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Characterization of Mitochondrial Small-Subunit Ribosomal RNAs from Holoparasitic Plants

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Abstract. Mitochondrial small-subunit (19S) rDNA sequences were obtained from 10 angiosperms to further characterize sequence divergence levels and structural variation in this molecule. These sequences were derived from seven holoparasitic (nonphotosynthetic) angiosperms as well as three photosynthetic plants. 19S rRNA is composed of a conservative core region (ca. 1450 nucleotides) as well as two variable regions (V1 and V7). In pairwise comparisons of photosynthetic angiosperms to *Glycine*, the core 19S rDNA sequences differed by less than 1.4%, thus supporting the observation that variation in mitochondrial rDNA is 3–4 times lower than seen in protein coding and rDNA genes of other subcellular organelles. Sequences representing four distinct lineages of nonasterid holoparasites showed significantly increased numbers of substitutions in their core 19S rDNA sequences (2.3–7.6%), thus paralleling previous findings that showed accelerated rates in nuclear (18S) and plastid (16S) rDNA from the same plants. Relative rate tests confirmed the accelerated nucleotide substitution rates in the holoparasites whereas rates in nonparasitic plants were not significantly increased. Among comparisons of both parasitic and nonparasitic plants, transversions outnumbered transitions, in many cases more than two to one. The core 19S rRNA is conserved in sequence and structure among all nonparasitic angiosperms whereas 19S rRNA from members of holoparasitic Balanophoraceae have unique extensions to the V5 and V6 variable domains. Substitution and insertion/deletion mutations characterized the V1 and V7 regions of the nonasterid holoparasites. The V7 sequence of one holoparasite (*Scybalium*) contained repeat motifs. The cause of substitution rate increases in the holoparasites does not appear to be a result of RNA editing, hence the underlying molecular mechanism remains to be fully documented.

Key words: Plant molecular evolution — Substitution rates — RNA editing — Transversions

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Introduction

Sequences of a number of mitochondrial genes have been extensively used in phylogenetic studies of animals; however, such is not the case for plants. This stems primarily from the vastly different structure and dynamics of these two genomes; in particular, the exceptionally low rate of nucleotide substitution in plants which is 40–100 times less variable than in animals (Palmer 1992). Understanding of plant mitochondrial genome evolution is limited, being based primarily on studies of crop species such as *Zea* (Fauron et al. 1995) and *Brassica* (Palmer 1988). The number of silent substitutions per site for mitochondrial DNA is one-third to one-quarter that of chloroplast DNA and is 12 times lower than that of nuclear DNA (Palmer 1990; Palmer 1992). With the exception of *coxIII* (Hiesel et al. 1994; Malek et al. 1996) mitochondrial genes have been used less frequently than those from the nucleus or plastid for inferring evolutionary relationships in plants.

Plant mitochondrial genomes contain three ribosomal RNA genes: the small-subunit rDNA and 5S, which are tightly linked, and the large-subunit rDNA which (in

Table 1. Angiosperm mitochondrial 19S rDNA sequences compared with *Glycine*[a]

Species	Total length	Core length	V1 length	V7 length	Transitions (TN)		Total TN
					TC	AG	
Photosynthetic monocots							
Secale cereale	1971	1454	151	366	5	4	9
Triticum aestivum	1949	1454	151	344	5	4	9
Zea mays	1961	1454	144	350	6	3	9
Photosynthetic dicots							
Lindera benzoin	2114	1454	171	489	3	2	5
Lepidoceras chilense	1919	1453	102	364	2	2	4
Oenothera berteriana	1897	1453	106	338	3	3	6
Lupinus luteus	2017	1453	103	461	1	0	1
Glycine max	1984	1454	103	427	—	—	—
Nicotiana tabacum	1891	1454	102	335	4	4	8
Asterid holoparasite							
Epifagus virginiana	>1969	1452	87	>430	6	6	12
Nonasterid holoparasites							
Cytinus ruber	1873	1446	97	330	5	6	11
Rafflesia pricei	1934	1457	183	294	5	4	9
Hydonora africana	1996	1458	89	449	5	4	9
Corynaea crassa	1829	1448[c]	75	271	13	6	19
Helosis cayennensis	1823	1447[c]	144	217	9	8	17
Scybalium jamaicense	2087	1456[d]	39	484	19	15	34

[a] Substitutions (TN, TV), percent divergence, insertions, and deletions calculated using the core sequences only (i.e., excluding the V1 and V7 regions)

[b] The first number reflects the number of indel events, the second number the lengths (in nucleotides) of these indels

