

Do nonasterid holoparasitic flowering plants have plastid genomes?

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Abstract

Past work involving the plastid genome (plastome) of holoparasitic plants has been confined to Scrophulariaceae (or Orobanchaceae) which have truncated plastomes owing to loss of photosynthetic and other genes. Nonasterid holoparasites from Balanophoraceae (*Corynaea*), Hydnoraceae (*Hydnora*) and Cytinaceae (*Cytinus*) were tested for the presence of plastid genes and a plastome. Using PCR, plastid 16S rDNA was successfully amplified and sequenced from the above three holoparasites. The sequence of *Cytinus* showed 121 single base substitutions relative to *Nicotiana* (8% of the molecule) whereas higher sequence divergence was observed in *Hydnora* and *Corynaea* (287 and 513 changes, respectively). Secondary structural models for these 16S rRNAs show that most changes are compensatory, thus suggesting they are functional. Probes constructed for 16S rDNA and for four plastid-encoded ribosomal protein genes (*rps2*, *rps4*, *rps7* and *rpl16*) were used in Southern blots of digested genomic DNA from the three holoparasites. Positive hybridizations were obtained using each of the five probes only for *Cytinus*. For *Sma*I digests, all plastid gene probes hybridized to a common fragment ca. 20 kb in length in this species. Taken together, these data provide preliminary evidence suggestive of the retention of highly diverged and truncated plastid genome in *Cytinus*. The greater sequence divergence for 16S rDNA and the negative hybridization results for *Hydnora* and *Corynaea* suggests two possibilities: the loss of typically conserved elements of their plastomes or the complete absence of a plastome.

Introduction

Holoparasitic flowering plants have lost photosynthetic function and are totally dependent upon their host plant for water, nutrients and reduced carbon. Parasitism in angiosperms has evolved independently at least nine times [28]; however, only four families (Balanophoraceae, Hydnoraceae, Lennoaceae, and Rafflesiaceae) are represented entirely by holoparasites. Within the past decade, holoparasites have received increasing attention since they serve as model organisms to study plastid genome (plastome) organization. The plastome of *Epifagus virginiana* (beechdrops, Scrophulariaceae s. l.), has been completely sequenced

[45] and is only 70 kb, i.e. half the size of most green plants. This truncated plastid genome, containing only 42 intact genes as opposed to 113 in tobacco, has lost essential all genes involved in photosynthesis. Despite these extensive losses, the vestigial plastome is expressed and is functional [9, 13]. Other holoparasitic Scrophulariaceae have been investigated such as *Orobanche hederaceae* [41], *Lathraea clandestina* [7], and *Conopholis americana* [5, 43]. Despite experiencing different patterns of plastid gene deletions, all members of holoparasitic Scrophulariaceae and Cuscutaceae have retained plastids and plastomes (reviewed in [8]). Thus the observation, first made by Dodge and Lawes [11] based upon ultrastructural evidence, that holoparasites retain plastids as storage organs and for other physiological functions, is supported by molecular genetic evidence [45].

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers U47845, U67745 and U67744.

Unlike Scrophulariaceae, which is clearly a component of Asteridae [6], modern classifications differ greatly on the placement of Balanophoraceae, Hydnoraceae and Rafflesiaceae, here referred to as the ‘nonasterid holoparasites’. Classification of these parasites has been hampered by the extreme reduction and/or modification of morphological structures as well as the acquisition of highly derived features that confound assessment of character homologies (see [4, 6, 39, 42] for differing views). Molecular phylogenetic work using nuclear 18S rDNA has only recently provided some insights into relationships of these plants, being impeded mainly by extremely high rates of substitution as compared with more typical angiosperms [29, 31]. That work proposed a magnoliid affinity for Hydnoraceae and supported the recognition of Cytinaceae as distinct from Rafflesiaceae, a result in agreement with at least one recent classification [39].

In contrast to Scrophulariaceae, essentially no molecular investigations have focused upon the plastomes of the nonasterid holoparasites. Indeed for most species, ultrastructural data are lacking that confirm the existence of plastids or plastid-like organelles. Exceptions include an ultrastructural investigation of *Cytinus* scale cells that showed they contained double-membrane organelles classified as amyloplasts (starch-storing, colorless plastids) [36]. Since these organelles did not contain any structures reminiscent of thylakoids, the authors concluded that *Cytinus* was totally nonphotosynthetic. For Balanophoraceae, leucoplasts (i.e. colorless plastids) were observed in vegetative tissues of *Thonningia* [24]. Investigations of tuber ultrastructure in other members of this family did not indicate the presence of plastids [16, 20]. Finally, a study of the endophyte system of *Pilosyles* (Rafflesiaceae) revealed organelles (referred to as plastids) within sieve elements [21]. Although such structural studies can provide visual documentation of the presence of plastid-like organelles, they cannot provide unequivocal evidence for a plastome. In this paper, we report results of molecular investigations (PCR and heterologous probe hybridizations to five plastid genes) to determine whether a plastome is present in three selected nonasterid holoparasites.

Materials and methods

Sources of plant material used for DNA extraction are shown in Table 1. Plant tissues were used fresh or were frozen on liquid nitrogen and stored at -80°C prior to

use. Total genomic DNA was isolated from these plants using a modified CTAB method [27]. *Hydnora* tissue contains high amounts of latex and other secondary compounds that often resulted in unusually large and dense precipitates using the typical $2\times$ CTAB method. DNA extraction from *Hydnora* required further purification using additional phenol-chloroform extractions and CsCl gradient centrifugation.

PCR reactions, gel purification of resulting products, and sequencing conditions have been described [27]. The PCR products from *Cytinus* and *Hydnora* were sequenced directly. Insufficient product was obtained from *Corynaea crassa* after the amplification of the 1.2 kb fragment to allow direct sequencing. This product was cloned prior to sequencing using the PCRII vector (TA cloning kit, Invitrogen).

