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Extensive Intraindividual Variation in Plastid rDNA Sequences from the Holoparasite *Cynomorium coccineum* (Cynomoriaceae)

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Abstract. Ribosomal genes are considered to have a high degree of sequence conservation between species and also at higher taxonomic levels. In this paper we document a case where a single individual of *Cynomorium coccineum* (Cynomoriaceae), a nonphotosynthetic holoparasitic plant, contains highly divergent plastid ribosomal genes. PCR amplification a nearly complete ribosomal DNA cistron was performed using genomic DNA, the products cloned, and the 23S rDNA genes were sequenced from 19 colonies. Of these, five distinct types were identified. Fifteen of the sequences were nearly identical (11 or fewer differences) and these were designated Type I. The remaining types (II–V) were each represented by a single clone and differed from Type I by 93 to 255 changes. Compared with green vascular plants, we found that there are more substitutional differences in the 23S rDNA sequences within a single individual of *Cynomorium* than among all sequenced photosynthetic vascular plants. Several trends of molecular evolution observed in 16S rDNA from other holoparasitic angiosperms and heterotrophic green algae have been also observed in *Cynomorium* 23S rDNA. Higher-order structures were constructed for representatives of the five clone types, and in all cases these possessed complete complements of the major structural elements present in functional plastid 23S rRNAs. These data indicate that such molecules may be subject to purifying selection, thus providing indirect evidence that they have retained some degree of functionality. This intraindividual polymorphism is probably a case of plastid heteroplasmy but translocation of ribosomal cistrons to the nucleus or mitochondria has not been tested and therefore cannot be ruled out.

Key words: 23S rRNA — Heteroplasmy — Ribosomal RNA — Chloroplast — Nonphotosynthetic — RNA structural model

Introduction

Within eukaryotic species, subcellular organellar genomes are generally assumed to be homogeneous (homoplasmic). However, heteroplasmy, the presence of different organellar genotypes within a single individual, is known for mitochondrial and plastid genomes. Heteroplasmy can arise by biparental inheritance, mutation of organelle genomes after zygote formation, or variation among uniparentally transmitted genes when vegetative segregation is incomplete (Chesser 1998). Most of the studies on organellar heteroplasmy have been centered on mitochondrial DNA (mtDNA), likely because most heteroplasmic mitochondrial mutations in animals (including humans) are pathogenic (Chinnery et al. 2000). With regard to mitochondrial heteroplasmy, issues involving the transmission genetics, population genetics, and evolution of animal mtDNA were reviewed by Rand (2001). In plants, evidence of intragenic recombination and therefore the existence

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of heteroplasmy in mtDNA has been described in *Silene acaulis* by Städler and Delph (2002). Hattori et al. (2002) documented seven types of mitochondrial *nad3–orf156* sequences in nucleus–cytoplasm hybrids of wheat and *Aegilops* and the data suggested differential amplification of the heteroplasmic copies between the hybrids and the parental lines. Plastid heteroplasmy has been detected in somatic chimeras, which arise by the sorting-out of normal and chlorophyll-deficient plastids, thus resulting in variegated plants with tissue sectors of homoplasmic cells (Börner and Sears 1986). Lee et al. (1988) and Johnson and Palmer (1989) demonstrated the existence of biparental inheritance and heteroplasmic cells with both chlorophyll-deficient and wild-type plastids in *Medicago*. In other cases, such as *Gossypium* (Lax et al. 1987), where uniparental inheritance occurs, heteroplasmy can occur when segregation of wild-type and mutant plastids is incomplete and heteroplasmic gametes are produced. Persistent plastid heteroplasmy can be transmitted by slow vegetative segregation as found by Gillham et al. (1991). Rice plants derived from anther culture contain plastids with complete genomes as well as ones with large-scale deletions that result in albino phenotypes (Harada et al. 1991; Yamagishi 2002). Nuclear mutations may induce chlorophyll-deficient phenotypes but also can be originated by exposure to inhibitors of plastid protein synthesis (Zubko and Day 1998). These authors found that exposure to spectinomycin leads to transmittable variegation in *Brassica napus*, resulting in heteroplasmic cells with normal and ribosome-deficient plastids.

As pointed out by Frey (1999), plastid DNA has great potential for accumulating mutations but the lack of phenotypic markers makes detection difficult, especially for pseudogenes and noncoding regions. In most cases, detection of intraindividual plastid DNA polymorphisms in coding genes involves point mutations, as is the case for a synonymous transition in the *psbC* gene in *Actinidia* (Chat et al. 2002) or the *psbA* gene that confers triazine resistance in *Senecio vulgaris* (Frey et al. 1999). Plastid 16S and 23S rDNA gene sequences are highly conserved, thus are less likely to show intraindividual polymorphisms. In this paper, we document a case of intraindividual variation in the highly conserved plastid rRNA genes of *Cynomorium coccineum*, a perennial holoparasitic (nonphotosynthetic) angiosperm.