[c] Lengths excluding the unalignable V6 region corresponding to a 35 and 15 base insertion in *Corynaea* and *Helosis* respectively

[d] Length excluding the V5 and V6 insertions accounting for 108 nucleotides

angiosperms) lies elsewhere in the genome. Plant small-subunit rDNA is generally longer than the nuclear or plastid counterpart (ca. 1.9–2.0 kb), hence, it will be referred to here as 19S rDNA to distinguish it from nuclear 18S rDNA (ca. 1.8 kb). Only six angiosperm 19S rDNA sequences have been published: *Zea*, *Triticum*, and *Secale* (Poaceae), *Oenothera* (Onagraceae), and *Lupinus* and *Glycine* (Fabaceae). This low number reflects the perception that the entirety of sequence variation in angiosperms has already been adequately documented in these six species. Among the ribosomal RNA genes found in the three subcellular genomes of plants, comparisons show that the greatest number of substitutions occurs in nuclear 18S, followed by plastid 16S, and finally mitochondrial 19S rDNA sequences. This trend is consistent with the observed overall rates of organellar and nuclear evolution in plants (Palmer 1990).

Plant mitochondrial 19S rRNAs are composed of three parts: (1) a ''core'' sequence (ca. 1450 nucleotides) that is conserved in length and sequence among all plants, (2) the variable 1 region (V1) associated with helix 6 of the mature rRNA, and (3) a second variable region (V7) associated with helix 43 (nomenclature of helices according to Van de Peer et al. 1994). In contrast to the core sequence, which exhibits greater than 98.6% sequence similarity among angiosperms, the V1 and V7 domains are so divergent as to preclude unambiguous alignment. Furthermore, alignment cannot be guided by secondary structural features since length variation and lack of covariance data preclude construction of universal models. The only published angiosperm higher-order structural model of the core 19S rRNA is that of *Zea* (Gutell 1994).

Among parasitic flowering plants, some lineages are holoparasitic, i.e., they have lost photosynthesis and rely on the host for water and organic nutrients. A number of molecular investigations have focused on one holoparasite, *Epifagus* (Scrophulariaceae or Orobanchaceae), that has clear phylogenetic affinity with photosynthetic relatives of Asteridae. Holoparasitism has evolved in a number of other families for which relationships are less certain, for example Balanophoraceae, Hydnoraceae, and Rafflesiaceae (Kuijt 1969). Recent studies of the latter so-called nonasterid holoparasites have revealed increased nucleotide substitution rates for both nuclear 18S and plastid 16S rDNA (Nickrent and Starr 1994; Nickrent and Duff 1996; Nickrent et al. 1998; Nickrent et al. 1997a). In comparison to nonparasitic plants, these families have rates increased by 3.5 times for nuclear 18S rDNA (Nickrent and Starr 1994) and even higher rates for plastid 16S rDNA (Nickrent et al. 1997b). Given these observations, it was of interest to determine whether the mitochondrial rDNA genes of holoparasitic plants had experienced similar rate increases. Relatively little work has focused on substitution rates in plant mitochondrial genes, an exception being a study that examined the divergence date between monocots and dicots (Laroche et al. 1995).

Table 1. Extended

Transversions (TV)				Total		Total			
AT	AC	TG	CG	TV	TN/TV	subst.	% Divergence	Insertions[b]	Deletions[b]
2	2	3	2	9	1.00	18	1.2	0	0
2	2	3	2	9	1.00	18	1.2	0	0
3	2	4	2	11	0.82	20	1.4	1 (13)	0
1	0	0	4	5	1.00	10	0.7	0	0
2	0	1	2	5	0.80	9	0.6	0	1 (1)
2	0	2	2	6	1.00	12	0.8	1 (1)	2 (2)
1	3	3	3	10	0.10	11	0.8	0	1 (1)
—	—	—	—	—	—	—	—	—	—
1	2	0	4	7	1.14	15	1.0	0	0
1	2	5	2	10	1.20	22	1.6	0	2 (2)
2	5	10	5	22	0.50	33	2.3	0	4 (10)
1	9	4	2	16	0.56	25	1.7	1 (4)	1 (1)
7	6	13	8	32	0.23	43	3.0	3 (4)	0
3	7	5	6	21	0.90	40	2.8	2 (34)	1 (1)
4	7	5	8	24	0.71	41	2.8	2 (12)	2 (4)
7	25	38	7	77	0.44	110	7.6	6 (112)	4 (5)

The present study reports 10 PCR-derived sequences of angiosperm mitochondrial 19S rDNA. These new sequences, representing seven holoparasites and three additional photosynthetic angiosperms, were characterized at the primary and secondary structural level. As will be shown, sequence evolution and structural changes in the holoparasite 19S rRNAs represent the most extreme yet reported among angiosperms, thus paralleling previous results with nuclear 18S and plastid 16S rDNA. In addition to higher rates, a transversional bias is documented for most pairwise comparisons of parasitic and nonparasitic angiosperms.