Total genomic DNA was used for restriction digests and Southern blotting. Attempts to isolate plastids from holoparasites such as *Epifagus* (CWD) and *Cytinus* (Thalouarn, pers. com.) were not successful, hence purified plastid DNA was not available for the holoparasites used in this study. Because of difficulties encountered obtaining high quality DNA (above), very limited amounts were available for Southern blot studies. Based upon the published sequences of complete plastomes (*Nicotiana*, *Epifagus*, *Oryza*) and relevant smaller regions (*Glycine*, *Cuscuta*), two restriction enzymes (*Hind*III and *Sma*I) were found that release a fragment containing both *rps7* and 16S rDNA. Digestions were performed using 0.5 to 2.0 μg of DNA (the lesser amount for the green plant *Aristolochia*) and 20–50 units of enzyme (New England Biolabs). Southern blot methods, as described [34], were performed using Hybond nylon membrane (Amersham). Five conserved genes distributed throughout the plastid genome [9, 38] were selected as probes for Southern blot analyses (Table 2). Each gene was found to be present in all parasitic [8] and nonparasitic [12] plant previously examined. PCR primers for the plastid ribosomal protein genes were designed after an alignment of a diverse set of angiosperm and nonangiosperm sequences. Probes were constructed for the five plastid genes by nick translation (using ^{32}P -dATP) of the PCR products amplified from genomic DNA of *Aristolochia* using the 16S primers 8 for and 1461 rev (Table 2) and the primers listed in Table 3. Hybridizations and washes were conducted at 50, 55, and 60 $^{\circ}\text{C}$ using Blotto (0.5% nonfat dry milk, 1% sodium dodecyl sulfate (SDS), $4\times$ SSC [34] as the hybridization buffer and wash buffer of $2\times$ SSC and 0.5% SDS. Radioactive signals were captured either with Kodak BioMax film

Table 1. Voucher information for plant materials.

Species	Family	Tissue	Collection number ^a	Native to	GenBank accession number for 16S rDNA sequences
<i>Cytinus ruber</i> Fritsch	Cytinaceae	Young fruits	Nickrent 2738	Mediterranean ^b	U47845
<i>Hydnora africana</i> Thumb.	Hydnoraceae	Rhizome	Nickrent 2767	South Africa ^c	U67745
<i>Corynaea crassa</i> Hook. f.	Balanophoraceae	Inflorescence	Nickrent 3011	Costa Rica	U67744
<i>Aristolochia elegans</i> M.T. Mast.	Aristolochiaceae	Leaves	Ouyang 94.1.	Brazil	not determined

^aVoucher specimens present at SIU (Nickrent) and Vanderbilt University (Ouyang).

^bPlant cultivated at Bonn Botanical Garden. Tissue kindly provided by W. Barthlott (Universität Bonn).

^cPlant cultivated and donated by S. Carlquist (Claremont, California).

Table 2. Plastid 16S rDNA primers.

Primer sequence (5' → 3')	Position on <i>Nicotiana</i>	Primer Name	Specificity ^a
GGA GAG TTC GAT CCT GGC TCA G	7–28	8 for	P, B
CGG GAA GTG GTG TTT CCA GT	68–87	63 for	P (green plants)
CCA CAC TRG GAC TG	282–295	281A for	P, B, M
CTG CTG CCT CCC GTA	314–328	343A rev ^b	P, B
CAG CAG TGG GGA ATT TTC CG	323–342	323 for	P
ACG ACT CTT TAG CCT	385–399	374 rev	<i>Corynaea</i> -specific
GTG CCA GCA GCC GCG G	462–477	530 for ^b	P, B, M
TGA TAC CCC AAC TTT CGT CGT TG	705–727	705 rev	<i>Corynaea</i> -specific
GCC GTA AAC GAT G	756–768	756 for	P, B, M
AAC TCA AAG GAA TTG	856–870	856 for	P, B, M
GCC CCC GWC AAT TCC T	863–878	915 rev ^b	P, B, M
CCA TGC ACC ACC TG	993–1006	993 rev	P, B, M
CGA GGG TTG CGC TCG TTG	1048–1065	1037 rev ^b	P, B
AGG GGC ATG ATG ACT TGA CG	1139–1158	1149 rev ^b	P, B, M
TTC ATG YAG GCG AGT TGC AGC	1259–1279	1259 rev	P
ACG GGC GGT GTG TRC	1339–1353	1392 rev ^b	P, B, M
GGT GAT CCA GCC GCA CCT TCC AG	1461–1483	1461 rev	P, B
AAG GAG GTG ATC CAG CC	1472–1488	1525 rev	P, B

^a Specificity refers to whether these primers will work for PCR and/or sequencing; P, plastids; B, bacteria; M, mitochondria.

^b Primer obtained from C. Woese.

and two intensifying screens or a Molecular Dynamics Phosphorimager with exposures ranging from 2 to 14 h.

The initial set of 16S rDNA primers was constructed after conducting a multiple sequence alignment contained cyanobacterial sequences (as outgroups) as well as plant nuclear and mitochondrial 18S rDNA sequences, thus permitting the construction of 16S-specific or plastid-specific primers (Table 2). Manual alignment, accomplished using SeqApp [17], was generally unambiguous and secondary structural features were used to guide alignment in variable regions. The species used (and GenBank accession numbers) were: *Anacystis nidulans*

(X03538), *Anabaena* sp. (X59559), *Microcystis aeruginosa* (U03402), *Marchantia polymorpha* (X04465), *Pinus thunbergii* (D17510), *Oryza sativa* (X15901), *Zea mays* (X86563), *Pisum sativum* (X51598), *Glycine max* (X06428), *Daucus carota* (X73670), *Cuscuta reflexa* (X72584), *Nicotiana tabacum* (Z00044), *Epifagus virginiana* (X62099), and *Conopholis americana* (X58864). The sequence of *Brassica* and *Vicia* were obtained from published accounts [14, 15]. To determine plastid-specific sites on 16S rDNA, another alignment was made using the above species as well as additional bacterial and nonasterid holoparasite sequences. This alignment included *Cynomorium coccineum*,

Table 3. Plastid ribosomal protein primers.

