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Evidence for RNA editing in mitochondria of all major groups of land plants except the Bryophyta

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ABSTRACT **RNA editing has been documented in mitochondria of higher plants, notably dicots and monocots. To determine the distribution of mitochondrial RNA editing in the plant kingdom, we have now undertaken a survey of evolutionarily distant plants. RNA editing occurs in all major groups of land plants except the Bryophyta, suggesting that this process is an ancient trait that was established before the radiation of kormophyte plants. No editing is observed in representatives of the green algae, suggesting that editing arose in early land plants after the split of the Bryophyta or has been lost selectively in both algae and mosses. In ferns several U → C changes are observed, one of which eliminates a genomically encoded UAA termination codon and creates a functional open reading frame.**

RNA editing has been observed in mitochondria and chloroplasts of angiosperm plants belonging to the Dicotyledonae and Monocotyledonae (1–3). In both organelles specific cytidines of the primary transcripts are altered to uridines in the mature mRNAs. Only a few instances of editing have been found in chloroplasts (4–6), whereas numerous editing events have been documented in mitochondria of higher plants for almost all mRNAs investigated (7–11). In the more thoroughly investigated higher plant species, all of the open reading frames encoding conserved functional polypeptides appear to be edited to some extent and the total number of editing sites documented amounts to 294 in wheat (11) and to >400 in *Oenothera* (10). The observed effect of this extensive editing on the encoded polypeptide sequences suggests that many of the higher plant mitochondrial genes would not encode functionally competent products without appropriate "correction" by RNA editing.

Mitochondrial RNA editing has been reported in a number of angiosperm species, including the monocots wheat and maize and the dicots *Oenothera, Sorghum, Petunia, Daucus*, and *Arabidopsis* (7–11). The observation of analogous RNA editing in both monocots and dicots suggests that the origin of this process predates the divergence of the two lineages and is generally present in angiosperms. Recently, RNA editing has also been described in the gymnosperm *Thuja* (12). However, none of the plant species presumed to be closer to the evolutionary origin of flowering plants has been investigated. The only mitochondrial sequence data available allowing any inference about the need of RNA editing in nonflowering plants are from two bryophytes.

Analysis of protein sequences deduced from the complete genomic nucleotide sequence of the liverwort *Marchantia polymorpha* (13) and comparison of several cDNA and genomic DNA sequences (14) did not show any evidence of RNA editing. Genomic sequence analysis of a mitochondrial gene in the moss *Physcomitrella patens* (15) allows a similar deduction, suggesting that RNA editing might not occur in mitochondria of the two bryophytes.

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To gain further information about the origin of RNA editing in plant mitochondria, representative species from taxonomic groups of land plants other than bryophytes and flowering plants have to be investigated for the presence or absence of this process.

To determine the distribution of RNA editing in mitochondria of the major lineages of land plants, we have now surveyed both genomic DNA and cDNA sequences in representative species from the Spermatophyta, Bryophyta, and Pteridophyta. Evidence for RNA editing is found in all kormophytes investigated, whereas bryophytes and green algae show no editing in the mitochondrial gene analyzed.†

MATERIALS AND METHODS

Plant Material. Green leaf tissues from the plant species examined were obtained from the Berlin Botanical Garden, purchased from local flower shops, and collected in Berlin forests. Species from the Botanical Garden were kindly identified by I. Hagemann. Green algae were obtained from the Sammlung für Algenkulturen, Pflanzenphysiologisches Institut der Universität Göttingen, and are identified as *Coleochaete* sp. SAG50.90 and *Stichococcus* sp. SAG107.80, respectively.

Isolation of Nucleic Acids. Total cellular nucleic acids were isolated as described (16–18). Purified preparations were divided and treated separately for DNA and RNA analyses. DNA was cleaned by exhaustive treatment with RNase A to remove all RNA. To obtain total cellular RNA, the nucleic acid preparations were digested with RNase-free DNase (Boehringer Mannheim).

Amplification of Nucleic Acids. Two primers were designed corresponding to two regions of the cytochrome oxidase subunit III gene (*coxIII*) that are conserved between angiosperms and the moss *Physcomitrella* (15, 19, 20). The first nucleotides of the primers 5′-GTAGATCCAAGTCCATGGCCT-3′ and 5′-GCATGATGGGCCCAAGTTACGGC-3′ align with nt 34 and 458, respectively, of the *Oenothera coxIII* sequence (20). The intervening sequence fragments were amplified from total DNA preparations and from first-strand cDNA, respectively, with 30 cycles of 1 min at 94°C, 2 min at 60°C, and 4 min at 72°C. Denaturation time at 94°C was 4 min in the first cycle only.

