J Mol Evol (2001) 53:670–679
DOI: 10.1007/s002390010254

JOURNAL OF MOLECULAR EVOLUTION

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Complex Patterns of Plastid 16S rRNA Gene Evolution in Nonphotosynthetic Green Algae

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Received: 29 December 2000 / Accepted: 18 May 2001

Abstract. This study provides a phylogenetic/comparative approach to deciphering the processes underlying the evolution of plastid rRNA genes in genomes under relaxed functional constraints. Nonphotosynthetic green algal taxa that belong to two distinct classes, Chlorophyceae (*Polytoma*) and Trebouxiophyceae (*Prototheca*), were investigated. Similar to the situation described previously for plastid 16S rRNA genes in nonphotosynthetic land plants, nucleotide substitution levels, extent of structural variations, and percentage AT values are increased in nonphotosynthetic green algae compared to their closest photosynthetic relatives. However, the mutational processes appear to be different in many respects. First, with the increase in AT content, more transversions are noted in *Polytoma* and holoparasite angiosperms, while more transitions characterize the evolution of the 16S rDNA sequences in *Prototheca.* Second, although structural variations do accumulate in both *Polytoma* and *Prototheca* (as well as holoparasitic plastid 16S rRNAs), insertions as large as 1.6 kb characterize the plastid 16S rRNA genes in the former, whereas significantly smaller indels (not exceeding 24 bp) seem to be more prevalent in the latter group. The differences in evolutionary rates and patterns within and between lineages might be due to mutations in replication/repair-related genes; slipped-strand mispairing is likely the mechanism responsible for the expansion of insertions in *Polytoma* plastid 16S rRNA genes.

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Key words: 16S rRNA — Plastid — Nonphotosynthetic — Insertion — Repair — *Polytoma* — *Prototheca*

Introduction

Evolution is often associated with loss of functions and/or structures. One of the most dramatic evolutionary losses is the loss of photosynthesis in green plants. Although "green" lineages are usually defined by their ability to photosynthesize, many nonphotosynthetic "green plants," both land plants and green algae, are known. Among such lineages are the parasitic nonphotosynthetic (holoparasitic) angiosperms (e.g., Scrophulariaceae/Orobanchaceae) and the strictly heterotrophic ("colorless") unicellular green algae (e.g., *Polytoma, Polytomella, Prototheca*). In both land plants and green algae, loss of photosynthesis has occurred more than once: at least five times in Scrophulariales (dePamphilis et al. 1997), at least four times among nonasterid plants (Nickrent et al. 1998), and at least four times in green algae (Rumpf et al. 1996; Huss et al. 1999).

The nonphotosynthetic land plants and green algae investigated to date have been found to contain degenerate plastids (leucoplasts) and/or plastid DNA (e.g., dePamphilis and Palmer 1990; Machado and Zetsche 1990; Haberhausen et al. 1992; Vernon 1996; Nickrent et al. 1997b). Leucoplast genomes in nonphotosynthetic plants are reduced in size and number of genes compared with their counterparts in photosynthetic relatives (e.g., Morden et al. 1991; Wolfe et al. 1992a; Nickrent et al. 1997b).

The retention of a degenerate but potentially active plastid genome in all secondarily nonphotosynthetic green plants is attributed to the presence of unidentified plastid genes whose protein products are thought to be indispensable for the well-being of the organism (e.g., Wolfe and dePamphilis 1998). It thus follows that the gene expression machinery is still present and may be active in these degenerate plastids. Genes coding for components of the leucoplast transcriptional/translational apparatus (e.g., rRNAs, tRNAs, elongation factors, ribosomal proteins) indeed have been found in many holoparasitic plants (e.g., dePamphilis and Palmer 1990; Wimpee et al. 1991; Wolfe et al. 1992a; Nickrent et al. 1997a; Lohan and Wolfe 1998) as well as nonphotosynthetic green algae (Vernon 1996; Vernon et al. in press). Although there is evidence that plastid genes are transcribed in some parasitic plants (e.g., Ems et al. 1995; Nickrent et al. 1997a), the actual cellular localization as well as the functionality of their products awaits further investigations. In addition, the transcription of plastid genes is dependent upon components of the cytosolic expression machinery (such as RNA polymerase), which substitute for the plastid-encoded counterparts whose coding regions became pseudogenes (Lusson et al. 1998).