Primer sequence (5' → 3')	Primer positions on <i>Nicotiana</i>	Gene length (bp)	Primer names
<i>rps2</i>			
ACC CTC ACA AAT AGC GAA TAC CAA	47	711	<i>rps2</i> -47 for
CTC GTT TTT TAT CTG AAG CCT G	661		<i>rps2</i> -661 rev
<i>rps4</i>			
AGC AAT TCA TTT ATT TKA RAC CGA	32	606	<i>rps4</i> 32 for
ATG TCR CGT TAC CGR GGA CCT M	585		<i>rps4</i> 585 rev
<i>rps7</i>			
ATG TCA CGT CGA GGT ACT GC	1	467	<i>rps7</i> 1 for
TAA CGA AAA TGT GCA AMA GCTC	446		<i>rps7</i> 446 rev
<i>rpl16</i>			
GGC ATT TTA GAT GCT GCT AKT GAA A	29	405	<i>rpl16</i> 29 for
AAA CAA CAT AGA GGA AGA ATG AAG G	351		<i>rpl16</i> 351 rev

Bdallophyton americanum, *Pilostyles thurberi* and *Mitrostema yamamotoi* (see [30]). Also included were a chrysophyte (*Pyrenomonas salina*, X55015), a chlorophyte (*Chlamydomonas reinhardtii*, X03269), a glaucophyte (*Cyanophora paradoxica*, X81840), low G+C gram-positive bacteria (*Clostridium botulinum*, X68185; *Mycoplasma capricolum*, X00921), high G+C gram-positive bacteria (*Bifidobacterium longum*, M58739; *Clavibacter xyli*, M60935; *Mycobacterium sphagni*, X55590; *Rhodococcus fascians*, X79186), Cytophaga/Flexibacter group bacteria (*Flavobacterium aquatile*, M62797), alpha subdivision of purple bacteria (*Rhodobacter sphaeroides*, X53853), and beta subdivision of purple bacteria (*Escheria coli*, J01695). Sequence motifs (of two or more consecutive nucleotides) were sought that were conserved among all land plants (including the nonasterid holoparasites) but were variable in one or more of the algal or bacterial sequences. This process resulted in the identification of a plastid-specific site for which the 323 forward primer was constructed.

The Basic Alignment Search Tool (BLAST [1]) was used to determine the degree of similarity between the nonasterid holoparasite 16S rDNA sequences and existing SSU sequences present in GenBank and EMBL. Numbers of transitions and transversions between pairwise comparisons of aligned sequences were obtained using MEGA [22]. The secondary structural models were constructed using Microsoft ClarisDraw Version 1.0 following a model for *Nicotiana* [19].

Results

16S rRNA sequences

In our initial experiments, 16S rDNA was successfully PCR amplified and sequenced from *Cytinus* (Cytinaceae) and *Hydnora* (Hydnoraceae). Based upon the number and type of mutations, these sequences represented the most divergent 16S rRNA genes yet seen in flowering plants (discussed below). The primer combination of 8 forward and 1461 reverse was used to PCR amplify 16S rDNA from these holoparasites as well as plastid SSU rDNA from a large diversity of green plants (Duff and Nickrent, unpublished data). In contrast, this primer combination did not amplify plastid rDNA from *Corynaea* but instead resulted in a product of bacterial origin. The complete 16S rDNA sequence of this bacterium was determined and, following a BLAST search, was found to be a unique sequence most similar to a high-GC gram-positive bacterium (*Brevibacterium epidermidis*, X76565). Remarkably, the genomic DNA of *Corynaea* was obtained from fresh, internal tissues that were frozen immediately on liquid nitrogen and no macroscopic indication of bacterial infection was apparent. That bacterial sequences have been amplified from *Corynaea* (and other holoparasites) can be explained by the extreme sensitivity of the PCR procedure and the lack of specificity of some 16S rDNA amplification primers. This result further suggested that extreme holoparasites such as *Corynaea* contain extremely low concentrations of plastid DNA and/or that the remaining genes are highly divergent in sequence.

With the goal of identifying plastid-specific priming sites, a multiple sequence alignment was constructed that included a range of diverse 16S rDNA sequences (see Materials and methods). This exercise was clearly limited since only 15 algal and eubacterial sequences were used; therefore, the eubacterial motifs would likely change given a broader sampling of prokaryotic diversity. Several sequence motifs specific to plastid 16S rDNA (i.e. 'signature sequences') were identified (Table 4). Relatively few plastid-specific sites can be identified given the high degree of variation in the holoparasites and since bacterial and plastid sequences are often conserved and variable in the same regions. The 16 motifs that were found, however, were present in all land plant plastid sequences, including the divergent holoparasites, thus these sites are likely conserved across a wide diversity of plants. A plastid-specific primer (323 forward) was subsequently developed that, in combination with 1461 reverse, yielded a 1.2 kb fragment after PCR amplification of *Corynaea* genomic DNA. This fragment was sequenced and a *Corynaea*-specific primer (374 reverse) was then synthesized. In combination with the 8 forward primer, a 0.37 kb PCR product was obtained and sequenced, thus completing the 16S rDNA sequence of *Corynaea*.

Given the difficulty encountered in obtaining these highly divergent holoparasite 16S rDNA sequences, their identity as plastid-encoded sequences may be questioned. Specifically, could these sequences be of mitochondrial origin or derive from incidental bacterial contaminants? All 16 signature sequences identified for plastid 16S rDNA were present on the three nonasterid holoparasite 16S rDNA sequences. When these sequences were subjected to a BLAST search, all were matched with plastid-encoded 16S rDNA sequences, but only *Cytinus* was matched first with an angiosperm. The smallest sum probabilities obtained for *Hydnora* were to *Juniperus* and *Ephedra* (gymnosperms), *Angiopteris* (a fern), *Spirogyra* (a green alga), and *Cytinus*. For *Corynaea*, the smallest probabilities were to *Chondrus* (a red alga) and *Chlorella* (a green alga). Phylogenetic analyses using parsimony also place all the holoparasite sequences on a clade within the angiosperms, not among algal or bacterial groups (results not shown). Plastid SSU sequences can be readily distinguished from those present on the mitochondrial genome by primary and secondary structural features. All plant mitochondrial SSU sequences are longer than nuclear and plastid SSU sequences (ca. 2000, 1800 and 1500 bp, respectively) thus allowing size differ-

ences to be visualized on agarose gels. For mitochondrial SSU rRNA, two regions associated with helix 6 and 43 account for most of the size increases whereas helix 9 is truncated and helix 10 is absent. Finally, distinct and characteristic SSU sequences from each of the three subcellular genomes have been obtained from *Cytinus*, *Hydnora* and *Corynaea* ([29]; Duff and Nickrent, unpublished). The base compositions of the nonasterid holoparasite mitochondrial SSU sequences are very similar to those of other vascular plants (mean of 16 species: A/T = 47%, C/G = 53%), thus it seems unlikely that the plastid SSU gene could have been transferred to the mitochondrion yet still retain a high A/T content in a relatively G/C-rich environment.