Molecular evolutionary studies of plastid-encoded ribosomal genes in nonphotosynthetic organisms have been limited exclusively to small-subunit (SSU, 16S) rRNA. In several lineages of nonasterid holoparasitic angiosperms, Nickrent et al. (1997a) showed an increased rate of nucleotide substitution in 16S rDNAs genes compared to photosynthetic angiosperms. The divergence in these holoparasites ranged between 7.3% in *Cynomorium* (Cynomoriaceae) to 35.9% in *Corynaea* (Balanophoraceae) compared to *Nicotiana*. This increase was accompanied by what the authors called an "A/T drift" phenomenon, whereby the A + T content of the SSU rDNA sequences of several lineages of holoparasitic angiosperms was increased compared to photosynthetic relatives. Together with A/T drift, an increase in transversional vs. transitional mutations was observed, especially in the most highly diverged sequences. Similar molecular evolutionary phenomena have been observed in some lineages of the nonphotosynthetic chlorophyte alga *Polytoma* (Nedelcu 2001; Vernon et al. 2001). Despite the high level of sequence divergence for such SSU rRNAs, indirect evidence that these molecules have retained functionality exists. Specifically, they retain most or all of the higher-order structures compared to Noller–Woese–Gutell structural models. Moreover, most variability occurs in regions that also vary in other organisms with fully functional rRNAs.

In this study, we analyzed the plastid large-subunit (LSU or 23S) rDNA sequences, inferred rRNA secondary structures obtained from a single individual of *Cynomorium coccineum*, and compared these with sequences and structures from other plants. The objective was to determine whether the molecular evolutionary trends observed in the 16S rRNA molecules also exist in these 23S rRNA molecules. Moreover, we wished to explore further the phenomenon of heteroplasmy in this parasitic plant.

Materials and Methods

A single specimen of *Cynomorium coccineum* L. was collected April 19, 1996, in Cádiz, Spain (Nickrent 4063; voucher at SIU). Total genomic DNA was obtained by grinding the fresh tissue to a powder on liquid nitrogen and extracting in 2× CTAB buffer as described by Nickrent (1994). Nearly the entire plastid rDNA cistron was PCR amplified using a TaKaRa long PCR kit (Takara Bio Inc., Shiga, Japan) with the SSU rDNA plastid-specific primer 323 for (Nickrent et al. 1997a) and the 23S rDNA 2749rev primer (Table 1). The reactions were conducted in a Perkin–Elmer Thermal Cycler with one cycle at 94°C for 3 min and 35 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 3 min, followed by a final extension at 72°C for 5 min. A 4.3-kb PCR product was obtained and gel purified using Gel Elute spin columns (Sigma Aldrich, Inc., St. Louis, MO). The PCR product was cloned using the PCRII vector from a TA cloning kit (Invitrogen Corporation, Carlsbad, CA) and 19 colonies were selected. Only one of the clones (Cyno0) was obtained from a different PCR reaction but from the same genomic DNA extraction. The plasmids were purified using the QIAprep Spin Miniprep Kit (QIAGEN Inc., Valencia, CA). The presence of inserts was checked by restriction digestion (*Hind*III) followed by electrophoresis of the fragments in an agarose gel. Manual sequencing was conducted using the SequiTherm EXCEL II DNA Sequencing kit (EpiCentre Technologies, Madison, WI) with the internal primers listed in Table 1.

Complete 23S rDNA sequences for 19 clones were obtained from the *Cynomorium* sample and deposited in the NCBI database (Table 2). For comparative analyses, additional plastid 23S rDNA sequences were obtained from GenBank. These were *Pinus thun-*

Table 1. Primers used to sequence plastid 23S rDNA

Primer sequence (5′→3′)	Position in *Nicotiana*	Primer name
GGT GGA TAC CTA GGC ACC CAG AG	22–44	42 for
CAA GGA CCA CCT TGC AAG GCT	420–440	420 for
TGG TTC TCC CCG AAA TGC GTT	815–835	815 for
CAT TGG TGA GAA TCC AAT GCC	1294–1314	1294 for
AAC TCT CTC TAA GGA ACT CGG	1686–1706	1686 for
GCG GAC TAC CTG CAC CTG GA	2044–2063	2044 for
CGG ATA AAA GTT ACT CTA GGG	2437–2459	2437 for
TAC TRA GAT GTT TCA STT C	174–192	175 rev
TCC CAT TTC GCT CGC CGC TAC	228–248	245 rev
GAA TAT TAA YCT ATT GTC CAT C	1394–1415	1394 rev
GAT GCT TTC AGC AGT TAT CC	2749–2768	2749 rev

Table 2. Pairwise number of substitutions between the different clones of *Cynomorium*

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C18	C19
Cyno1 (AY330870)[a]	—																	
Cyno2 (AY330871)	5	—																
Cyno3 (AY330872)	5	2	—															
Cyno4 (AY330873)	6	3	3	—														
Cyno5 (AY330874)	189	186	186	187	—													
Cyno6 (AY330875)	8	5	5	6	186	—												
Cyno7 (AY330876)	4	1	1	2	184	4	—											
Cyno8 (AY330877)	4	1	1	2	185	4	0	—										
Cyno9 (AY330878)	7	4	4	5	188	7	3	3	—									
Cyno10 (AY330879)	109	106	106	107	197	109	105	105	108	—								
Cyno11 (AY330880)	9	6	6	7	190	9	5	5	8	108	—							
Cyno12 (AY330881)	9	6	6	7	190	9	5	5	8	110	6	—						
Cyno13 (AY330882)	4	1	1	2	185	4	0	0	3	105	5	5	—					
Cyno14 (AY330883)	8	5	5	6	189	8	4	4	7	109	9	9	4	—				
Cyno15 (AY330884)	10	7	7	8	185	10	6	6	9	109	11	11	6	10	—			
Cyno16 (AY330885)	220	217	217	218	186	218	215	216	218	255	220	221	216	218	220	—		
Cyno18 (AY330886)	5	2	2	3	186	5	1	1	4	106	6	6	1	5	7	217	—	
Cyno19 (AY330887)	8	5	5	6	189	8	4	4	7	108	8	9	4	8	10	220	5	—
Cyno0 (AY330869)	173	170	170	171	93	171	168	169	172	179	173	174	169	173	169	162	170	172

[a] Accession numbers in parentheses.

bergii (D17510), *Oryza sativa* (X15901), *Zea mays* (Z00028), *Oenothera elata* (AJ271079), *Alnus incana* (M75722), *Amaranthus tricolor* (AF254866), *Spinacia oleracea* (AJ400848), *Arabidopsis thaliana* (AP000423), *Nicotiana tabacum* (Z00044), and two asterid holoparasites, *Conopholis americana* (X59768) and *Epifagus virginiana* (X62099). These sequences were entered into the program SeA1 version 1.0 (Rambaut 1996) where a multiple sequence alignment was conducted manually.