Materials and Methods

The following six plant mitochondrial 19S rDNA sequences were obtained from GenBank: *Secale cereale* (Poaceae, Z14059, Coulthart et al. 1993), *Triticum aestivum* (Poaceae, Z14078, Coulthart et al. 1993), *Zea mays* (Poaceae, X00794, Chao et al. 1984), *Glycine max* (Fabaceae, M16859, Grabau 1995), *Lupinus luteus* (Fabaceae, Z11512, Augustyniak et al. 1992), and *Oenothera berteriana* (Onagraceae, X61277, Brennicke et al. 1991). Collection numbers for voucher specimens (of DLN, deposited at SIU) and Genbank accession numbers for the new sequences reported here are as follows: (1) photosynthetic nonparasites: *Lindera benzoin* (Lauraceae, 2901, U82646), *Nicotiana tabacum* (Solanaceae, 2917, U82638); (2) asterid holoparasite: *Epifagus virginiana* (Scrophulariaceae, 3016, U82642); (3) nonasterid holoparasites: *Corynaea crassa* (Balanophoraceae, 3011, U82636); *Helosis cayennensis* (Balanophoraceae, 3017, U82640); *Scybalium jamaicense* (Balanophoraceae, 3021, U82656); *Cytinus ruber* (Cytinaceae, 2738, U82639); *Hydnora africana* (Hydnoraceae, 2767, U82637); and *Rafflesia pricei* (Rafflesiaceae, 4034, U96694).

Total genomic DNA was obtained by grinding the fresh tissue to a powder on liquid nitrogen and extracting it using the 2X CTAB method, as described in Nickrent (1994). Amplification and sequencing primers developed for plant plastid 16S rDNA were used (Nickrent et al. 1997b) in addition to primers specific to plant mitochondrial 19S rDNA. For the latter, three forward primers (positions correspond to *Glycine*) were used: GAGTTTGATCCTGGCTCAGA (8), GCCGCTTGTAAAGCTC (434), and CTGCATGGCTGTCGTC (1035). Reverse primers (5′ to 3′ direction) were CAGCCACACTGCRACT (318), GCGTAAAGGGCACGTAGG (557), ACTGGAGGAAGGTGG (1567), and AGCCGTAGGGGAACCTGTGGC (1949). PCR amplification and product purification methods were as described in Nickrent (1994). The 19S rDNA products were sequenced directly using Sequenase® (U.S. Biochemical), except for *Corynaea* which was cloned prior to sequencing using the PCRII™ vector (TA cloning kit, Invitrogen, Inc.).

The conserved cores of the 19S rRNAs for the 16 species shown in Table 1 were manually aligned using the computer program SeqApp (Gilbert 1993). The V1 and V7 regions were excluded since they resulted in ambiguous alignment due to length mutations and sequence divergence. Similarly, insertions on helix 29 and 37 unique to Balanophoraceae were also excluded. This resulting core alignment was essentially unambiguous and was aided by reference to higher-order structures constructed for each sequence. (Alignments can be obtained from the authors upon request.) Structures were constructed using ClarisDraw following the model of *Zea* (Gutell 1994). Separate alignments were also made for sequences corresponding to the V1 (16 sequences) and V7 (15 sequences) regions (nucleotides 60–203 and 1167–1518, respectively, on the *Zea* model). Substitutional differences among the core sequences were determined using MEGA (Kumar et al. 1993). Relative rate tests (Sarich and Wilson 1967; Wu and Li 1985; Nickrent and Starr 1994) were used to quantify substitution rate differences between the parasitic and nonparasitic lineages.

Results

Comparisons of nonparasitic plants to the photosynthetic dicot soybean (*Glycine*) yielded divergence values for the core mitochondrial 19S rDNA sequences ranging from 0.6 to 1.4% (Table 1). The higher values were