The 16S rDNA sequence of *Nicotiana* (tobacco) is representative of nearly all angiosperms, hence it will be used as an exemplar for comparative purposes. In comparisons with tobacco, *Cytinus* shows 121 base substitutional changes, i.e. 8% of the molecule (Table 5). This number represents three times that seen when tobacco is compared to the more distantly related pine. The sequences of two holoparasitic Scrophulariaceae, *Epifagus* and *Conopholis*, show approximately the same number of substitutions as in a monocot to dicot comparison. More extreme divergence is seen when the 16S rDNA sequences of *Hydnora* and *Corynaea* are compared with tobacco. *Hydnora* differs from tobacco at 287 sites (19% of the molecule) and *Corynaea* at 513 sites (35.9%). Transitional mutations outnumber transversions, however, the ratio varies widely depending upon the pairwise comparison. For tobacco compared with pine, the ratio is 3.75:1 whereas the ratio is 2.28:1 when compared with rice. For *Cytinus* and *Hydnora*, the ratio is about 2:1 but in *Corynaea* the ratio drops to 1.3:1 indicating an increasing trend towards transversions. Compositional bias (unequal proportions of A, T, C, and G) becomes pronounced in the three nonasterid holoparasites (Table 5). Comparisons of 13 green plant 16S sequences shows that the proportions seen in *Epifagus* are typical. Conversely, *Cytinus*, *Hydnora* and *Corynaea* show a bias towards increasing proportions of A and T. This trend becomes extreme in *Corynaea* where 72% of the bases are A or T.

Structural analysis of Cytinus 16S rRNA

Given the large number of substitutions in the 16S rDNA sequences of *Cytinus*, *Hydnora* and *Corynaea*, it is possible that the plastid ribosomes of these holoparasites are no longer functional and that these sequences

Table 4. Signature sequences for plastid 16S rDNA.

Positions ¹	Land plant motifs ²	Eubacterial/algal motifs ³	Comments ⁴
29–31	GAT	RWM	5' helix 3
45–46	AT	DK	helix 4
110–114	AAGAA	RDSHR	helix 8
223–224	TT	WH	5' helix 12
244–245	AA	DW	3' helix 12
277–280	ATCA	WBCV	bulge of helix 13
407–409	TTT	WKK	multistem loop of 17, 18, 19
484–485	AG	RK	helix 21
500–501	AT	RW	3' helix 3
770–772	ATA	VBR	5' helix 28
823–825	TAT	YVB	3' helix 28
962–964	AAA	RRR	part of tetraloop of helix 37-1; missing in <i>Pilostyles</i> ⁵
1007–1009	CT	YY	helix 38
1152–1154	TGC	BSS	helix 38
1198–1199	AA	RN	loop between helix 45 & 46
1286–1288	ATC	RTY	helix 33

¹ On *Nicotiana* 16S rDNA.

² Sequence present in all 21 land plant (including holoparasite) plastid 16S rDNAs. Not necessarily exclusive of all bacterial motifs.

³ Sequence present in 15 eubacterial, glaucophyte and algal sequences.

⁴ Helix numbering according to Neefs *et al.* [26].

⁵ See Nickrent *et al.* [30].

Table 5. Comparison of plastid 16S rDNA sequences.

	Nucleotide composition				Total	Transitions (TN) ^a		Total TN	Transversions (TV)				Total TV	Total % substitutions
	%A	%T	%C	%G		AG	TC		AT	AC	TG	CG		
<i>Nicotiana</i> ^b	24.6	18.9	24.1	32.4	1489	–	–	–	–	–	–	–	–	–
<i>Pinus</i>	25.2	18.5	24.5	31.8	1491	13	17	30	2	1	4	1	8	38 (2.5)
<i>Oryza</i>	25.2	19.1	23.9	31.8	1491	14	18	32	6	2	3	3	14	46 (3.0)
<i>Epifagus</i>	25.0	19.5	23.4	32.1	1492	11	15	26	3	7	5	0	15	41 (2.7)
<i>Conopholis</i>	24.5	20.0	23.6	31.9	1487	12	15	27	1	5	9	2	17	44 (2.9)
<i>Cytinus</i>	27.9	22.1	21.4	28.5	1497	42	38	80	10	11	17	3	41	121 (8.0)
<i>Hydnora</i>	32.4	24.5	18.4	24.7	1504	118	80	198	31	22	26	10	89	287 (19.0)
<i>Corynaea</i>	39.8	32.2	12.1	15.9	1452	168	121	289	64	73	77	10	224	513 (35.9)

^a Transitions and transversions calculated relative to *Nicotiana*.

^b See Materials and methods for GenBank accession numbers to published 16S rDNA sequences.

represent pseudogenes. To investigate the effect of each type of mutation on the higher-order structure of the 16S rRNA molecule, models for *Cytinus*, *Hydnora* and *Corynaea* were constructed. The structural model for *Cytinus* is shown in Figure 1. Models for the other two species differed little from *Cytinus* in overall features, despite extensive sequence variation. Detailed analyses of the above three and other nonasterid holoparasite 16S rRNA sequences have been reported [30].

All helical regions found in the 16S rRNA molecule of *Nicotiana* are present and of approximately the same length in *Cytinus* (Figure 1). Fully compensated changes occur when each paired base in a helix changes but canonical or noncanonical base pairing is retained. Fully compensated changes can be found in 14 pairs representing 28 mutational events. Compensated changes, when one member of a base pair in a helix changes, yet canonical or noncanonical pairing