Higher-order structural models for *Cynomorium* were constructed manually using ClarisDraw version 1.0 following covariance-based Noller–Woese–Gutell structural models (Gutell et al. 2002). Reference models and online RNA sequence and structure information were obtained from the Comparative RNA Web (CRW) site (www.rna.icmb.utexas.edu) (Cannone et al. 2002). At present, the most recent angiosperm chloroplast 23S rRNA structural model is for the monocot *Zea* but no updated structures for dicots are present on the CRW. For this reason, we constructed a higher-order structural model for *Nicotiana*, which was generally straightforward given the high degree of sequence conservation between the two species. This model was then used as a starting point for constructing the *Cynomorium* models. Changes that resulted in the disruption of a Watson–Crick (A–U or G–C) or a wobble (G · U) base pair were considered noncanonical and drawn with symbols used in models present on the CRW (A∘G, C•A, etc.).

Results

A PCR product ca. 4.3 kb in length was obtained following long PCR amplifications from the genomic DNA. As determined later from sequencing, this product included most of the plastid rDNA cistron and missed the first 320 bp at the 5′ end of the 16S rDNA and ca. 40 bp of the 3′ end of the 23S rDNA molecule. Restriction digests of the plasmids and agarose gel electrophoresis of the products showed variation in the overall size of the inserts as well as variation in cut sites. These digestion patterns suggested that there were differences in the sequences between the clones, which was confirmed by sequencing. Sequence variation was seen in both 16S and 23S rDNA and in the 16S–23S spacer, which was reduced to only 400 bp and is missing the two transfer RNAs typically found in plastids of green plants. The 23S rRNA molecules from *Cynomorium* varied in size from 2689 to 2754 bp (depending upon the clone).

Fig. 1. One of the five shortest equally parsimonious trees derived from a maximum parsimony analysis of plastid 23S rDNA sequences from 19 clones of *Cynomorium coccineum*, 10 photosynthetic and 2 nonphotosynthetic outgroups. Numbers above the lines indicate branch lengths (number of substitutions).

This is shorter than the molecules seen in most green plants, e.g., 19 to 84 bp shorter than *Nicotiana* (2773 bp).

To demonstrate graphically the amount of sequence divergence between the *Cynomorium* clones and the nonparasitic plants, 23S rDNA sequences were analyzed using maximum parsimony. Five trees of length 1179 were obtained, which differed only in terms of the deeper nodes of the green plant clade. In one of the resulting phylograms (Fig. 1), branch lengths, which are proportional to the number of substitutional differences, are considerably longer leading to the five types of *Cynomorium* clones than branches among more distantly related species of nonparasites. Some indication of rate heterogeneity is seen by examining the branches leading to the asterid holoparasites *Conopholis* and *Epifagus*, but not of the magnitude of that seen in *Cynomorium*. There are more substitutional differences in the 23S rDNA sequences within a single specimen of *Cynomorium* than between all photosynthetic vascular plants analyzed. The variation reflected here is probably only indicative of the actual variation that exists in a single plant, considering that this variation was obtained by sequencing only 19 clones.

Pairwise distances between the 19 *Cynomorium* clones (Table 2) show that 14 of the sequences are nearly identical (with 11 or fewer differences) and these were designated Type I. Three of these (clones 7, 8, and 13) had identical sequences. Differences between the other Type I clones are small enough to fall within the realm of Taq incorporation or sequencing errors but could also be real. Type II is represented only by clone 10 and is different from Type I sequences by 105–110 substitutions. Type III is represented only by clone 16; it is the most distinct