Generally, it is believed that the plastid translational machinery in nonphotosynthetic plants is subjected to less stringent selection because, subsequent to the loss of photosynthesis in these lineages, translation levels drop (e.g., Morden et al. 1991; Wolfe et al. 1992b; dePamphilis et al. 1997). Indeed, some leucoplast genes that feature accelerated rates of DNA sequence evolution, have undergone major structural changes and/or have become pseudogenes (e.g., Wolfe et al. 1992b, c; dePamphilis et al. 1997; Nickrent et al. 1997a; Wolfe and dePamphilis 1998). However, a study on the evolution of the gene encoding the plastid ribosomal protein S2 (*rps2*) in the Scrophulariaceae/Orobanchaceae lineage (which contains free-living and parasitic plants) revealed that not all holoparasites appear to have accelerated rates of *rps2* evolution; on the other hand, some hemiparasites (i.e., photosynthetic parasites) do show increased rates (dePamphilis et al. 1997). In addition, different mechanisms seem to be independently acting in the Scrophulariaceae/Orobanchaceae lineage: lessening of functional constraints might be responsible for the increased rate of nonsynonymous substitutions, but variations in mutational or repair mechanisms are likely responsible for the observed differences in synonymous rates (dePamphilis et al. 1997). Furthermore, the increased rate of nonsynonymous substitutions was shown to be lineage specific (Nickrent et al. 1998). Lineage-specific processes may also be responsible for differences in the retention of a photosynthetic gene, *rbcL*, among the holoparasite *Orobanche* species examined by Wolfe and dePamphilis (1997, 1998).

Although information on plastid genes in nonphotosynthetic plants is currently accumulating (e.g., Wolfe and dePamphilis 1997, 1998; dePamphilis et al. 1997; Young et al. 1999), the forces and the mechanisms involved in the evolution of leucoplast genes are not fully understood. The questions addressed in this study include (i) What are the patterns and trends that characterize the evolution of plastid 16S rRNA genes in nonphotosynthetic green algal lineages? (ii) Are these evolutionary patterns and trends similar or different among heterotrophic green algal groups and between green algae and parasitic angiosperms? and (iii) Which are the potential evolutionary forces and mechanisms underlying the evolution of plastid genes in nonphotosynthetic green algal lineages?

A comparative/phylogenetic approach can provide information on these evolutionary processes and help distinguish between general and lineage-specific phenomena. It is in this light that in the present study nonphotosynthetic green algal lineages from two green algal groups, *Polytoma* and *Prototheca*, have been investigated. The former belongs to the class Chlorophyceae, whereas the latter is included in the Trebouxiophyceae (*sensu* Friedl 1995). *Polytoma* consists of *Chlamydomonas*-like biflagellate algae that affiliate with at least two distinct chlamydomonadalean clades (Rumpf et al. 1996), whereas *Prototheca* species are nonflagellate algae that are related to *Chlorella* species (Huss et al. 1999).

Materials and Methods

The classification, species names, source, and strain numbers of the taxa investigated, as well as the accession numbers of the sequences analyzed in this study, are summarized in Tables 1 and 2. Algal cultures were grown in the media indicated in Table 1. Cultures were axenic and checked for bacterial contamination before nucleic acid extraction. Total DNA and RNA were extracted as described previously (Nedelcu et al. 1996, 2000). Total DNA from *P. oviforme* and the *rrn16* sequence of *P. obtusum* were kindly provided by Dawne Vernon and C. William Birky, Jr. PCR amplifications were performed using a Stratagene Robocycler and Platinum Taq DNA Polymerase High-Fidelity, Elongase Enzyme Mix, and SuperScript One-Step RT-PCR System (Life Technologies). PCR primer sequences and their positions relative to the *Escherichia coli* counterpart are summarized in Table 3. Where more than one PCR product was obtained, the major bands were individually excised, and the DNA was extracted, purified, and reamplified. The PCR products were cleaned using a Qiagen Gel Extraction Kit and/or PCR Purification Kit and sequenced on both strands using the automated sequencing facilities at the Laboratory for Molecular Systematics and Evolution, University of Arizona, Tucson (http://www.arl.arizona.edu/lmse/). Sequences were aligned using Clustal V, and the alignments were refined manually, based on conserved secondary structure features. Phylogenetic analyses were performed using PAUP version 4.0 (Swofford 1998); gaps and unalignable regions were manually excluded from the analyses using MacClade version 4.0 (Madison and Madison 2000). Bootstrap analyses (100 replications) were conducted using the default settings in PAUP. Homology searches were performed via BLAST (Altschul et al. 1990) at the National Center for Biotechnology Information (NCBI). Base composition, transition/