obtained when comparing monocots or asterids to soybean, thus indicating that the modest sequence variation present within 19S rDNA does, at least to some degree, reflect phylogenetic differences. The asterid holoparasite *Epifagus* showed a sequence divergence of 1.6%, a value moderately increased over that obtained when another asterid (*Nicotiana*) is compared with soybean. The number of substitutions was increased in all nonasterid holoparasite mitochondrial 19S rDNA sequences (Table 1). *Rafflesia* (Rafflesiaceae) exhibited the smallest degree of sequence divergence (1.7%) followed by *Cytinus* (2.3%) and *Hydnora* (2.8%). With the exception of *Scybalium* (7.6%), members of Balanophoraceae showed substitution percentages comparable to *Hydnora.* Whereas insertion and deletion mutations (indels) were virtually absent from photosynthetic angiosperms, the sequences of the nonasterid holoparasites were characterized by a higher frequency of indels. Core sequence length varied by only two nucleotides among the photosynthetic dicots (and *Epifagus*) and was invariant among the three grass genera. The range in size among the nonasterid holoparasites was 14 nucleotides excluding the V5 and V6 regions that are unique to Balanophoraceae. It is noteworthy that this family contained greater variation in core length than was found among all other reported angiosperms.

Among all the nonasterid holoparasite sequences, transversions outnumbered transitions (Table 1). At the extreme, *Hydnora* showed nearly a 1:4 TN/TV ratio. Not all substitution types were equally represented. In a comparison of *Glycine* to *Scybalium,* the A to G and T to C transitions outnumbered the G to A and C to T types 19:2, respectively. For transversions, two of the four types (AC, TG; Table 1) were most frequent. Specifically, the C to A transversion was more frequent than A to C (17:1) and the G to T was more frequent than T to G (24:5).

Relative Rate Tests

Implementation of parametric relative rate tests presented several problems with the plant mitochondrial 19S rDNA sequences. As pointed out by Wu and Li (1985), to minimize the errors in substitutions per site, the reference should be an unambiguous yet closely related outgroup to the test organisms. Since the phylogenetic position of these holoparasites within the angiosperms is uncertain, assignment of an appropriate outgroup is problematic (Nickrent and Starr 1994; Nickrent and Duff 1996). Given this, and that these holoparasites are angiosperms, it might be expected that a gymnosperm could be used as a reference taxon. Tests using *Juniperus* (Cupressaceae) as a reference showed that the number of substitutions per site (K) from it to taxon one (K_{13}) and to taxon two (K_{23}) were disproportionately greater than comparisons between any two angiosperms (data not shown). Tests were then conducted using *Zea,* a monocot, as the reference to the nonasterid holoparasites. It is not clear, however, that all monocots diverged prior to the holoparasite lineages, hence the three-taxon requirement may be violated. Furthermore, the number of substitutional differences between any two photosynthetic dicots was less than 15 and less than 20 between monocots and dicots. For statistical significance, the Wu and Li (1985) test requires at least 20 substitutions, thus explaining the result that no significant tests were obtained among all photosynthetic dicots. The asterid holoparasite *Epifagus* had a positive K_{13}–K_{23} value; however, this value did not represent a statistically significant rate increase. In contrast, all the nonasterid holoparasites exhibited significant rate increases.

Higher-Order Structures

Higher-order structures constructed for the core portion of the mitochondrial 19S rRNA for all photosynthetic plants showed very little deviation from the model for *Zea.* Although canonical and noncanonical pairings differed in detail in variable helices (such as helix 49), all major structural elements were present. Despite the higher numbers of substitutions in the nonasterid holoparasites, most mutations resulted in compensatory changes that did not obviously disrupt secondary structure. Given the very high number of substitutions in the mitochondrial 19S rRNA of *Scybalium,* a secondary structural model was constructed to examine the specific effects of mutations on this molecule (Fig. 1). In contrast to all known photosynthetic plant 19S rRNAs, the model for *Scybalium* showed large insertions in the V5 (46 nucleotides) and V6 (56 nucleotides) regions. As was done with the V7 region, these insertions are represented in Fig. 1 only as blocks of sequence since no covariance data exist to propose a structure. The other two members of Balanophoraceae, *Helosis* and *Corynaea,* also had insertions in the V5 region (10 and 31 nucleotides, respectively) but did not have insertions for V6, thus suggesting the former structural feature is a synapomorphy for the family. Another unique feature to the *Scybalium* 19S rRNA is a V1 region truncated to only 36 nucleotides, i.e., much shorter than the mean (126 nucleotides) for nine nonparasitic angiosperms. The larger size of the *Scybalium* 19S rRNA (2,087 nucleotides as compared with *Zea* with 1,961 nucleotides) is primarily due to the V5 and V6 extensions and to a larger than average V7 region (484 nucleotides).