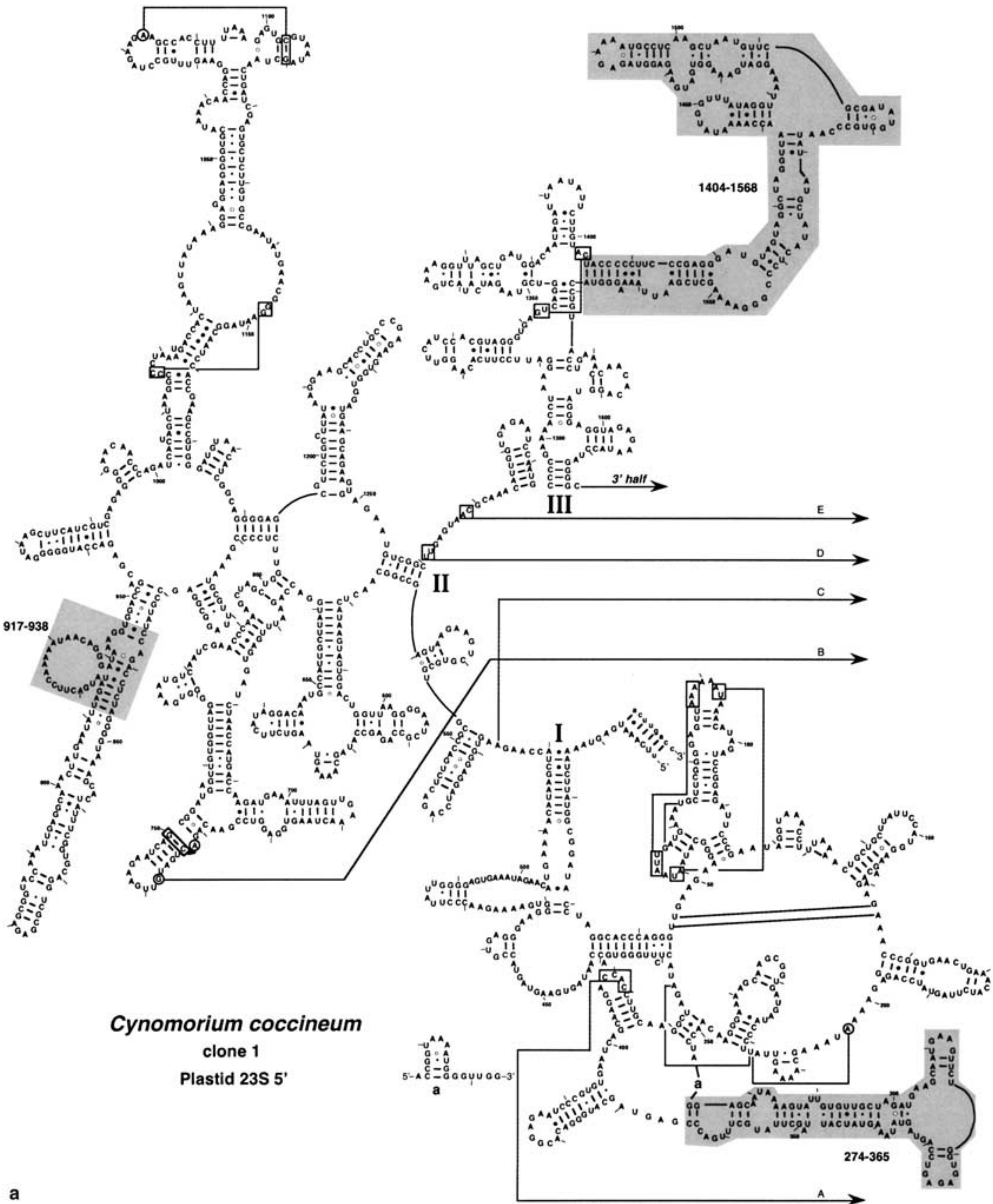

Fig. 2. a, b Higher-order structural model for the 23S rRNA from clone Cynol of *Cynomorium coccineum* following conventions of the Noller–Woese–Gutell comparative structural model of *E. coli* and *Zea mays*. Lowercase bases at the 3′ and 5′ ends of the molecule were not determined because they occur at priming sites (sequence shown is of *Nicotiana*). The shaded variable regions are shown in detail in Fig. 3 and discussed in the text.

and differs from others by 162 to 255 substitutions. Type IV is represented by clone 5 and it is most similar to clone Cyno0 (Type V), but it differs from that clone by 93 substitutions. The Cyno0 clone, generated by a separate PCR reaction than the other 18, forms a sister group to all the others. It is also basal on the tree, thus more closely related to the green plant sequences.

Higher-order structural models were constructed for representatives of the five clone types. Clone Cyno1 was selected as representative of Type I and its structural model is shown in Fig. 2. The complete secondary structures of the other four types are available at The Parasitic Plant Connection Web Page (www.science.siu.edu/parasitic-plants/rRNA_structures.html). For all five types, the *Cynomorium*