Table 1. Classification, species name/strain number, source, and growth media for the taxa whose plastid 16S rRNA sequences were obtained in this study

Classification	Taxon	Source/strain[a]	Medium[b]
Chlorophyceae			
Polytoma uvella clade	*Polytoma uvella*	UTEX 964	PM
	Polytoma mirum	SAG 62-3	PM
	Chlamydomonas applanata	UTEX 225	mM
Polytoma oviforme clade	*Polytoma oviforme*	SAG 62-27	PM
Outgroup	*Scenedesmus obliquus*	UTEX 78	BM
Trebouxiophyceae			
Prototheca clade	*Prototheca wickerhamii*	UTEX 1533	MM

[a] UTEX, Culture Collection of Algae at the University of Texas at Austin; SAG, Culture Collection of Algae at the University of Gottingen, Gottingen, Germany.
[b] BM, basal medium (Oh-Hama and Hase 1980); PM, *Polytomella* medium (Burton and Moore 1974); mM, minimal medium (Starr and Zeikus 1993); MM, malt medium (Wolff and Kuck 1990).

Table 2. Accession numbers of the sequences used in this study

	Accession number[a]	
Taxon	16S rDNA	18S rDNA
Chlamydomonas applanata/humicola	AF394204*	U13984
Chlamydomonas moewusii	X15850	U41174
Chlamydomonas reinhardtii	J01395	M32703
Chlorella ellipsoidea	X12742	—
Chlorella protothecoides	X65688	X56101
Chlorella vulgaris	AB001684	X13688
Polytoma obtusum	AF374187	U22935
Polytoma oviforme	AF394207*	U22936
Polytoma mirum	AF394203*	U22934
Polytoma uvella	AF394208*, AF394209*	U22943
Polytomella parva	—	D86497
Prototheca zopfii	X74006	X63519
Prototheca wickerhamii 1533	AF394205*	—
Prototheca wickerhamii 263-11	X74309	X74003
Nephroselmis olivacea	X74754	AF137379
Scenedesmus obliquus	AF394206*	X56103

[a] Asterisks indicate sequences obtained in this work.

transversion ratios, repeated sequence analyses, and nucleotide substitutions per site analyses were done using Gene Runner (Hastings Software, Inc., Hastings, NY) and the Molecular Evolutionary Analysis (MEA) package (Moriyama and Powell 1997). Nucleotide substitutions per site were estimated based on the two-parameter method of Kimura (1980). Transition/transversion ratios were assessed using a Tamura and Nei (1993) method in MEA.

Results

PCR Amplification of Plastid 16S rDNA Sequences

This work reports six new plastid 16S rDNA sequences belonging to four nonphotosynthetic taxa (*Polytoma mirum* SAG 62-3, *Polytoma uvella* UTEX 964, *Polytoma oviforme* SAG 62-27, *Prototheca wickerhamii* UTEX 1533) as well as to two photosynthetic relatives (*Chlamydomonas applanata* UTEX 225 and *Scenedesmus obliquus* UTEX 78). The first five sequences were obtained from PCR amplifications of total DNA, whereas the sixth came from the amplification of a cDNA.

Of the six species whose plastid 16S rRNA genes (*rrn16*) were amplified in this work, two, i.e., *P. oviforme* and *P. uvella*, yielded PCR products of a larger size than expected. Attempts to amplify (from both genomic DNA and total RNA) plastid *rrn16* sequences from another nonphotosynthetic green algal taxon, *Polytomella parva*, proved unsuccessful.