Substitutions can be categorized as (1) fully compensated when both members of a base pair change yet pairing is retained or (2) compensated when only one member changes but pairing is maintained (permitting the noncanonical pair G · U). Comparing the core sequence of *Scybalium* with the published sequence and secondary structure of *Zea,* 71 of the 99 total substitutions are transversions. These transversions were evenly

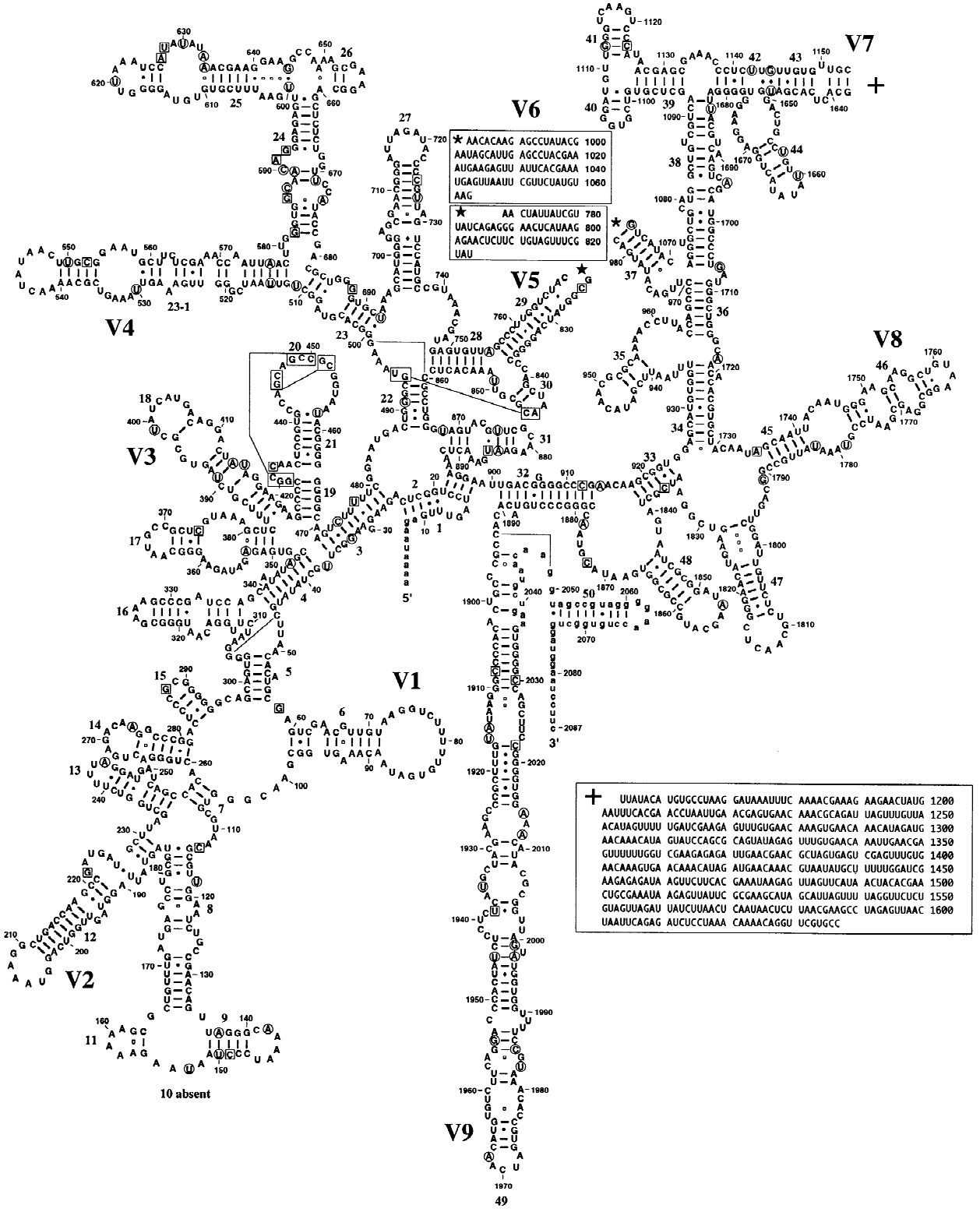

Fig. 1. Higher-order structural model for ''core'' mitochondrial-encoded small-subunit ribosomal RNA for *Scybalium jamaicense* (Balanophoraceae). Base pairing follows the covariance model proposed for *Zea* by Gutell (1994). Lowercase bases at the 5′ and 3′ end of the molecule were not determined because they are near priming sites (*Zea* sequence shown for clarity). Transitions (28 relative to *Zea*) are indicated by bases enclosed by boxes whereas transversions (71) are shown by bases enclosed by circles. Secondary structures are not known for the V5, V6, and V7 sequences enclosed within boxes. Among angiosperms, the V5 extension is found only in Balanophoraceae and the longer V6 is unique to *Scybalium.* Conversely, the 39 nucleotide V1 region of *Scybalium* is shorter than the mean for photosynthetic angiosperms (126 nucleotides).

distributed among stem and loop regions (37 and 34, respectively). Of the 99 total substitutions, 20 (10 pairs) involved fully compensated changes, 27 were compensated changes, 11 resulted in disruption of base-pairing (compared with *Zea*), and 41 were located in unpaired (loop) regions. Ten indels were noted that included six insertions of 112 nucleotides and four deletions of 5 nucleotides. These substitutions did not result in any obvious alterations of the secondary structure.