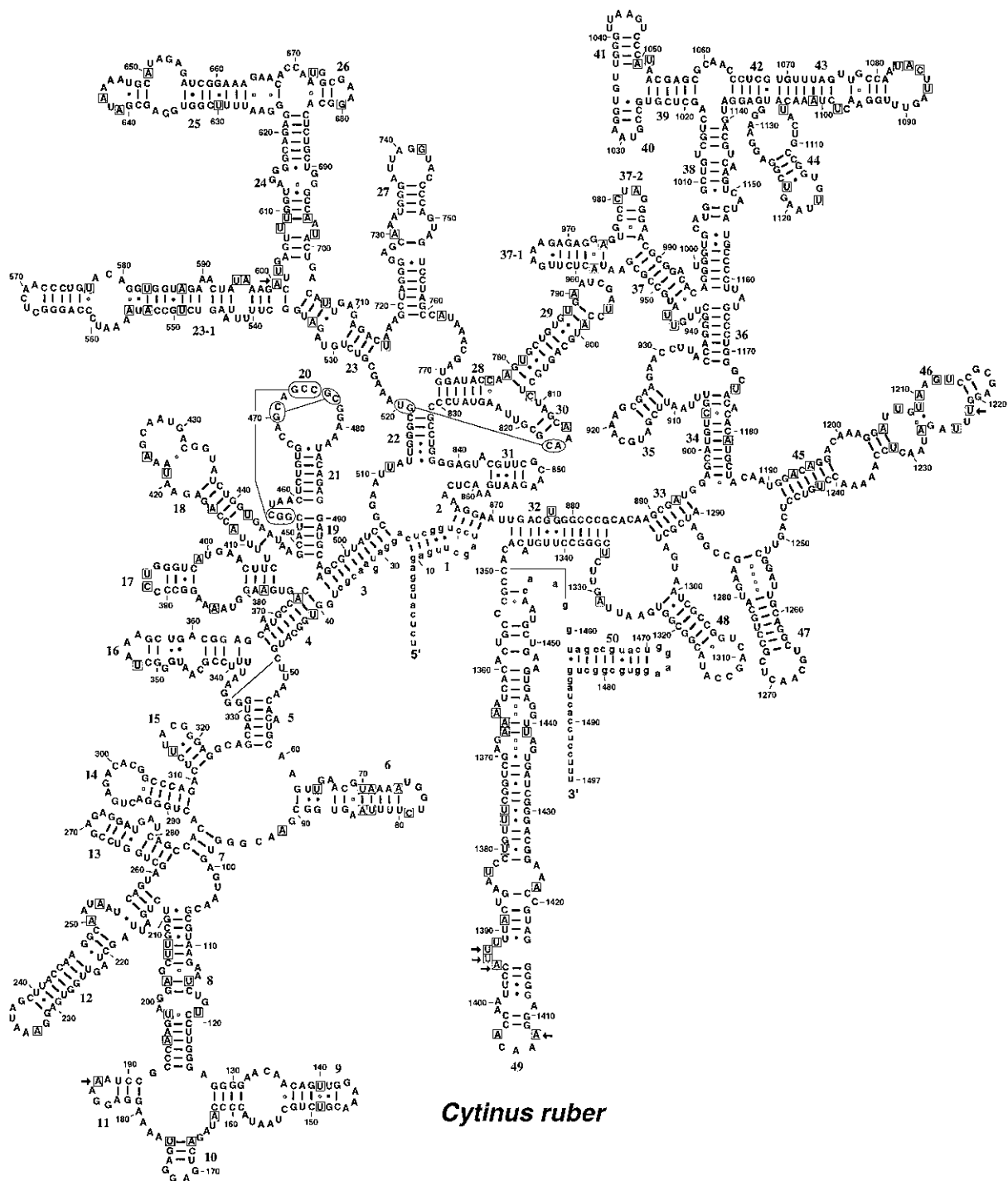


Figure 1. Secondary structural model for plastid-encoded 16S rRNA from *Cytinus ruber* (Cytinaceae or Rafflesiaceae). This model was constructed from the DNA sequence using the structure of *Nicotiana* as a reference which was obtained from the rRNA structural database (<http://pundit.colorado.edu:8080/RNA/16S/16s.html> [18]). Noncanonical pairings are included to coincide with covariance data obtained for all plastid SSU rRNA sequences. The numbering of helices follows that proposed by Neefs *et al.* [26] for prokaryotic SSU rRNAs. Bases in lower case letters (tobacco shown for display purposes) were not determined since they occur at or near the 5' and 3' priming positions. The 121 substitutional mutation in *Cytinus* relative to tobacco are indicated by bases enclosed in boxes. The seven insertion mutations are marked with arrows.

is maintained (e.g. G-C → G • U), was observed at 29 sites. Among these, six changes resulted potential (rare) noncanonical base pairs (e.g. U • C, C • A, C • C, or U • U). Changes in unpaired regions (loops and bulges) were the largest class of mutation, constituting 64 changes. Seven insertion mutations and no deletions were observed. These results show that, despite frequent mutation, the majority do not result in obvious disruptions of the secondary structure.

Hybridization to plastid gene probes

Hybridizations to Southern blots containing digested genomic DNA yielded strong signal from the green plant control (*Aristolochia*) but signals of different intensities from the three holoparasites. For 16S rDNA (Figure 2A), strong hybridizations were obtained on blots of *Sma*I and *Hind*III digested genomic DNA of *Aristolochia* and *Cytinus*. The major *Sma*I fragment for *Aristolochia* was 18 kb in size as are simulated digestions of other angiosperms for which a complete plastome sequence is known. The *Sma*I fragment for *Cytinus* was ca. 20 kb in length. Hybridizations to *Corynaea* and *Hydnora* DNA resulted in weak bands representing a range of fragment sizes. For *Hind*III, *Cytinus* showed a strongly hybridizing band at 3 kb as compared with 6 kb for *Aristolochia*. Multiple faint bands were detected in *Corynaea* ranging in size from 4 to 15 kb.

For the *rps7* probe used with *Sma*I-digested genomic DNA, *Aristolochia* showed very strong hybridization at ca. 18 kb. Strong hybridization to a 20 kb fragment was obtained for *Cytinus*. *Corynaea* and *Hydnora* showed hybridization at the higher molecular weight position (possibly uncut genomic DNA). For the *Hind*III digestions, the blot showed strong hybridization to a 1.7 kb fragment for *Cytinus* and weak hybridization at 20 kb for *Corynaea* (*Hydnora* not analyzed). Among the three holoparasites, only *Cytinus* showed definite hybridization to the remaining ribosomal protein probes (Figure 3A, B, C). For *Sma*I digests of *Cytinus* DNA, hybridization to a 20 kb fragment was seen in *rps2*, *rps4* and *rpl16*. The *Hind*III digest probed with *rps2* resulted in a fragment of 0.8 kb with minor bands at higher molecular weights. For *rps4*, two weakly hybridizing bands of ca. 3.0 and 3.8 kb were seen. Finally, a light band of ca. 6.0 kb was obtained using the *rpl16* probe which is similar in size to the *Aristolochia* control.

