193</td>
<td>Loss</td>
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<td>MP</td>
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Abbreviations: MP, mispair; bMP, mispair at the base of a stem; dMP, mispair on the distal side of a stem; B, bulge; B opp, bulge in the opposite strand; SL, stem-loop; Irr, irregular folding; ND, no data; sh, shifted symmetry in the EBS1 loop (see Supplementary Data Figure 1; Michel, Umesono, and Ozeki 1989; Toor, Hausner, and Zimmerly 2001).

Distinct mispairs and bulges in different positions are denoted by one to five ticks.

* All mispairs in this column are distinct.

** The missing nucleotide in J5/6 coincides with an extra bp in D6. These structures could be redrawn with a predicted less stable GG in J5/6 combined with an unusual loop at the bulged A in D6. In either case, the structure would deviate from the mitochondrial consensus.

*** It is not possible to exclude that a theta-like or eta-like interaction is supported by the diverged sequence.
In contrast, the loss of the α-α' pairing in Chaetosphaeridium cannot be dated because no close relatives are available. A second category of deviation is general to a phylogenetic subgrouping, indicating that a change occurred in a common ancestor. This type of deviation includes the loss of the θ motif in vascular plants and the gain of a stem-loop in angiosperms between ID(i) and I(ii). Third, some RNA structural features are shared among all trnK intron RNAs, but differ from the consensus of ML introns, suggesting that these features were present in the common ancestor of trnK introns. Examples include the loss of the D2 tetraloop with the η-η' interaction and a γ-γ' pairing of AU, whereas most ML introns have a GC pair. Finally, in addition to substantial changes in sequences and motifs, there are a multitude of minor changes that are observed in both conserved and nonconserved regions, such as mispairs, small insertions, or minor sequence changes (see Supplementary Data Figures 1 and 2, Supplementary Material online). It should be noted that C to U RNA editing may correct some of these deviations and re-store pairing to a more conventional structure, as has been shown in the case of domain 6 of the wheat nadH4 intron (Farré and Araya 1999); however, this possibility would apply to only a small proportion of the single mispair deviations and not to the more numerous indels or extended mispairings.

Based on these differences, we can reconstruct the evolution of the intron RNA structure during plant evolution. This reconstruction is summarized briefly in Figure 5A in detail in Figure 2 in Supplementary Data (Supplementary Material online). The distant ancestor is predicted to be a typical mobile group II intron of the ML clade, having a standard A1 RNA structure and self-splicing activity (fig. 5A). The last common ancestor of trnK introns had an RNA structure with the following modest modifications from the ML consensus: the η-η' and β-β' interactions were lost, as was the internal loop motif of domain 3; the γ-γ' pairing was A-U; and the base pair at the base of domain 5 was A-U, whereas most ML introns have a G-C pair (fig. 5B). Among the early-branched charophytes, there were many significant deviations from the standard structure that suggest compromised RNA structure. The Chaetosphaeridium intron lost the α-α' pairing and the ID(iii)2 motif. The Chara intron acquired weakened I(i) and α-α' pairings, lost stable pairing for ID(ii)1, gained two mispairs in domain 5, and gained large insertions in domains 3 and 6. In Tolypella, all pairing in domain I(i) was lost, while both Tolypella and Nitella lost the 0-0' interaction. Together, these changes suggest a compromised structure compared to the ancestral intron, with the changes being acquired independently in each charophyte lineage.