Characteristics of the V1 and V7 Regions

The results of sequencing additional angiosperms confirms that nearly all the length variation in mitochondrial 19S rRNA occurs in the V1 and V7 regions. This and high sequence divergence precludes unambiguous alignment, thereby impeding the construction of higher order structures for these regions. Sequences from both photosynthetic and holoparasitic plants have significantly expanded the known size ranges for the V1 and V7 regions. With the exception of *Lindera,* the V1 region for photosynthetic dicots is conserved in sequence and length (Table 1). The only significant length variation found in photosynthetic plants occurs in *Lindera* and monocots, the latter because of a single large insertion. In contrast, the parasitic plants are characterized by higher numbers of substitutions and a wide range in length. Some of the holoparasite V1 sequences are similar in sequence and in length to photosynthetic dicots (*Cytinus* 97, *Mystropetalon* 95, and *Epifagus* 87 nucleotides, respectively) whereas others are truncated (*Corynaea* 75, *Langsdorffia* 65, and *Scybalium* 39 nucleotides, respectively) or expanded (*Helosis* 144 nucleotides and *Rafflesia* 183 nucleotides). The large V1 of *Rafflesia* results from a unique 82 nucleotide insertion.

Among the photosynthetic dicots, the shortest V7 region was found in *Nicotiana* (335 nucleotides) and the longest in *Lindera* (489 nucleotides) which had a unique 142 nucleotide insertion compared with other angiosperms. Despite length variation, large portions of the V7 region can be aligned among the monocots and dicots (data not shown). The sequences from *Epifagus* and *Cytinus* were more similar in sequence to the photosynthetic dicots than to the nonasterid holoparasites. The shortest angiosperm V7 region sequences were those from *Helosis* (217 nucleotides) and *Corynaea* (271 nucleotides). These sequences lacked nearly all the conserved motifs found in photosynthetic plants but contained large segments that were mutually alignable. Among Balanophoraceae, the V7 region of *Scybalium* was the longest (484 nucleotides) and contained almost no sequence in common with *Corynaea* and *Helosis.* In addition, the *Scybalium* sequence was found to include several repetitive motifs. Two 39 base repeats were separated by 84 nucleotides of unique sequence. Included in each repeat unit were three eight-base subrepeats (TGAACAAA), two of which were in tandem and the third only four nucleotides downstream. Two additional repeats of the eight-base sequence were also found dispersed in the V7 region. In addition, a different 12-base sequence occurred twice with the V7 with each motif separated by 75 nucleotides.

Discussion

The ubiquitous ribosomal RNAs have been well characterized for prokaryotes, eukaryotes, and subcellular organelles and remain the most widely used macromolecule for inference of phylogenetic relationships. Among the three subcellular genomes in plants, comparatively little is known about mitochondrial ribosomal RNA genes. The 19S rDNA sequences presented here more than doubles the number previously available for angiosperms and allows further insight into sequence variation and evolution. The following discussion will compare and contrast these mitochondrial 19S sequences with nuclear 18S and plastid 16S rRNAs in terms of patterns and rates of substitution and the effect these have on the structure of the mature rRNA. Given the degree of sequence and structural variation observed in these normally conserved mitochondrial sequences, questions are raised concerning the biochemical and molecular evolutionary factors that are responsible for such change.