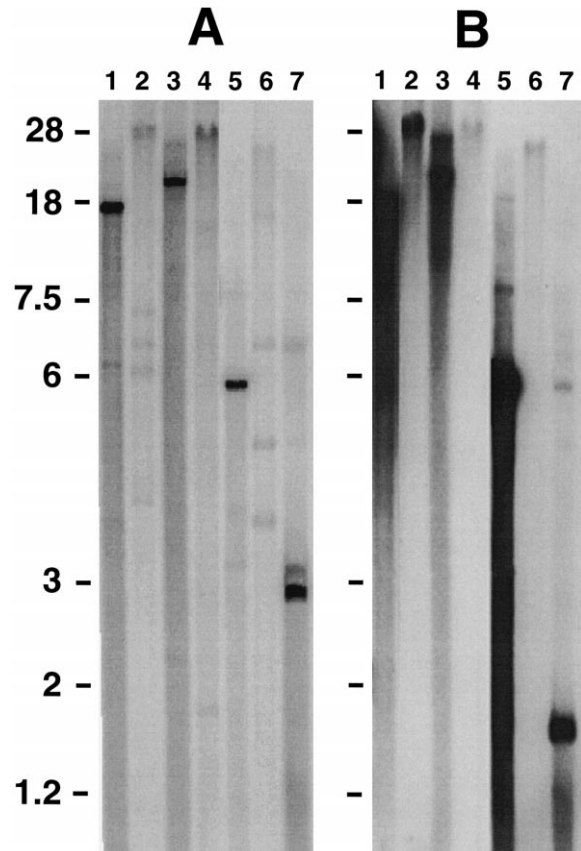


Figure 2. Results of Southern blot analyses of nonasterid holoparasites and a green plant control (*Aristolochia*). A. Blot using a probe to plastid-encoded 16S rDNA (2-day exposure). B. Blot using a probe to plastid-encoded *rps7* (7-day overexposure to detect possible light signals). Lanes 1 and 5, *Aristolochia* (Aristolochiaceae); 2 and 6, *Corynaea* (Balanophoraceae); 3 and 7, *Cytinus* (Cytinaceae); 4, *Hydnora* (Hydnoraceae). Total genomic DNA (0.5–2 µg) was completely digested with *Sma*I (lanes 1–4) and *Hind*III (lanes 5–7), electrophoresed 18 cm on 1% agarose gels, transferred to filters, and hybridized to probes constructed (via PCR) to plastid genes. Hybridizations and washes were at 60 °C; lower-stringency hybridizations and washes (to 50 °C) did not affect the number of fragments observed. Fragment sizes (in kb) are based upon a standard ladder. See text for discussion of these results.

Discussion

Sequence data for plastid-encoded SSU rDNA and positive hybridizations to four plastid ribosomal protein genes represents the first direct evidence that suggests retention of a plastid genome in *Cytinus*. At present, only SSU rDNA sequence data have been obtained for *Corynaea* and *Hydnora*, thus the presence of a plastome in these holoparasites remains unconfirmed. The following discussion will focus on the question

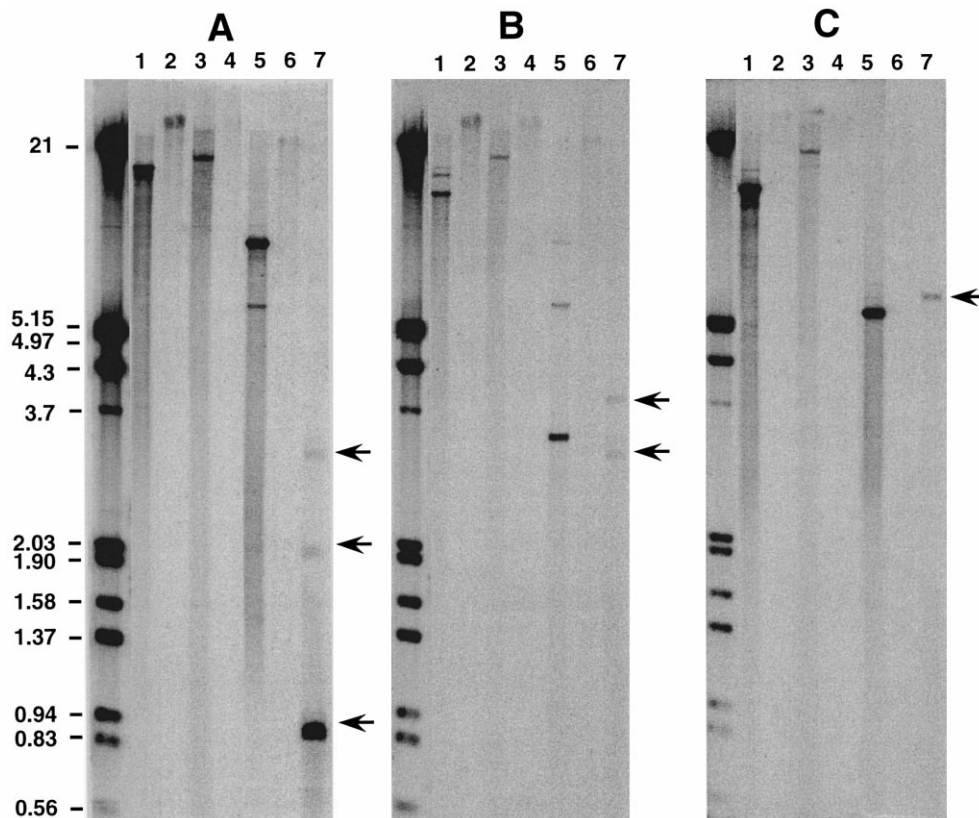


Figure 3. Results of Southern blot analyses of nonasterid holoparasites and a green plant control (*Aristolochia*). Phosphorimager scans to plastid-encoded genes: *rps2* (panel A), *rps4* (panel B), and *rpl16* (panel C). Samples (lanes), enzyme digestions, and electrophoretic and hybridization conditions as in Figure 2. Fragment sizes (in kb) of a standard ladder are shown. Positive hybridizations were obtained only for *Aristolochia* and the holoparasite *Cytinus*. For the latter, major and minor bands are indicated with arrows.

posed in the title, i.e. ‘do nonasterid holoparasitic flowering plants have plastid genomes’. The evidence for and against the presence of a plastome will be examined in the light of the data obtained to date and with respect to the limitations imposed when working with extremely divergent holoparasite genomes.