Table 3. PCR primers used to amplify chloroplast 16S rDNA sequences

Primer	Sequence (5′ to 3′)	*E. coli* coordinate
Forward		
ms-5′	GCGGCATGCTTAACACATGCAAGTCG	*E.c.* 39
A	GTTTGATCCTGGCTCAC	*E.c.* 11
B	GAGGCGAAAGCGCTCTACTAG	*E.C.* 722
E	CGCTGAGAGACGAAAGCTATGG	*E.c.* 754
964/5′*a* ins	TCCGACGATTCATCGGT	—
Reverse		
ms-3′	GCTGACTGGCGATTACTATCGATTCC	*E.c.* 1362
C	CGCTACCATAGCTTTCGTCTC	*E.c.* 780
D	ACGGGCGGTGTGTAC	*E.c.* 1392
F	CCTAGTAGAGCGCTTTCGGCC	*E.c.* 742
964/ins3′	CTCGCCGCATTTCTAATGCGGTG	—
62-27/ins3′	GGCTACCTATAGGTAAACGTTGCCCGC	—

Phylogenetic Analyses

Phylogenetic analyses using nuclear 18S rDNA (*rrn18*) (1344 sites) as well as plastid *rrn16* sequences (1062 sites) were performed to assess the phylogenetic position of the taxa investigated and to identify their closest green relatives. In addition to the sequences obtained in this work, a number of sequences already available in GenBank were used (Table 2). *P. parva* was included in these analyses as the third nonphotosynthetic chlorophycean lineage and *S. obliqutus* as a representative of the non-chlamydomonadalean chlorophycean clade; the prasinophyte *Nephroselmis olivacea* sequences were used to root the trees. The trees were constructed using neighbor-joining and parsimony analyses and produced roughly the same results (data not shown), with one exception (discussed below). Figure 1 shows the relative affiliations of the taxa, as suggested by nuclear *rrn18* and plastid *rrn16* sequences (Figs. 1A and B, respectively). The phylogenetic analyses presented here are intended to provide a phylogenetic framework for this work and to