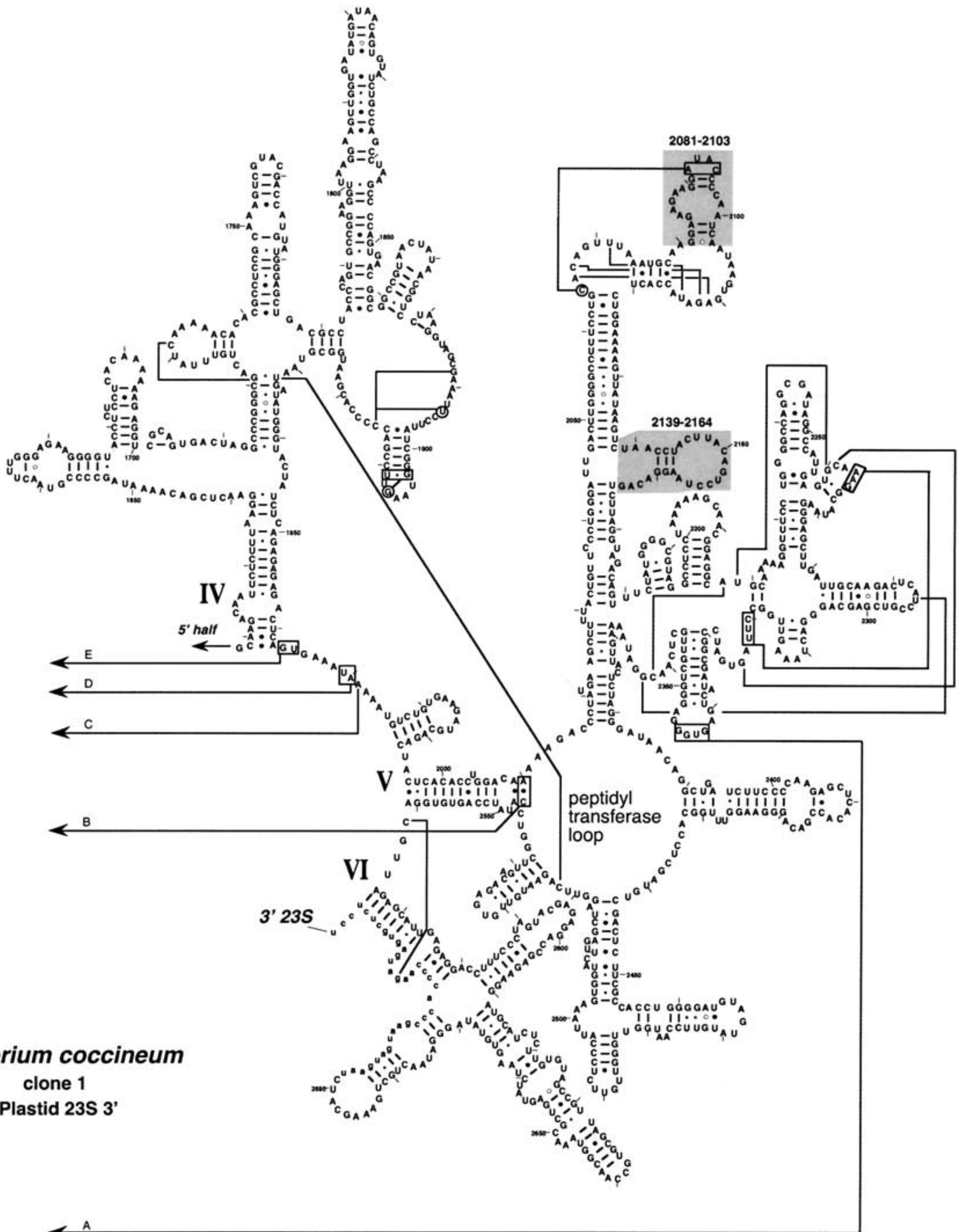

Fig. 2. Continued.

sequences could be folded into higher-order structures and all possessed a complete complement of structural elements present in functional plastid 23S rRNAs. In the following section, we discuss only the most variable regions, especially where most of the sequence length variation occurs (Fig. 3). These regions are shaded in the Cyno1 structure, which also contains the sequence numbering which is used as a reference for all structures throughout the text.

Positions 274–365. Sequences for this region range in length from 91 nt in Cyno18 (Type I) to 130 nt in clone Cyno0 (Type V) compared with 107 nt for *Nicotiana*. Unique indels include Cyno5, which has a 12-bp insertion resulting in a bulge loop at ca. position 280, or the 26-bp duplication in Cyno0, which results in a large terminal loop (no structure was inferred). Conversely, in other clones (e.g., 1 and 10), deletions of 10–11 nt occur at this same terminal

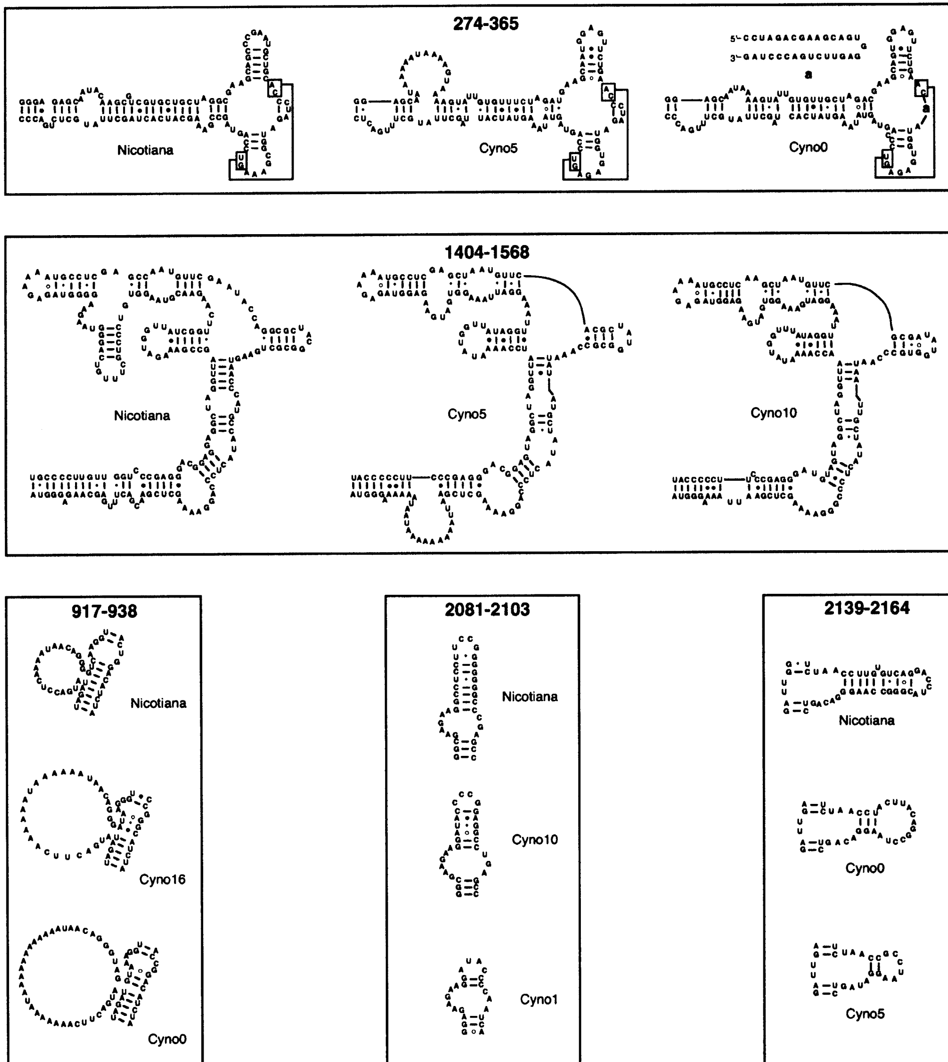

Fig. 3. Structural models of selected variable regions seen among the five clone types of *Cynomorium* compared to *Nicotiana*. The numbers indicate the variable regions shaded in Fig. 2.