Nucleotide Substitutions, Composition Bias, and RNA Editing

Although all rRNAs are mosaics of conserved and variable domains, plant mitochondrial rRNA represents an extreme case when the conserved ‘‘core’’ is compared with the V1 and V7 variable domains. As with nuclear 18S (Nickrent and Starr 1994) and plastid 16S rRNA (Nickrent and Duff 1996; Nickrent et al. 1997a), nucleotide substitution rates in mitochondrial 19S rDNA are accelerated in Balanophoraceae, Hydnoraceae, and Rafflesiaceae. Given previous work on plastid genome evolution in holoparasites, a mechanistic explanation for such rate increases is not immediately apparent. For the nonphotosynthetic *Epifagus,* whose plastid genome has lost the majority of photosynthetic and chlororespiratory genes, relaxation of selection pressure on the translational function of the ribosome may permit increased fixation of mutations in rDNA and ribosomal protein genes (Wolfe et al. 1992). Additionally, an underlying increase in the mutation rates of plastid genes may be a result of changes in DNA replication and repair (dePamphilis et al. 1997; Nickrent et al. 1997a,b). Such explanations appear reasonable when applied to plastid genomes that have lost photosynthetic genes. Extending this idea to the mitochondrial genome would

suggest that, similarly, it has lost genes and has decreased ribosome translation efficiency. In contrast to plastid genomes that can exist without photosynthetic genes, it is not clear how many of the 16 respiratory genes encoded by the mitochondrion can be lost from this genome. Unlike photosynthesis, respiration is not dispensable in holoparasitic plants, hence it will be informative to determine which mitochondrial genes are present, their subcellular location, and their substitution rates.

Two genera, *Helosis* and *Corynaea,* differ in their core 19S rDNA sequences by 23 substitutions, i.e., more than that obtained when comparing a monocot with a dicot. This is remarkable because these genera are clearly very closely related, as reflected by morphology-based classifications (within tribe Helosieae (Hansen 1980)) and molecular phylogenetic analyses of nuclear 18S rDNA (Nickrent and Duff 1996; Nickrent et al. 1998). Furthermore, *Scybalium* is classified in the same subfamily (Scybalioideae) as *Corynaea* and *Helosis,* yet its 19S rDNA exhibits the most extreme increase in substitutions of any angiosperm and differs from the above two genera by 99 and 110 substitutions, respectively. These results demonstrate that substitution rates in the typically conservative mitochondrial rDNA core sequence can vary widely even among closely related taxa.

If all substitutional changes were equally probable, a transition or transversion bias would be indicated by a deviation from the theoretical rate ratio of 1TN:2TV. A strong bias towards transitional mutations is often observed for molecular datasets that have been analyzed in a comparative framework (Wakeley 1996). A rate ratio of 2TN:1TV was reported for nuclear 18S rDNA sequences following analysis of over 200 species (Soltis and Soltis 1998). Plastid 16S rDNA sequences also exhibit approximately the same ratio (Nickrent and Duff, unpublished data). These examples contrast with plant mitochondrial 19S rDNA sequences where transversions frequently outnumber transitions. In particular cases (*Hydnora* and *Scybalium*), transversions were more than twofold greater than transitions, thereby resulting in a transversion bias. For those plants where transitions outnumbered transversions (cf. *Nicotiana* and *Epifagus*), the weak transition bias was less than expected based upon data obtained from other small-subunit rDNAs.

The 19S rDNA sequences of nonasterid holoparasites have a nucleotide composition bias that differs from those reported for both nuclear and plastid small-subunit rDNA sequences for the same plants. The mean G/C content of nuclear 18S rDNA from approximately 200 dicots is 49.4% (±2%). Among the nonasterid holoparasites, G/C content for nuclear 18S rDNA is lower (mean 46.8%), reflecting a higher T and lower C percentage (Nickrent and Starr 1994). For the plastid 16S rDNA, G/C content varies little from the mean of 55.6% among a wide range of green plants. In contrast, all the nonasterid holoparasites exhibit a lower plastid 16S rDNA G/C content ranging from 52% in *Cytinus* to 28.6% in *Corynaea.* For these plants, both G and C are replaced by A and T, a process referred to as the A/T drift phenomenon (Nickrent et al. 1997a). With the exception of *Scybalium,* this A/T bias was not observed among any of the mitochondrial 19S rDNA core sequences examined. Here, base composition is even more invariant than in nuclear 18S and plastid 16S rDNA with the G/C content varying by less than 1% from the mean (53.7%). Only *Scybalium* deviates significantly with a G/C content of 51.4%.

Though RNA editing is recognized as a significant process in many mitochondrial genes (Arts and Benne 1996; Bowe and dePamphilis 1996; Sper-Whitis et al. 1996), the holoparasite small-subunit sequences reported here are unlikely to be significantly affected by this process. Only one case of RNA editing in plant mitochondrial ribosomal RNA genes is known, i.e., the 26S rRNA gene of *Oenothera* (Schuster et al. 1991), but no editing has been demonstrated for the small-subunit rRNAs in plants. We have obtained direct evidence that indicates editing is not responsible for the observed pattern of substitutions in the holoparasite 19S rDNAs. A cDNA sequence that included the V1 region was obtained (via reverse transcriptase PCR) for *Pilostyles* (Rafflesiaceae). This sequence was identical to that obtained directly from rDNA (Duff and Nickrent, unpublished data), thus indicating the absence of editing, at least in this species and for this variable domain.