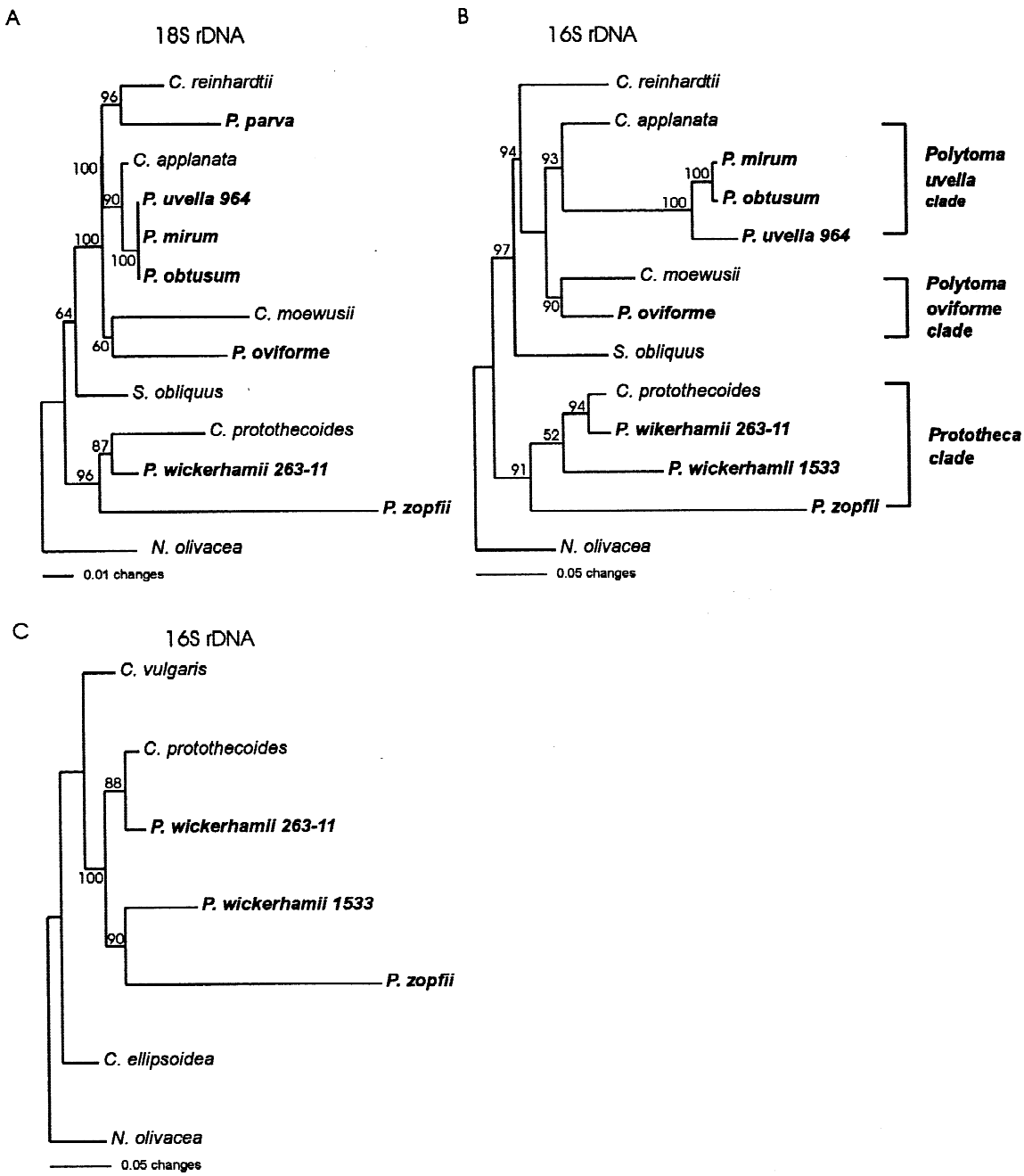

Fig. 1. Neighbor-joining phylogenetic trees using nuclear 18S rDNA (**A**) and chloroplast 16S rDNA (**B** and **C**) sequences. Full species names are indicated in Table 2; nonphotosynthetic taxa are in *boldface.* Numbers indicate bootstrap values (maximum parsimony, 100 replications, heuristic search). Branch lengths correspond to evolutionary distances.

facilitate the discussion of patterns and trends in the evolution of plastid 16S rRNA genes in these nonphotosynthetic lineages; most of the affiliations in these trees have been previously supported or discussed by others—see below. In addition, these phylogenetic analyses prove the algal plastid nature of the sequences PCR-amplified in this work (i.e., they exclude the possibility of a mitochondrial or bacterial contaminant). Overall, these phylogenetic analyses suggest that photosynthesis was lost independently at least three times in the evolution of the chlorophycean group and at least twice within the trebouxiophycean clade.

The relationships among chlorophycean taxa depicted in Figs. 1A and B are consistent with affiliations previously suggested by other phylogenetic studies using nuclear 18S rRNA sequences (e.g., Rumpf et al. 1996; C. W. Birky, Jr., and P. Mackowski, personal communication). Such relationships include the following: (i) *P. parva* is more closely related to *C. reinhardtii* than to other *Chlamydomonas* taxa (from 18S rRNA data); and (ii) the two *Polytoma* clades, *P. uvella* and *P. oviforme*, appear to be more closely related to *C. applanata* and *Chlamydomonas moewusii*, respectively, than to each other. It should be noted that the nuclear and chloroplast

Table 4. Comparison of plastid *rrn16* features from nonphotosynthetic strains relative to their closest known green relative (in boldface)

	Strain[a]									
Trait	**Capp**	*Pmir*	Pobt	Puve	**Cmoe**	*Povi*	**Cpro**	*Pw263-11*	*Pw1533*	*Pzop*
AT%	48.7	53.4	53.6	54.1	50.4	51.6	51.2	53.6	54.45	63.1
Substitution per site		0.162	0.161	0.173		0.109		0.045	0.112	0.321
SD		0.012	0.012	0.013		0.010		0.006	0.010	0.020
TS/TV		1.518	1.466	1.191		1.378		1.097	1.196	1.514
Insertions/deletions										
Small insertions (1–5 bp)		4	5	5		4		2	4	9
Small deletions (1–24 bp)		6	8	7		0		0	7	10
Large insertions (86–1600 bp)										
Number		0	1	2		1		0	0	0
Size (bp)		—	228	86/1600		1197		—	—	—
AT%		—	83.3	88.3/81.7		67.2		—	—	—

[a] **Capp**, *C. applanata*; *Pmir*, *P. mirum*; ***Puve***, *P. uvella*; **Cmoe**, *C. moewusii*; **Cpro**, *C. protothecoides*; *Pw263-11*, *P. wickerhamii* 263-11; *Pw1533*, *P. wickerhamii* 1533; *Pzop*, *P. zopfii*.

trees in Figs. 1A and B are congruent. Moreover, another plastid gene, *tufA*—coding for the elongation factor Tu—suggests the same affiliations between the four *Polytoma* taxa and their green relatives (unpublished data).