position. Most of the substitutions compared to *Nicotiana* are C→T transitions that increase the number of noncanonical base pairs. This region is variable in size and in sequence not only in *Cynomorium* but also in other angiosperm plastids 23S rRNAs.

Bulge Loop in Positions 917–938. This adenine-rich region is variable in size in plastid 23S rRNAs of many different organisms. It is 22 nt long in all *Cynomorium* Type I clones, Cyno5 and Cyno10, but longer in Cyno16 (29 nt) and Cyno0 (39 nt). The increase in length is caused by insertions (compared to Cyno1): $AT(A)_{12}$ in Cyno0 and $T(A)_6$ in Cyno16.

Positions 1404–1568. The helix formed by positions 1404–1438 (5′ side) and 1528–1568 (3′ side) shows great divergence in size and sequence that affect both paired and unpaired zones. At ca. position 1508, some clone types have insertions composed almost exclusively of adenines (Cyno5), whereas others (Cyno10) have deletions in this same region. In the asterid holoparasite *Conopholis* there is a 13-bp insertion in this region. All *Cynomorium* clones show a deletion of 18 nt at position 1475 compared to the *Nicotiana* sequence. In plastid 23S structures of other vascular plants, these nucleotides form a hairpin that is missing in *Cynomorium*. This hairpin is also missing in other 23S rRNA structures such as that of *E. coli* and eukaryotes (Schnare et al. 1996). A common deletion to all the clones occurs at positions 1511–1512, the same region where *Pisum* has a 6-nt duplication. The helix formed by positions 1512–1527 is conserved in *Cynomorium* but in *Oryza* and *Zea* there are 67 additional nucleotides that significantly increase the length of this helix.

Table 3. Nucleotide composition of plastid 23S rDNA sequences from *Cynomorium* clone types compared with vascular plants

	A %	C %	G %	T %	A+T %	No. sites
Cyno0	30.0	20.4	28.5	21.1	51.1	2754
Cyno1	31.2	19.6	26.8	22.4	53.6	2690
Cyno5	30.7	19.5	27.8	22.0	52.7	2737
Cyno10	31.1	19.4	27.0	22.5	53.6	2696
Cyno16	30.8	19.8	27.5	21.9	52.7	2716
Mean Cyno	30.7	19.7	27.5	22.0	52.7	2718
Alnus	26.6	23.6	31.5	18.3	44.9	2774
Amaranthus	26.7	23.4	31.5	18.4	45.1	2772
Arabidopsis	26.3	23.4	31.8	18.5	44.8	2773
Conopholis	27.7	22.6	30.3	19.4	47.1	2773
Epifagus	27.0	22.9	31.0	19.1	46.1	2767
Nicotiana	26.4	23.4	31.7	18.5	44.9	2773
Oenothera	26.3	23.4	31.7	18.6	44.9	2772
Oryza	26.8	23.3	31.4	18.5	45.3	2848
Pinus	26.9	23.3	31.1	18.7	45.6	2766
Pisum	27.4	22.8	30.9	18.9	46.3	2775
Spinacia	26.6	23.2	31.6	18.6	45.2	2772
Zea	26.7	23.4	31.3	18.6	45.3	2848
Mean green plant[a]	26.6	23.3	31.4	18.5	45.2	2787

[a] Excluding *Conopholis* and *Epifagus*.

Positions 2081–2103 and 2139–2164. These regions represent examples of the reduction in helix length in different clone types. The first region forms a helix that is variable in size among plastid and eukaryotic 23S-like rRNA structures. All the type I clones have a 15-nt deletion, whereas this deletion involves only 7 nt in the other four types. In *Conopholis* and *Epifagus*, an 8-nt deletion also occurs at these positions, ones that are conserved in the other angiosperm sequences. At positions 2139–2164, the 18-nt deletion in Cyno5 and 9-nt deletion in the rest of the clones reduce the length and structure of this helix compared to *Nicotiana*.

Positions that are highly conserved in functional 23S rRNA molecules are also conserved in those from *Cynomorium*. Among 52 plastid 23S sequences analyzed in the CRW, more than 1400 positions are conserved at 90% or more. For the Cyno1 clone, 1305 (93%) of these positions are also conserved, representing more than half the total length of the molecule.

Nucleotide frequencies for 23S rDNA sequences from the *Cynomorium* clone types are compared to sequences from various green plants in Table 3. Base composition is uniform across the different lineages of green plants and the mean A + T content is 45.2%. In contrast, the mean A + T in *Cynomorium* is 52.7%, an increase greater than 7%. The two asterid holoparasites, *Epifagus* and *Conopholis*, also show an increased A + T content but not to the extent seen in *Cynomorium*.