Analysis of cDNA Sequences. Reverse transcription and first-strand cDNA synthesis were done with N_6-random primers by standard procedures (21) or with a cDNA kit (Boehringer Mannheim). Control reactions of the cDNA amplifications were routinely done in parallel except that RNase A replaced reverse transcriptase. Amplification products were purified by agarose gel electrophoresis and cloned in modified pBluescript vectors by using the *Nco* I site of the 5′ primer and the *Apa* I site created by one mismatch in the

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†The sequences reported in this paper have been deposited in the GenBank data base (accession nos. X76270–X76282).

3′ primer. Sequences were determined by the chain-termination method (22).

RESULTS

Selection of Plant Species. To investigate the extent and distribution of RNA editing in mitochondria of the major lineages of land plants, we analyzed genomic DNA and cDNA sequences coding for subunit III of cytochrome oxidase (*coxIII*) in representative species from Spermatophyta, Bryophyta, and Pteridophyta. Within the Spermatophyta, angiosperms were represented by *Oenothera berteriana* for the Dicotyledonae, and data for another species, belonging to the Monocotyledonae, *Triticum aestivum*, were taken from the literature (19). Representatives for the gymnosperms were *Ginkgo biloba, Picea abies*, and *Cycas revoluta*. For the Pteridophyta, *Equisetum arvense, Psilotum nudum, Osmunda claytoniana*, and *Asplenium nidus* were analyzed. Also from the Pteridophyta, *Selaginella elegans* and *Lycopodium squarrosum* were investigated as representatives of the Lycopodiates. The bryophyte *Sphagnum palustre* cDNA and genomic sequences were compared with the available genomic sequence data from *Physcomitrella patens* (15) and *Marchantia polymorpha* (13). As representatives for the green algae, *Coleochaete* sp. and *Stichococcus* sp. were analyzed.

Selection of the Diagnostic Coding Region. The well-conserved mitochondrial gene coding for subunit III of the cytochrome *c* oxidase (*coxIII*) was chosen for analysis. At the initiation of this investigation, this was the only gene for which sequence information was available from a plant species outside the angiosperms (the archegoniate *Physcomitrella*). With the completion of the entire mitochondrial genome sequence of the liverwort *Marchantia* (13), other genes are now similarly amenable for selection. A second reason for selecting the *coxIII* coding region for an investigation of RNA editing was the observation of numerous editing sites in this gene in both monocot and dicot plants (ref. 19 and unpublished results).

Two conserved regions in the open reading frame were identified in sequence alignments of *coxIII* genes that could be used to produce oligonucleotide sequences for amplification. A 381-nt fragment can be amplified between the two oligonucleotide primers corresponding to the two evolutionarily highly preserved domains (Fig. 1). The genomic and cDNA sequences were amplified from total DNA or RNA and analyzed in several independent clones. Nucleotide differences observed in more than one of the clones were considered RNA editing sites.

Genomic and cDNA Sequences Are Identical in Green Algae. The occurrence of RNA editing outside of land plants was investigated in the green algae *Coleochaete* and *Stichococcus* as representatives of the Charophyceae. Charophyceae are discussed as potentially close to the ancestors of land plants and are, therefore, candidates in which to investigate mitochondrial RNA editing, particularly in regard to whether this process arose before the divergence of land plants.

When genomic and cDNA sequences of the mitochondrial *coxIII* coding region from these algae were compared, no difference was observed. Thus RNA editing is not involved in expression of this gene and may be absent entirely from mitochondria of these algae. Likewise, no evidence for mitochondrial RNA editing has been found in the *Chlorella*-like alga *Prototheca wickerhamii* (G. Wolff, U. Kück, I. Plante, B. F. Lang, G. Burger, personal communication), suggesting that RNA editing may not occur in green algae.