The phylogenetic relationships among the treboux-iophycean taxa investigated here are, nevertheless, less clear. Consistent with previous suggestions (e.g., Huss et al. 1999), in both nuclear and plastid trees *Prototheca* species appear to be more closely related to *Chlorella protothecoides* than to other *Chlorella* species. In nuclear trees, the relative position of the *Prototheca* taxa, however, changes with the number and type of species as well as the type of analysis; the closest relative of *C. protothecoides* is either *Prototheca zopfii* [in parsimony analyses (data not shown; Huss et al. 1999), with low support] or *Prototheca wickerhamii* 263-11 (with low support) (Fig. 1A). In plastid trees, on the other hand, the closest relative of *C. protothecoides* is always *P. wickerhamii* 263-11; in the same trees, however, *P. zopfii* branches either at the base of the clade (Fig. 1B) or as a sister lineage to *P. wickerhamii* 1533 (Fig. 1C). The instability of the *Prototheca* lineages in both nuclear and plastid trees might be related to the relatively long branches featured by *P. zopfii* nuclear and chloroplast rDNA sequences, *P. wickerhamii* 1533 chloroplast *rrn16*, as well as *C. protothecoides* nuclear *rrn18* (Fig. 1), and can be attributed to the "long-branch attraction" phenomenon (Felsenstein 1988; Huss et al. 1999). Interestingly, the two *P. wickerhamii* strains that were used in this study, 263-11 and 1533, are very divergent in plastid *rrn16* sequence and they do not form a monophyletic group (Figs. 1B and C). A shared common ancestry of the three *Prototheca* species and *C. protothecoides* (to the exclusion of *C. vulgaris*) is strongly supported by the presence of a 20-bp insertion (relative to *C. vulgaris*) in all four taxa (around *E. coli* 1015), as well as 15 sites where the four taxa share the same change relative to *C. vulgaris* (either substitution or indel; data not shown).

Gene Sequence and Structure of Plastid rrn16 *in Nonphotosynthetic Green Algae*

In the analyses presented below, the nonphotosynthetic green algae are organized in three groups comprising both photosynthetic and nonphotosynthetic species. The clade designation and composition are as follows: *P. uvella (P. uvella, P. obtusum, P. mirum,* and *C. applanata), P. oviforme (P. oviforme* and *C. moewusii*), and *Prototheca (P. wickerhamii* 1533, *P. wickerhamii* 263-11, *P. zopfii* and *C. protothecoides)* (Fig. 1B). Plastid *rrn16* sequences have been aligned separately for each clade (alignments are available from the author). For consistency, the sequences in the three alignments spanned the same region of the gene, that is, the sequence that corresponds to *E. coli* 87-1324 coordinates. Table 4 summarizes features of these genes in comparison with their counterparts in the closest known green relatives (the latter are presented in boldface in Table 4).

P. uvella Clade

With the exception of the 86-bp and 1.6-kb insertions in *P. uvella* and the 1.2-kb insertion in *P. obtusum*, the three sequences can be easily aligned with a set of chloroplast homologues. In addition, the three *Polytoma* sequences can be folded into secondary structures that reveal all the conserved regions known to be important for proper function (Vernon et al. in press; unpublished data).

The plastid *rrn16* in this clade varies greatly both in base composition and nucleotide sequence. The AT content of the three *Polytoma* plastid *rrn16* sequences is higher than that of the *C. applanata* homologue with the most AT-rich sequence being that of *P. uvella.* The number of estimated nucleotide substitutions per site is also high in all three *Polytoma* taxa (i.e., up to 18.5% higher than the value obtained when the counterpart regions of

Table 5. AT%, TS/TV ratios, and transitions and tranversions per site in the *P. uvella*, *P. oviforme*, and *Prototheca* clades (relative to *C. applanata*, *C. moewusii*, and *C. prototheco*ides, respectively)[a]