The number and kinds of substitutions in paired and unpaired regions in the five *Cynomorium* clone types (compared to *Nicotiana*) are shown in Table 4. The most common transition types in paired and unpaired regions are G→A and C→T and the most common transversion types are C→A and G→T, in both cases leading to an increase in A + T content. In all the clone types there is an increase in the percentage of G→T transversions in unpaired regions compared to paired regions. The Tn/Tv ratio is higher in unpaired regions, especially in clone Cyno0 (3.6 to 1). The increase in the number of transversions in unpaired regions is caused by the increase in G→T substitutions. The substitution rate is slightly higher in paired than in unpaired regions. Compared to *Nicotiana*, there is 0.13 substitution/site in Cyno1 but 0.09 substitution/site in paired regions.

Together with the A/T drift seen in *Cynomorium* 23S rDNA, there is an increase in A–U pairings in helical regions. In the five clone types there are 24 positions in conserved zones where double-compensated changes occur compared to *Nicotiana*. In the most variable regions, up to six additional double-compensated changes could occur. In 23 of these 24 positions, the change is from a G–C pair to an A–U pair, representing a maximum of 46 substitutions, and only one double-compensated change occurs from C–G to G–C in the 1701:1705 lone base pair in Cyno10. In 13 of the 23 C–G to A–U double-compensated changes, intermediate states have been observed in one or several clones. In nine of these, the double-compensated change occurred through a wobble G · U pairing, two of them through an A∘G pairing, and three through a C•A noncanonical base pairing (one of these also with a G · U intermediate). This indicates that a C→T transition followed by a G→A transition is the most common substitution pathway leading to double-compensated changes in *Cynomorium*. The double-compensated changes are through two transitions, except when A∘G intermediates

Table 4. Substitutions in paired and unpaired regions of the five types of *Cynomorium* clones compared to *Nicotiana*[a]

	Cyno0			Cyno1			Cyno5			Cyno10			Cyno16		
	Paired	Unpaired	Total	Paired	Unpaired	Total	Paired	Unpaired	Total	Paired	Unpaired	Total	Paired	Unpaired	Total
A→G	4	4	8	5	8	13	4	4	8	3	7	10	8	8	16
G→A	35	20	55	60	33	93	38	25	63	63	32	95	55	32	87
T→C	8	4	12	12	8	20	10	3	13	14	8	22	14	5	19
C→T	40	15	55	61	24	85	57	20	77	64	26	90	57	20	77
Total Tn	87	43	130	138	50	188	109	52	161	144	73	217	134	65	199
A→C	0	0	0	2	2	4	0	0	0	2	1	3	2	5	7
A→T	0	3	3	1	3	4	0	3	3	2	6	8	2	2	4
C→A	9	14	23	16	14	30	13	16	29	13	16	29	19	15	34
C→G	2	1	3	5	0	5	2	0	2	7	1	8	5	0	5
G→C	2	0	2	4	2	6	4	0	4	5	2	7	4	0	4
G→T	6	22	28	13	23	36	9	24	33	13	24	37	15	23	38
T→A	4	2	6	5	2	7	4	3	7	5	3	8	5	3	8
T→G	1	1	2	4	2	6	2	1	3	4	2	6	4	2	6
Total Tv	24	43	67	50	48	98	34	47	81	51	55	106	56	50	106
Tn/Tv	3.63	1.00	1.94	2.76	1.52	1.92	3.21	1.11	1.99	2.82	1.33	2.05	2.39	1.30	1.88

[a] Variable positions 298–327, 1520–1592, and 2200–2223 were excluded from this analysis.

occur; in that case the change is through two transversions (C→A and G→T). In 9 of the 10 double-compensated changes where no intermediates have been found, the pathway toward the double-compensated change is through two transitions. The pattern of double-compensated changes through G · U intermediates was observed between different species of *Drosophila* in variable zones of nuclear large-subunit rRNA by Rousset et al. (1991). In addition to the double-compensated changes, the increase in the number of A–T pairings is also because of G · U changing to A–U pairings. Thirteen of the wobble G · U pairings in *Nicotiana* are A–U in clone Cyno5, 14 in clone Cyno0, 15 in Cyno16, and 20 in Cyno1.

Discussion

By comparing sequences and higher-order structures from functional plastid 23S and eukaryotic 23S-like rRNA (Schnare et al. 1996), those from the different clone types of *Cynomorium* appear to remain functional despite high sequence divergence. The positions that are highly conserved in functional rRNAs are also conserved in the *Cynomorium* sequences, whereas most of the substitutions and indels are in universally variable positions. The mosaic of conserved and variable regions observed in functional rRNA is also present in *Cynomorium* 23S, indicating that they are still subject to purifying selection and therefore have at least some degree of functionality. However, the increase in A–T base pairings and noncanonical interactions could affect helix stability and therefore the rate of translation and/or the assembly of the ribosome. Even if the ribosomes are less efficient in translation, such levels could be sufficient if a lower rate of protein synthesis is tolerated in *Cynomorium* plastids; alternately, if the rate is the same, fewer genes have to be transcribed and translated than in chloroplasts.

Some molecular evolutionary trends previously observed in plastid 16S rDNAs from heterotrophic organisms also occur in *Cynomorium* 23S rDNA. The increase in A + T content in the plastid genome is a general phenomenon in the evolution of parasitic and/or nonphotosyntetic organisms (Nickrent et al. 1997a). As indicated in Table 4, most of the substitutional changes in *Cynomorium* are either transitions (especially G→A and C→T) or C→A and G→T transversions leading to an increase in the A + T content. The same trends were observed for chloroplast noncoding regions in angiosperms (Bakker et al. 2000). As pointed out by these authors, a possible explanation might come from mechanisms ensuring fidelity in DNA replication based on geometric selection (Goodman 1997). During replication, G:T, G:A, and A:C mispairings have a closer geometry to Watson–Crick pairing, thus allowing the formation of mispairs that are repaired by either transitions or C↔A and G↔T transversions.