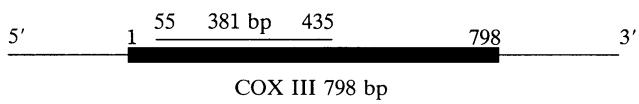

FIG. 1. Location of the *coxIII* coding region analyzed in genomic and cDNA sequences. Coding sequences were amplified between two oligonucleotide primers designed for two regions of the *coxIII* gene conserved in higher plants and mosses. The 381 nt analyzed are located between nt 55 and 435 of the 798-nt *Oenothera coxIII* coding sequence (7).

RNA Editing Is Not Seen in Bryophytes. From the *coxIII* genomic sequence in *Physcomitrella* and the complete mitochondrial genome sequence of *Marchantia*, there seems to be little or no need for RNA editing to adjust the encoded amino acid sequences to the conservative requirements deduced in interspecific comparisons of animals, fungi, and higher plants (13, 15). These observations suggest that mitochondrial RNA editing is absent from these species. Investigation of several potential editing sites in the liverwort *Marchantia* has indeed given no evidence for such mRNA sequence alterations (14).

Since liverworts (Hepaticophytina) and leafy mosses (Bryophytina) are phylogenetically rather distant, representatives of the Bryophytina should be included in the analysis of RNA editing. We have thus investigated genomic and cDNA sequences of *coxIII* in the moss *Sphagnum* and found that both derived sequences were identical (Fig. 2). This suggests that RNA editing does not occur in this species, at least not in the sequence analyzed. These direct data from *Sphagnum* and *Marchantia* and the conclusion from the genomic analysis of *Marchantia* and *Physcomitrella* mtDNAs suggest RNA editing is very rare or even completely absent from the entire lineage of the Bryophyta.

RNA Editing in Lycopodiaceae. In the lycopsid *Selaginella*, genomic and cDNA sequences amplified from DNA or RNA to produce the 381-bp PCR products were also identical. The analyzed region, however, surprisingly showed the highest similarity to the sequence of the alga *Coleochaete* with 72.4% nucleotide identity between the two species. Similarity between the *coxIII* genes of this lycopsid and the other plant species investigated was in all instances <72.4%, whereas sequence identity among the other land plants was always >78% (for details, see ref. 23). In the second lycopsid investigated, *Lycopodium*, PCRs of DNA yielded two products, one of the expected size of 381 bp and a second of 1155 bp. The sequence of the small product is identical to that of *Selaginella*, reproducibly so with independent DNA isolates and PCRs. The other *Lycopodium* DNA product of 1155 bp encodes a 774-bp group II intron (for details, see ref. 23) and adjacent "typical" mitochondrial sequences, with >80% nucleotide identity with *coxIII* sequences of other plants. Amplification of cDNA from RNA yielded only sequences corresponding to the spliced product of this gene, further evidence of correct and exclusive reverse transcription from RNA in the cDNA syntheses. Comparison of genomic exon and cDNA sequences reveals a single editing site in the region analyzed.

These results suggest that the *coxIII* sequences are nuclear encoded in *Selaginella* and present in both nuclear and mitochondrial genomes in *Lycopodium*. The actively transcribed intron-containing copy in *Lycopodium* is probably located in the mitochondrion, whereas the nuclear genome in this plant encodes a presumably silent sequence. The single editing event observed in *Lycopodium* shows that this process indeed occurs in mitochondria of Lycopodiaceae, although possibly to a lesser extent than in other plants.

RNA Editing in Ferns. The *coxIII* sequences of all the other plants investigated are clearly of mitochondrial origin and show numerous RNA editing sites, mostly C → U transitions (Fig. 2). However, several U → C alterations were found in the two true ferns analyzed. In *Asplenium* two such "reverse" RNA editing events are observed, one of which alters a genomic TAA translational stop codon to the CAA glutamine triplet conserved in this position in all other plants. The