	Taxon						
Measure	*Pmir*	Pobt	Puve	*Povi*	*Pw263-11*	*Pw1533*	*Pzop*
AT%	53.4	53.6	**54.1**	51.6	53.6	54.45	**63.1**
TS/TV	**1.518**	1.466	1.191	1.378	1.097	1.196	**1.514**
TS/site	**0.0981**	0.0963	0.0946	0.0635	0.0240	0.0613	**0.1953**
GA	0.0412	0.0396	**0.0421**	0.0338	0.0132	0.0333	**0.0742**
CT	0.0429	**0.0430**	0.0388	0.0237	0.0099	0.0222	**0.0700**
GA vs CT	GA<CT	GA<CT	**GA**>CT	GA>CT	GA>CT	GA>CT	**GA**>CT
TV/site	0.0646	0.0657	**0.0793**	0.0461	0.0219	0.0512	**0.1290**
GT	0.0168	0.0168	**0.0219**	0.0093	0.0058	0.0137	**0.0282**
CA	0.0185	0.0168	**0.0202**	0.0102	0.0058	0.0068	**0.0179**
GT vs CA	GT<CA	GT = CA	**GT**>CA	GT<CA	GT = CA	GT>CA	**GT**>CA

[a] The highest values in each clade are in boldface.

two very distant photosynthetic *Chlamydomonas, C. applanata* and *C. moewusii,* are compared; data not shown). At 0.173 substitution per site, the *P. uvella* plastid *rrn16* sequence is the most divergent within the clade; the level of nucleotide substitutions in another plastid translational gene, *tufA,* is also the highest in *P. uvella* (unpublished data). Interestingly, there is an inverse correlation between AT content and substitution levels, on the one hand, and transitions/transversions (TS/TV) ratios, on the other (due to an increase in the number of transversions), with *P. uvella* plastid *rrn16* showing the highest substitution level but the lowest TS/TV ratio (Table 4). Furthermore, a relative increase in G-to-A vs C-to-T transitions and G-to-T vs C-to-A transversions can be noted in this clade (Table 5).

The number and type of indels are also variable among the *Polytoma* plastid *rrn16* from this clade. Up to 13 1-bp indels (relative to the *C. applanata* counterpart) are present in *Polytoma* plastid *rrn16;* in addition, a 5-bp insertion is present in *P. uvella.* Notably, most of these insertions involve A's and T's. Of the three *Polytoma* species, only *P. obtusum* and *P. uvella* contain insertions that are larger than 5 bp (up to 1.6 kb) in their plastid *rrn16* (Table 4). Moreover, while *P. obtusum* plastid *rrn16* has only one such insertion [i.e., of 228 bp (Vernon et al. in press)], *P. uvella* contains one insertion of 87 bp and another of ca. 1.6 kb; the ca. 200 bp-central sequence of the latter insertion is incompletely sequenced due to sequencing and assembly difficulties associated with the presence of complex repeated sequences (see below). All these insertions are very high in AT- content, the most AT-rich (i.e., 88.3%) being the 87-bp insertion of *P. uvella* plastid *rrn16* (Table 4). The 228-bp insertion in *P. obtusum* contains a number of repeated sequences with a TACGA motif separated by long stretches of multiple T's and A's. Likewise, the *P. uvella* 1.6-kb insertion contains two types of short repeated sequences: one has a CG-rich core that contains a TCCGACGAT motif, and the other is made strictly of AT, i.e., AATATTATTATATATTAAA(A/T)TATA.

The 86-bp, 228-bp, and 1.6-kb insertions are located in variable regions of 16S rRNA secondary structure and do not show typical features of group I or II introns, in terms either of insertion sites or of secondary structure or conserved sequence motifs. Whether these insertions are excised or spliced out of the transcript awaits further investigation; preliminary analyses using Northern blot hybridizations and RT-PCR failed to identify a plastid 16S rRNA transcript in *P. uvella* (unpublished data). The 87-bp insertion in *P. uvella* plastid *rrn16* (*E. coli* coordinate 140; Fig. 2A) can be folded into a cloverleaf tRNA-like structure (data not shown) and is 5′-adjacent to the variable region V2; interestingly, this region represents a discontinuity site in the fragmented and scrambled mitochondrial *rrn16* of *Chlamydomonas eugametos* (Denovan-Wright and Lee 1994). Moreover, the 1.6-kb insertion in *P. uvella* (*E. coli* 746) is inserted at exactly the same site as the 228-bp insertion in *P. obtusum,* which is also the site where a 1-bp insertion, specifically a T, is found in *P. mirum* (Fig. 2A). Notably, these insertions are situated in regions that also feature structural changes in the plastid counterparts from nonphotosynthetic angiosperms (Nickrent et al. 1997a). For example, the 87-bp insertion present in *P. uvella* plastid *rns* (*E. coli* 140) is situated in a region in which a 7-bp insertion is found in the plastid *rrn16* of the holoparasite *Hydnora africana.* Likewise, the ca. 1.6-kb and 228-bp insertions in *P. uvella* and *P. obtusum,* respectively, are located at the same site (*E. coli* 746) as a 5-bp insertion (affecting both sides of the helix) that is present in the plastid *rrn16* of the holoparasite *Pilostyles thurberi.* Finally, a 6-bp insertion in *P. uvella* plastid *rrn16* is situated on a helix that is completely missing in the *P. thurberi* counterpart.