In land plants, different deviations occurred compared to charophytes (fig. 5C). As a group, land plant trnK introns acquired an AA in place of the conserved AC bulge in domain 5. The introns also diverged individually, for example, by losing domain IC2 (Porella, Physcomitrella, and Plagiomnium), gaining a large insertion in IC2 (Anthoceros) or domain 3 (Sphagnum, Anthoceros), losing pairing in ID(iv) (Pellia), or gaining a mispair in domain 5 (Anthoceros). As a group, the main change among vascular plants was the loss of the 0-0' interaction (fig. 5D), while there were many individual changes. Hornwort, fern allies, and conifers obtained substantial insertions in IC2, while all except Equisetum obtained a large insertion in domain 3. Pinus gained an insertion within ID(iii), and Psilotum gained an insertion between ID(i) and I(ii). Among the clade of angiosperms, the intron gained a conserved A-A mispair in domain 5 and an insertion of a stem-loop between ID(i) and I(ii) (fig. 5E). Again, individual changes are seen, which occurred recently, including a change in the conserved GUGYG sequence to GUAAG in Zea and loss of ID(ii)1 in Arabidopsis. It should be noted that all trnK introns except four had a variety of mispairs in ID3(ii) (EBS1 stem) but in different places (table 1). Such mispairs are not common in other group II introns, and the variety seen for the trnK introns suggests a need for flexibility in positioning EBS1. We also note that the earliest branching introns (charophytes) appear more defective than introns in higher plants, a generalization that seems to apply to other introns as well (R. Olson, I. Johnson, S. Zimmerly, unpublished data).

Together, the data illustrate that RNA structural deviations accumulate during plant evolution, from a predicted ancestor that resembles a self-splicing intron. While some changes introduce new structures (e.g., the ID(i)/I(ii) stem in angiosperms), most changes are losses of motifs associated with ribozyme activity. A second major type of change comes from insertions or deletions in peripheral regions; however, the newly inserted sequences do not form conserved structures that might contribute to ribozyme function (e.g., the D3 insertion in conifers cannot be folded into a common structure); hence, we argue that motif loss is the prevailing trend during trnK intron evolution. Mispairs within stem structures are also very common among individual introns, even within the highly conserved domain 5. While it is impossible to conclude the biochemical consequences of all the individual changes, it is hard to imagine that cumulatively they do not compromise ribozyme function of the trnK intron, considering that many changes are in conserved regions and important motifs (e.g., mispairs in domain 5; loss of α-α', κ-κ', θ-θ', and η-η' interactions). This conclusion is supported by mutational studies of some of these motifs in other introns. Mispairing of the α-α' interaction in the yeast al5γ intron blocked splicing (Harris-Kerr, Zhang, and Peebles 1993); point mutations in the highly conserved residues of the κ motif also blocked splicing (Boudvillain and Pyle 1998); and mutations in the θ motif of the yeast al5γ and cox/I1 introns decreased efficiency of splicing, while mutations of the η motif had modest effects (Costa et al. 1997).

The pattern of RNA deviations for the trnK intron agrees with previous studies on the evolution of the petD intron in angiosperms and gymnosperms (Löhne and Borsch 2005). Mutations did not accumulate uniformly throughout the intron during evolution but were concentrated in domains II, III, and IV (less critical regions). Domains Ic and II were the most variable in sequence, with hotspots occurring in loops rather than stems. The rpl16 intron of Myoporeaceae similarly showed heterogeneous substitutions, with highest levels in domain 2 (Kelchner 2002).

The pattern of ongoing degeneration of the trnK11 RNA structure during plant evolution is consistent with
Fig. 5.—Summary of evolution of the trnK11 secondary structure. Folded introns were analyzed for differences, and an evolutionary progression was reconstructed as described in the text. The brief summary here outlines the most important changes, while a detailed depiction can be found in Supplementary Data Figure 2 (Supplementary Material online). Features in blue are changes found for an individual intron or localized subsets of introns. Features in red indicate differences retained by all descendant introns. A comparison of the panels illustrates an accumulation of deviations from the ancestral, putatively self-splicing structure. Abbreviations are as follows: Chaeto, Chaetosphaeridium globosum; Chara, Chara connivens; Nit, Nitella opaca; Toly, Tolypella nidifica; March, Marchantia polymorpha; Pel, Pellia borealis; Pori, Porella baueri; Sphag, Sphagnum inanumatum; Phys, Physcomitrella patens; Plag, Plagiomnium insign; Antho, Anthoceros formosae; Equi, Equisetum arvense; Psi, Psilotum nudum; Arab, Arabidopsis thaliana; and Zea, Zea mays.
the scenario that the self-splicing function of group II introns came to require host-splicing factors in organelles to alleviate deficiencies in ribozyme function (Lambowitz et al. 1999; Toor, Hausner, and Zimmerly 2001; Barkan 2004). Splicing factors appear to have been adapted independently in different organisms, often from proteins with other known functions (Lambowitz et al. 1999; Barkan 2004). It follows that as splicing factors emerged in organelles to alleviate ribozyme defects, further RNA structure degeneration would be tolerated, until eventually the splicing reaction would be under the control of the host cell. If this scenario is true, it is interesting to consider that patterns of intron structure degeneration may correspond to the specific splicing factors available. Introns dependent on different factors may consequently have distinct patterns of RNA structure irregularities.