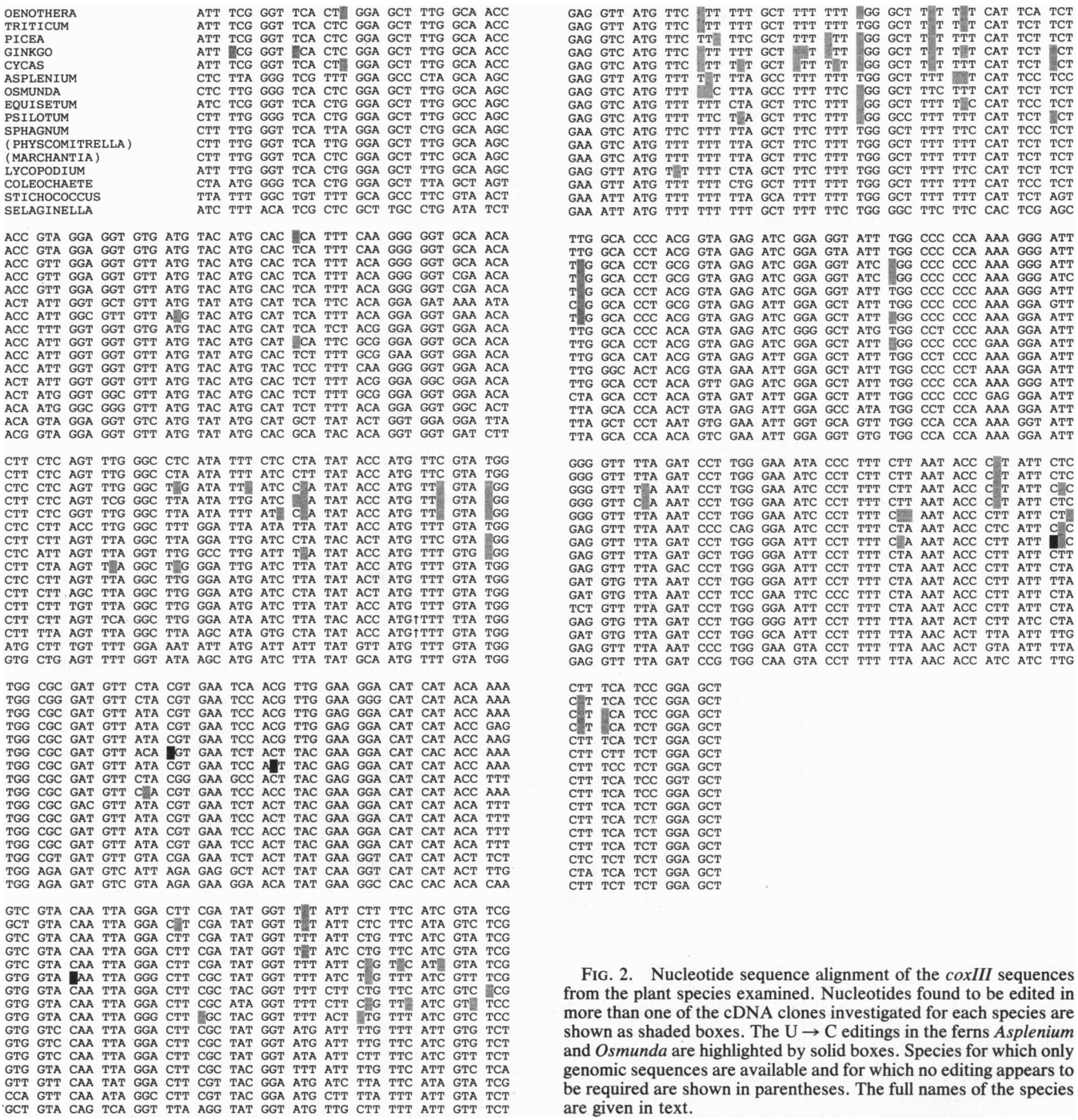

FIG. 2. Nucleotide sequence alignment of the *coxIII* sequences from the plant species examined. Nucleotides found to be edited in more than one of the cDNA clones investigated for each species are shown as shaded boxes. The U → C editings in the ferns *Asplenium* and *Osmunda* are highlighted by solid boxes. Species for which only genomic sequences are available and for which no editing appears to be required are shown in parentheses. The full names of the species are given in text.

second reverse editing event in this fern changes a TGT cysteine codon to a CGT arginine triplet, which is likewise conserved in other plant species. Although we cannot formally rule out the presence of another gene copy with an intact open reading frame, we consider this unlikely, since such a sequence should have been detected in the genomic DNA amplification. Furthermore, one cDNA clone was found to be unedited at this stop codon but was edited at many of the other sites—including the second reverse editing event—suggesting that this clone represents an intermediate mRNA transcribed from this genomic sequence. These two reverse RNA editing events in *Asplenium* are very frequently edited, the first observed in five of the six and the latter observed in six of the six cDNA clones analyzed. The two reverse editing events found in the second fern, *Osmunda*, are located in nonhomologous positions, one immediately adjacent to a forward C → U alteration (Fig. 2). Both reverse editings in *Osmunda* are altered in all cDNA clones and are thus also edited frequently. This very efficient editing and the high conservation of the edited triplets confirms the importance of these editing events for functional protein products. The identification of these essential U → C RNA editing events in plant mitochondria further corroborates that this reverse editing is an integral biochemical pathway of RNA editing in this organelle (19, 24).