Mitochondrial Genomes and Structural Features of 19S rRNA

A number of processes unique to plant mitochondrial genomes must be considered when seeking explanations for the presence of the 19S rRNA variable domains. Detailed studies of *Zea* (Fauron et al. 1995), *Brassica* (Palmer 1992), *Nicotiana* (Bland et al. 1985), and *Marchantia* (Odo et al. 1992) mitochondrial genomes have shown that structural organization and recombinational dynamics are complex. Despite having low substitution rates for most genes, intra- and intergenomic recombination rates are high (Levings and Brown 1989). These recombinational events are associated with the presence of homologous repeats that have been documented in many plants such as grasses, *Spinacia, Phytolacca,* and *Brassica* but are absent in *Marchantia* and *Brassica hirta* (Stern and Palmer 1984). Such recombining-repeat sequences exhibit little homology compared with both distantly and closely related plants (Palmer 1992). Evidence for repeated rDNA genes has been obtained only for *Oryza, Secale, Triticum,* and *Zea* (Coulthart et al. 1993); however, such information is not available for the plant sequences generated in the present study. In maize, intermolecular recombination also occurs that generates a complex variety of substochiomet-

ric molecules. Intramolecular recombination between homologous repeats generates isomeric forms of the master chromosome or smaller subgenomic circular DNA molecules (Fauron et al. 1995). The frequent incorporation of foreign DNA derived from the nucleus, plastids, and plasmids is also a prominent feature of plant mitochondrial genomes (Schuster and Brennicke 1994).

Assuming a background of frequent recombination both within and between mitochondrial chromosomes, length variants are expected. When ribosomal genes are involved in recombination, length and sequence variants that produce functionally compromised rRNAs might be eliminated by selection. This idea is supported by the observation that essentially no sequence or length variation exists in the core 19S rDNA. If the V1 and V7 regions are under relaxed functional constraint, length mutations generated via recombination may be retained. Evidence for the lack of functional constraints on V1 and V7 sequences among photosynthetic plants comes not only from the lack of sequence conservation but also from the presence of large deletions and insertions. This concept is supported by the observation that the V1 regions for the gymnosperm *Ephedra* is 29 nucleotides in length and the V7 region for the seedless vascular plant *Isoetes* is 8 nucleotides in length (Duff and Nickrent unpublished data). Similarly, the V1 region of *Scybalium* is also significantly shorter than the other members of its family that have been examined. With the exception of a 13 nucleotide insertion in *Zea* (see below), the six published angiosperm 19S sequences showed length variation only in the V1 and V7 regions, thus suggesting that only these regions are under relaxed constraint. Generation of additional photosynthetic plant 19S rDNA sequences supports this concept; however, the variability encountered in the V5 and V6 domains from the nonasterid holoparasites indicates that other regions may be released from functional constraint (Table 1).

As in *Zea*, which has a repetitive motif in the apex of helix 29 (three sets of CUACG), short (8 base) tandem repeats are observed in the V7 region of *Scybalium*. Cryptic simplicity, i.e., scrambled, repetitive sequence motifs that are the products of replication slippage, have been reported in both small- and large-subunit nuclear rDNA (Tautz et al. 1986; Hancock and Dover 1988; Hancock 1995). A complex history of multiple recombination events would effectively obscure sequence similarity, thus providing a possible explanation for the differences seen between even closely related species of Balanophoraceae. Although this is the first report of repetitive motifs in plant mitochondrial rDNA, this result is not unexpected given the high rate of recombination in this genome.

Conclusions

This study expands the number of available angiosperm mitochondrial small-subunit (19S) RNA sequences, confirms the highly conserved nature of its core region, further characterizes the sequence and length heterogeneity of the V1 and V7 domains, and documents for the first time repetitive motifs and a transversion bias. The core 19S rRNA sequences reported herein, the first obtained from any holoparasitic angiosperm, exhibit both the highest substitution rates as well as the most divergent structural features seen in flowering plants. Because these holoparasite lineages have similarly accelerated substitution rates in nuclear and plastid-encoded small-subunit rRNA genes, it appears that heterotrophy (and changes associated with this trophic mode) profoundly affects the molecular evolution of all three subcellular genomes. It is presently unknown whether these results with rRNA are indicative of similar changes occurring in other mitochondrial genes, hence further study of these plants represents a unique opportunity to investigate this poorly understood genome.

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