Evidence from ribosomal genes

The ribosomal RNA cistron is possibly the most conservative suite of genes in the plastid genome, hence they were the focus of our initial experiments aimed at detecting plastid genes in the nonasterid holoparasites. As shown in Table 5, there is a progressive increase in the number of mutations at the 16S rDNA locus from *Cytinus* to *Hydnora* and *Corynaea*. Motifs specific to plastid SSU rDNA can be identified (Table 4) and, in combination, can readily distinguish land plant SSU sequences from all others. The presence

of these specific motifs, results of BLAST searches, and phylogenetic analyses all indicate that the holoparasite SSU sequences are indeed most similar to other land plant plastid sequences. The retrieval of ‘hits’ to nonangiospermous plants following BLAST searches underscores the wide divergence of the holoparasite sequences as compared with others present in GenBank. This result further indicates that such searches cannot be used in lieu of formal phylogenetic analyses.

Although we have not obtained direct experimental evidence for the functionality of these unusual rRNAs, the locations and types of mutations do not appear to have resulted in the formation of pseudogenes. Given the evolutionary pressure toward a compact genome, pseudogenes in cpDNA are rare; however, two tRNA genes and several photosynthetic genes in *Epifagus* have become pseudogenes [25, 40, 46]. Only one report exists of a plastid small-subunit (SSU) rRNA pseudogene, i.e. that of the nonphotosynthetic euglen-

oid *Astasia longa* [37]. If plastid SSU rRNAs were not utilized in plastid ribosomes, it would be predicted that these rRNA genes would be free to accumulate random, neutral mutations and that structural components important in translation would be lost. Such does not appear to be the case with the nonasterid SSU sequences obtained to date. The results reported here indicate that although SSU rRNA contains more sites that are free to vary than expectations derived from previous sequence comparisons of photosynthetic plants, the majority of these are compensatory substitutions, thus retaining all the major structural components of the rRNA molecule (Figure 1 and [30]). Evidence now exists that the most structurally divergent SSU rRNA seen to date (from *Pilostyles*) is transcribed [30]. Since the translational demands of the plastid have been greatly reduced following the loss of photosynthetic genes, relaxation of selection against particular mutations may have occurred [25, 44].

Our data do not disprove the possibility that these divergent plastid SSU rDNAs have been translocated to the nucleus or mitochondrion, however, such movements seem improbable. Based upon our supposition that these rDNA genes are functional, it would seem unlikely that they exist in other organelles. Few examples exist where genes that have moved to a different subcellular compartment retain functionality [3]. Although transfer to the nucleus cannot be discounted, cases of functional gene transfers, such as *tufA* [2], are extremely rare. If plastid SSU sequences have moved to other organelles, this event would have to have occurred independently several times since the nonasterid holoparasites are not closely related [28, 29]. Moreover, three distinct SSU rDNA sequences have been obtained for each of the three holoparasites and these sequences retain base composition biases characteristic of those derived from separate subcellular organelles. Evidence for the presence of a reduced plastome in *Cytinus* includes the linkage data for the plastid genes that are widely spaced around the genome of photosynthetic plants. Additional empirical evidence is the recent generation of sequence for 23S rDNA and the 16S/23S spacer for *Cytinus* (Duff, unpublished). The spacer is 200 bp in length as compared with ca. 2000 bp in tobacco. These data indicate that the entire rDNA cistron is present and that it is colinear with tobacco. At least for *Cytinus*, the translocation hypothesis seems even less likely since it would require that the entire rDNA cistron be moved intact to another organelle.

Evidence from hybridizations to plastid-encoded genes

Multiple, weakly hybridizing bands were detected on the 16S rDNA Southern blots of *Corynaea*. At least two possibilities exist that could explain these results. As described above, the *Corynaea* genomic DNA contains bacterial DNA that could weakly hybridize to the probe. A second explanation is that the rDNA probe hybridized poorly to the uniquely diverged plastid rDNA genes and/or to nuclear and mitochondrial rDNA that is also present in the genomic DNA sample.

Hybridization experiments with all other known lineages of holoparasitic plants (Orobanchaceae, Lenoaceae, Cuscutaceae) and several groups of non-photosynthetic mycotrophic plants (Monotropaceae, Orchidaceae) yield strong hybridization signals to each of the plastid gene probes tested (Ouyang and dePamphilis, unpublished data). These data indicate that the probes are sufficiently similar to plastid DNA of most flowering plants to result in hybridization. To determine whether the lack of hybridization in *Hydnora* and *Corynaea* was the result of using a heterologous (green plant) probe, additional experiments were conducted using homologous 16S rDNA probes. A probe, constructed via PCR amplification of *Cytinus* DNA, hybridized only to *Cytinus* genomic DNA, not to either *Hydnora* or *Corynaea*. This result indicates that the amount of sequence divergence between *Cytinus* and the other two holoparasites is approximately the same as between these holoparasites and any green plant (such as *Aristolochia*). For this reason, the use of a green plant probe was appropriate. Generation of homologous probes to other plastid-encoded genes for *Hydnora* and *Corynaea* is hampered by lack of sequence information to construct PCR primers.

Hybridization to the same fragment using at least two plastid-encoded gene probes is required to establish linkage and thereby the presence of a plastid genome. Of the three holoparasites, evidence for linkage was obtained only for *Cytinus*. Given that all five plastid gene probes hybridized to a fragment of ca. 20 kb in length, it is possible that this fragment represents most or all of the plastid DNA molecule. Information from the sizes of the *HindIII* restriction fragments can potentially give further information relevant to the size of the *Cytinus* plastome. The following interpretation of the data should be considered highly preliminary at this stage. In tobacco, 16S rDNA and *rps7* are present on the inverted repeat and are separated by 2754 bp, i.e. the smallest distance among the gene

probes tested here), however, linkage was not detected in *Cytinus* suggesting a restriction site exists between them. Potential linkage can be suggested between *rps7* and *rpl6* (6 kb fragment) and between *rps2* and *rps4* (3 kb fragment). Assuming colinearity of the *Cytinus* and tobacco genomes, the *Hind*III data suggest a genome of ca. 18.3 kb, i.e. similar to the *Sma*I results. The difference in size could be accounted for by the release of fragments that did not hybridize to any of the probes.