RNA Editing Occurs in All Vascular Plants. The representative plant species investigated from all the other major groups of land plants show the C → U type of RNA editing exclusively. Most extensive editing in the investigated *coxIII* region is observed in *Ginkgo biloba* with 21 alterations in the 381-nt sequence (Fig. 3). The two other gymnosperms investigated also showed a comparatively high degree of editing

	OEN	TRI	PIC	GIN	CYC	ASP	OSM	EQU	PSI	LYC
OEN	9									
TRI	5	7								
PIC	3	3	16							
GIN	6	6	12	21						
CYC	4	2	6	9	19					
ASP	1	1	2	2	2	8				
OSM	2	1	5	5	5	3	12			
EQU	2	1	3	4	4	2	2	7		
PSI	2	0	3	3	3	0	2	1	10	
LYC	0	0	0	0	0	0	0	0	0	1

FIG. 3. Editing sites in the *coxIII* sequences from various species analyzed. The diagonal gives the number of editing sites observed in the respective species, and the number of editing events common to various species is listed below the diagonal. OEN, *Oenothera berteriana*; TRI, *Triticum aestivum*; PIC, *Picea abies*; GIN, *Ginkgo biloba*; CYC, *Cycas revoluta*; ASP, *Asplenium nidus*; OSM, *Osmunda claytoniana*; EQU, *Equisetum arvense*; PSI, *Psilotum nudum*; LYC, *Lycopodium squarrosum*.

with 19 and 16 sites in *Cycas revoluta* and *Picea abies*, respectively. If the many RNA editing events observed in the *coxI* coding region of the conifer *Thuja* (12) are also considered, then RNA editing may generally be more frequent in gymnosperms than in angiosperms. Significant species-specific differences in editing were, however, also seen among various angiosperms, and to identify a general tendency, more genes need to be investigated in these plants.

The representative species investigated for the Equisetaceae (*Equisetum arvense*) and Psilotaceae (*Psilotum nudum*) show a number of RNA editing events, all but one at different nucleotide positions in these two nonflowering plants (Fig. 3).

DISCUSSION

Evolution of RNA Editing Activities in Fern Mitochondria. The observation of comparatively frequent reverse editing in the two fern species may be significant with respect to the evolution of RNA editing in plant mitochondria. The functional requirement for a competent and efficient U → C editing reaction possibly indicates a somewhat different editing mechanism than that in the flowering plants with altered reaction kinetics that more readily allow editing activities in both directions. This interpretation implies that a more recent common ancestor of the flowering plants has evolved a once bidirectional editing mechanism to the preferentially unidirectional RNA editing mechanism observed today in these plants or vice versa.

RNA Editing Specificity Is Not Inherently Determined by the Neighboring Nucleotides. The species-specific differences of edited nucleotide positions in the highly conserved *coxIII* sequences in this wide range of plants allow extension and generalization of deductions on the specificity requirements of the editing activity. Comparisons of RNA editing patterns between the two species have previously allowed the conclusion that the specificity of RNA editing may not be solely and intrinsically located in the primary sequence information immediately surrounding an editing site (25, 26). This inference can now be further extended and qualified with more species-specific data available for a single conserved region in a larger number of plant species.

The last (silent) editing site in *Cycas* for example is not observed in *Triticum* or *Oenothera* although relative to this editing site 15 nt downstream and 6 nt upstream before and 14 nt after editing in the angiosperms are identical (Fig. 2). Analogously, the first (also silent) editing site in both *Cycas* and *Triticum* is not altered in *Oenothera* or *Picea*, although at least 14 nt upstream and 42 nt downstream are identical between any pair of plants that are asymmetrically edited. These observations show that a nucleotide to be edited is not singled out solely by the immediate sequence context *per se* but that additional factors must be involved that are independent of the adjacent primary sequence.