To test whether the observed patterns of RNA structure evolution extend to other group II introns, we examined the nad1I4 (nad1I728) intron of plant mitochondria, which encodes the MatR protein. In some respects, this intron is the mitochondrial equivalent of the trnK intron because it is the only intron in its organelle to encode an ORF. The intron is found in all land plants diverging after liverworts (Qiu et al. 1998; Qiu and Palmer 2004), and like MatK, the function of MatR is not completely clear. The ORFs have two insertions totaling nearly 300 amino acids, located between domains 4 and 5, and 7 and X, and the ORFs are also missing domains 0–1 (Zimmerly, Hausner, and Wu 2001). It is tempting to suggest that the function of MatR might also be as a generalized splicing factor because it is the only potential maturase encoded in the mitochondrion, but there is no specific evidence.

The RNAs of the five angiosperm nad1I4 introns were folded, along with Sphagnum and Notothylas (hornwort) introns (Supplementary Data Figure 3, Supplementary Material online). All introns were found to have lost domain IC2, to contain mispairings in the epsilon motif region, and to have acquired inserted nucleotides in the single-stranded flanks of I(i), together suggesting that these deviations were present in the nad1I4 ancestor (ancient changes). Deviations for individual introns were also found (recent changes) and include the loss of pairing within 1D2 in Notothylas, severe mispairing in I(i) of Sphagnum, and IB irregularities in angiosperm structures. Thus, the nad1I4 structures support our hypothesis of ongoing RNA structural degeneration for plastid introns.

We also considered additional introns in mitochondria and chloroplasts that do not encode ORFs. The introns trnA1I and trnI1I are highly conserved in chloroplasts, and the introns were folded and compared for Coleochaete orbicularis (green alga) and tobacco (not shown). The C.o. trnA1I and C.o. trnI1I lack the motifs ID(ii)1 and IA, respectively, while tobacco have intact motifs, indicating loss of the features in C. orbicularis since their divergence. In plant mitochondria, the related introns Anthoceros punctatus nad5I1 and M. polymorpha coxl2 were folded and found to differ by minor indels and by a mispair in D5 (M.p. coxl12) and disruptive bulge in D6 (M.p. coxl2; not shown), again indicating degeneration in liverwort since their common ancestor. Similarly, the homologous introns of liverwort nad2I1 and Arabidopsis nad2I3 were compared. The Arabidopsis intron differs by a large insertion in D6 and by mispairs in D5 and other stems in the structure (not shown), which represents a deviation in Arabidopsis since the split of the liverwort and Arabidopsis ancestors. Therefore, within multiple intron families across plants, and in both organelles, there are clear examples of both shared (ancient) and sporadic (recent) deviations from a presumed ancestral structure, suggesting that the patterns of ongoing RNA structural degeneration seen for trnK1I are general to plants.

Overall, the picture of RNA structure evolution that emerges in this study is consistent with our previous model for evolution of group II introns (Toor, Hausner, and Zimmerly 2001). Specifically, we predicted that organelle group II introns are degenerate versions of mobile bacterial introns, in which the introns often lost their ORFs and the RNA structures frequently degenerated in multiple ways. While this study does not address the issue of ORF loss, it does provide clear evidence for ongoing RNA structural degeneration in a chloroplast intron, which we argue is a general phenomenon for introns in both organelles. An interesting question is this: given the many degenerated forms of introns in plant organelles, why are there so many copies (~20 each in mitochondria and chloroplasts)? Was there an ancient explosion of mobile introns in organelles followed by massive loss of ORFs? Or are the degenerated introns still mobile in some way, perhaps with the help of cellular factors?

Supplementary Material

Supplementary Data Figures 1–3 are available at Molecular Biology and Evolution online (http://www.mbe.oxfordjournals.org/).

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