Results with other holoparasites

Previous work has demonstrated that photosynthetic genes have been selectively lost from the genome of *Epifagus* (beechdrops), yet the organization of its remaining genes is colinear with tobacco [9, 25, 45]. For genes such as rDNA, substitution rates are only slightly increased in contrast to being highly increased in holoparasites such as *Cytinus* [29]. In *Epifagus*, strong hybridization signals were obtained for ribosomal DNAs and other plastid genes such as *rps7* and *rpl2* [9] indicating that sequence divergence has not been so great as to compromise the use of heterologous probes constructed from tobacco. These and other data suggest that despite dramatic losses of plastid genes, the reduced plastome of *Epifagus* is functional [8, 13, 45]. From an evolutionary perspective, it is also likely that such events have taken place relatively recently in *Epifagus* as compared with plants such as *Cytinus*, *Hydnora* and *Corynaea*. Alternately, processes of accelerated sequence evolution and structural alterations that are seen in *Epifagus* have occurred at a greatly accelerated pace in the nonasterid holoparasites.

Although progress has been made in our investigations of plastome structure for *Cytinus*, such has not been the case with other holoparasites thought to be its relatives. Neither PCR amplifications of 16S rDNA nor Southern blots using heterologous 16S or plastid ribosomal protein probes were successful for large-flowered members of Rafflesiaceae such as *Rafflesia* or *Rhizanthus* (data not shown). These negative results suggest much greater sequence divergence than seen in *Cytinus* or possibly that the genome has been lost entirely. For small-flowered members of Rafflesiaceae, a highly divergent plastid 16S rDNA sequence has been obtained for *Pilostyles* [30] but not for the closely related *Apodanthes* thereby illustrating high variation between even closely-related genera.

For Balanophoraceae, we have obtained partial 16S rDNA sequence data for *Helosis cayennensis*, a close relative of *Corynaea*, and *Scybalium jamaicensis* (data not shown). The sequence of *Helosis* is most similar to *Corynaea* followed by *Scybalium*. In contrast to these New World members of the family, we have not been successful in PCR amplifications involving Old World genera such as *Balanophora*, *Dactylanthus* and *Rhopalocnemis* nor *Langsdorffia* (found in the Old and New World tropics). Using the mutation rate increase observed for nuclear 18S rDNA as an indicator of overall sequence divergence, the Old World genera show a higher substitution rate than New World genera, hence our negative PCR results are not surprising. As with the large-flowered Rafflesiaceae, the plastomes in these holoparasites may be so divergent as to escape molecular detection or they may be absent altogether.

Retention of plastids and plastomes in heterotrophic plants

The results of these investigations suggest that if the nonasterid holoparasites retain a plastome, it is either present in very low copy number and/or is highly diverged relative to photosynthetic plants and other previously examined holoparasitic plants. Small amounts of plastid DNA could result from low numbers of plastids and/or few copies of the chromosome per plastid. As shown with photosynthetic and heterotrophic *Ochromonas* (Chrysophyta), wide variation in number of plastids per cell (1 to greater than 8) and genomes per plastid (7 to greater than 200) occurs, where the lowest number of plastids per cell was found in dark-grown cultures [23]. As these authors point out, the total loss of plastid DNA has never been described for any plant. Explanations for retention of plastids, even in nonphotosynthetic plants such as *Epifagus* and *Conopholis*, usually involve maintenance of nonphotosynthetic processes such as the synthesis of lipids, aromatic amino acids, porphyrins, pyrimidines, chlororespiration, and reduction of nitrates and sulfates [8, 9, 23, 45]. It has also been suggested that hydrophobic membrane proteins which cannot easily be imported across the plastid outer membrane are reason for retention of a plastome [33]. Plastome redundancy may be required to allow genetic exchange and repair, especially in plants with uniparental plastid inheritance [23]. High levels of substitution, as seen in nonasterid holoparasites, may be a result of their lacking sufficient copies of plastid DNA to affect recombination.

The work presented here provides insights that may guide further investigations of plastome structure in nonasterid holoparasites. As discussed above, it has been suggested that the size of the plastome is being maintained by selection to retain certain genes [32]. The smallest plant plastid genome described to date (43 kb) is that of *Conopholis* (squawroot) [5] which, unlike *Epifagus*, has lost a copy of the inverted repeat. It is not known how small plastomes may become and still maintain functionality. Normally duplicated sequences are predicted to show an increase in substitutions when present in a genome where they are not duplicated [32]. Such appears to be the case with 16S rDNA sequences of *Vicia* and *Pisum* that show as many changes since their divergence from *Glycine* as occurs on the *Epifagus* and *Conopholis* clade [29]. High substitution rates in rDNA genes for nonasterid holoparasites could be indirect evidence that they have lost a copy of the inverted repeat. The mechanisms underlying mutation rate increases in plastid genes are presently not known, but may be due to changes in enzymes involved in DNA replication or repair [10, 30]. Further investigation of the striking nucleotide composition bias seen in nonasterid holoparasites is warranted. Sequence data on protein-coding genes is crucial to determine whether the nucleotide bias has affected amino acid composition. The interaction between nuclear and plastid genomes requires investigation since it is known that particular nuclear mutator genes can cause an increase in mutations in organelles [35].

Future prospects

Future molecular studies would benefit by having ready access to fresh tissues, hence attempts will be made to establish cultured specimens of *Cytinus*. Improved methods, tailored specifically to nonphotosynthetic holoparasites, need to be developed to isolate plastids and/or plastid DNA. Given the reduced size of holoparasite plastomes, complete sequencing is feasible and will be first pursued in less extreme holoparasites such as *Cytinus*. The presence of contaminating (or symbiotic?) microorganisms in and on the tissue of plants such as *Corynaea* necessitates caution when using PCR to screen for plastid-encoded genes. Despite the inherent difficulties, holoparasitic plants represent an excellent resource for genetic work. Their uniquely divergent genomes are naturally occurring genetic mutants [8, 35] whose study promises to illuminate the biochem-

ical and evolutionary mechanisms involved in shaping plastid structure and function.

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