In *Cynomorium*, a change in substitution dynamics in paired and unpaired regions was observed. Transitions are the most common substitutional pathway in paired regions, whereas there is an increase in transversions in unpaired regions caused by an increase in G→T substitutions. The increased number of transitions observed in paired regions could be related to selection toward double-compensated changes that proceeds predominantly through transitions, as found by Vernon et al. (2001) for 16S rRNA in *Polytoma*. The increase in number of transitions in paired regions was also observed by Rousset et al. (1991) in *Drosophila*. However, Springer et al. (1995)

found that in mammalian mitochondrial 12S rRNA the increase in the number of transitions in helical regions was not accompanied by an increase in the rate of nucleotide substitutions since substitution rates were higher in unpaired regions.

Cases of highly divergent plastid and mitochondrial ribosomal sequences between related organisms have been documented for holoparasitic angiosperms (Duff and Nickrent 1997; Nickrent et al. 1997a), however, the polymorphism reported here for *Cynomorium* is the first case of highly divergent plastid ribosomal genes occurring within an individual plant. Given that only 19 clones were examined, and that clones derived from different PCR reactions were the most divergent (compare Type V with Types I–IV), it is probable that additional variation remains to be sampled. The levels of plastid genetic polymorphism could be higher considering that genomes with identical ribosomal genes could be polymorphic for other genes with more relaxed selection or ones that are subject to spontaneous mutations without selection, as would be the case for pseudogenes or noncoding regions. The retention of different rRNAs suggests that the plastid genomes are not under strong selection, possibly because (1) only a few genes remain in the plastid genomes that must be transcribed and/or (2) mistranslation of plastid protein genes can be tolerated. This could increase the probability of fixation of different genetic versions of plastid ribosomal genes, leading to the observed intra-individual heterogeneity. Among the nonasterid holoparasite 16S rDNA sequences compared by Nickrent et al. (1997a), *Cynomorium* showed the lowest level of sequence divergence. Thus, if this same phenomenon exists in other holoparasites with higher sequence divergence (e.g., Balanophoraceae, Hydnoraceae, and Rafflesiaceae), it would be expected that the levels of intraindividual heterogeneity might be even greater.

Type I 23S rDNA is much more abundant than the others. This could represent the result of intracellular purifying selection toward Type I or it could reflect the original variation that was already present in the plant's zygote. There appears to be no obvious reason from structural characteristics why a functional constraint toward Type I might exist. Studies at the populational level might determine whether the difference in the proportion of each Type is a consequence of the vegetative segregation that leads to the maternal and/or paternal gametes. Further sampling would be necessary to confirm that overrepresentation of Type I is not an artifact originated by a preferential PCR amplification or cloning of this Type, to find additional intraindividual variation, and to check whether this polymorphism is a general feature in the species.

One question that arises from this study is whether the plastid ribosomal genes sequenced in *Cynomorium* are part of plastid genomes or have been transferred either to the nucleus or to the mitochondria. Many studies indicate the presence of plastids and plastomes in holoparasitic Orobanchaceae (see references, e.g., in Young and dePamphilis 2000). For nonasterid holoparasites, Nickrent et al. (1997b) showed through Southern blotting that probes constructed for 16S rRNA and for four plastid-encoded ribosomal proteins resulted in positive hybridizations in *Cytinus*, a genus that shows similar levels of 16S rRNA sequence divergence as *Cynomorium*. This, together with the existence of plastids characterized by a lack of thylakoids (Severi et al. 1980), provided evidence that suggested the presence of a plastome in *Cytinus*. No ultrastructural studies have been conducted showing the existence of plastids in *Cynomorium* and the existence of a plastome remains unproven. Martin et al. (2002) showed that thousands of cyanobacterial sequences exist in the nuclear genome of *Arabidopsis* and therefore the presence of the cistron in the nucleus cannot be ruled out. Numerous cases of chloroplast sequences in the mitochondrial genome of angiosperms have been reported since Stern and Lonsdale (1982) first reported a 12-kb chloroplast sequence in the mitochondrial genome of *Zea mays*. In most cases, the chloroplast sequences found in the mitochondrial genome are small pieces and represent pseudogenes that have lost their original functions. Sometimes these fragments are larger, such as the 6.8-kb sequence that was found in the mitochondrial genome of rice (Nakazono and Hirai 1993). Cummings et al. (2003) discovered five independent transfers to the mitochondrial genome of the chloroplast gene *rbc*L in angiosperms and the data suggest that they are evolving as pseudogenes without functional constraint. The conservation of the structural elements of functional plastid ribosomal DNA in *Cynomorium* provides evidence that these genes probably have not been transferred to the nucleus, where they would be expected to become pseudogenes. The same fate would be expected if they were transferred to the mitochondria, where retention of functionality is unlikely. Seven of the nine tRNAs of chloroplast origin found in the mtDNA of rice are transcribed, edited, and functional in translation in the mitochondria (Miyata et al. 1998), but this has not been yet documented for plastid-derived ribosomal genes. Most probably, these ribosomal types are part of very reduced and derived plastid genomes as evidenced by the unsuccessful amplification of other plastid genes using general primers.

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