How Old Is RNA Editing in Plant Mitochondria? The results of this investigation show that RNA editing occurs in all vascular land plants and, thus, probably predates the evolution of the kormophytes (Fig. 4). Most plants show several editing sites in the region investigated, with no editing observed in bryophytes and only one single editing site found

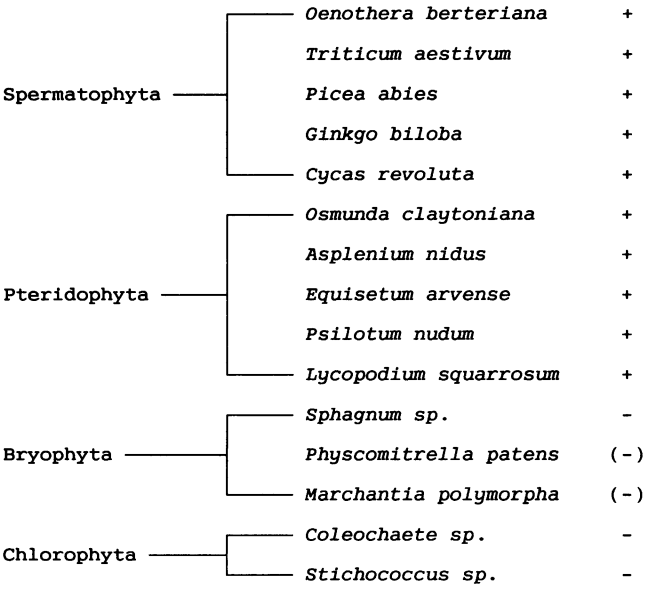

FIG. 4. Distribution of RNA editing in the plant kingdom. Species in which RNA editing has been observed are indicated by +, and species in which no editing has been seen are marked with a −. A (−) indicates species for which no editing is apparently required as deduced from the genomic sequences but for which no corresponding cDNA data are available.

in the lycopsid *Lycopodium* at a position different from any of the other plants investigated (Figs. 3 and 4). At all sites edited in other plant species, the lycopsid and bryophyte genomic sequences encode nucleotides corresponding to the edited sequences.

Two possible scenarios could explain this observation with respect to the origin of the RNA editing process in plant mitochondria. In the first, it is assumed that the common ancestors of present-day lycopsids and bryophytes were plants with mitochondrial RNA editing and that there was (and possibly still is) an evolutionary tendency to loose RNA editing within these branches. The trend would apparently be completed within the lineage leading to the bryophytes, where no editing is observed, and would have reduced the level of RNA editing in lycopsids. The second scenario supposes the reverse order in the evolution of RNA editing in plant mitochondria, in which RNA editing has arisen and spread during the evolution of the kormophytes. In this evolutionary order, RNA editing has never been present in bryophytes (and of course algae) and was established after the split of the bryophyte lineage from the ancestors of the kormophytes. Depending on how easily RNA editing can be lost once it is established, this latter scenario could be the more likely interpretation.

The fundamental difference in these two interpretations lies in the divergent timing of the invention of the plant mitochondrial RNA editing. The first alternative suggests that RNA editing is an old trait carried over from the ancestors of land plants. The second alternative suggests a more recent evolution of the editing process after plants had arrived on land and the ancestors of the bryophytes had split off but before lycopsids and the other kormophytes had separated.

In any case do the observations and deductions reported here imply that the appearance of mitochondrial RNA editing predates the divergence of all taxonomic groups of kormophyte land plants (Fig. 4). Further genes need to be investigated, especially in lycopsids—and of course green algae—to extend and generalize these conclusions.

The potential occurrence of mitochondrial RNA editing in nonland plants—i.e., algae and particularly those algal lineages thought to be most closely related to the ancestor(s) of land plants (e.g., Charophyceae)—has to be analyzed in more detail. Investigation of the representatives from this group of algae with the conserved primers for the mitochondrial *coxIII* sequence reported here shows that an analogous approach should be feasible also for species other than those investigated here. Since presently only few mitochondrial sequences are available from green algae, especially from the Charophyceae, RNA editing may nevertheless occur in these algae, although it is not apparent or does not seem to be required in the single gene in the two species investigated herein. A complete absence of editing in green algae would support an evolutionary appearance of mitochondrial RNA editing at the level of the first land plants, gaining in importance between *Lycopodium* and higher plants.

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