P. oviforme Clade

A high AT content is also observed in *P. oviforme* plastid *rrn16;* nevertheless, at 51.6%, the value is lower than that of its counterparts in the nonphotosynthetic members of *P. uvella* and *Prototheca* clades (Table 4).

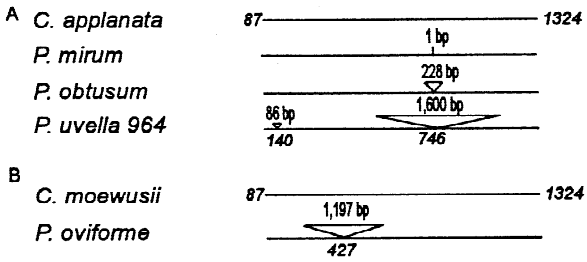

Fig. 2. Relative position of the large insertions found in the plastid 16S rRNA genes from *P. uvella, P. oviforme,* and *Prototheca* clades; *numbers below lines* are *E. coli* coordinates.

Moreover, the increase (relative to the *C. moewusii* homologue) is not as high as that observed in the nonphotosynthetic lineages within the *P. uvella* clade; this could be due in part to the fact that *C. moewusii rrn16* is also more AT-rich than the *C. applanata* counterpart. Likewise, the level of nucleotide substitutions is lower in *P. oviforme rrn16* [as well as in *tufA* (Vernon et al. in press)] than in the *P. uvella* clade.

Only three 1-bp and one 2-bp insertions, and no deletions (relative to the *C. moewusii* homologue), are present in *P. oviforme* plastid *rrn16.* However, an insertion of ca. 1.2 kb is found at *E. coli* coordinate 427 (Fig. 2B); at 62.7%, the insertion is more AT-rich than the rest of the sequence (51.6% AT) but considerably less AT-rich than the 228-bp insertion in *P. obtusum* and the 86-bp and 1.6-kp insertions in *P. uvella* (Table 4). This insertion does not show typical features of group I introns, moreover, it is situated in a variable region, V3, which is interrupted in the fragmented and scrambled mitochondrial *rrn16* of *C. reinhardtii* (Boer and Gray 1988). Furthermore, the helix that is affected by this insertion in the *P. oviforme* plastid *rrn16* represents a site of structural change in the holoparasite *P. thurberi* counterpart; however, in the latter the change involves a deletion rather than an insertion (Nickrent et al. 1997a).

Prototheca Clade

Compared with *C. prototheco*ides, as their closest known photosynthetic relative, the three *Prototheca* species investigated here have *rrn16* sequences that show highly increased values of AT% (with as much as 11.9%). However, in contrast to the situation observed in the *P. uvella* clade, there is a proportional correlation among AT content, substitution levels, and TS/TV ratios, the highest values being noted in the *P. zopfii* lineage (Table 4). Among the three *Prototheca* taxa, *P. wickerhamii* 263-11 plastid *rrn16* appears to be the least diverged: there are only two 1-bp insertions and the value of substitutions per site is very low (i.e., 0.045). At the other extreme is *P. zopfii rrn16,* with 19 indels and a value of substitutions per site of 0.321. Furthermore, an increase in the number of transitions relative to transversions and a relative increase in the number of G-to-T vs C-to-A transversions can be noted in this clade (Table 5). Interestingly, all the insertions involved A and T. Also, no insertions or deletions larger than 24 bp are present in the plastid *rrn16* of any of the three *Prototheca* lineages, which is in marked contrast to the situation observed among *Polytoma* counterparts.

Discussion

General and Specific Trends in the Evolution of Plastid rrn16 *rRNA Genes in Nonphotosynthetic Green Algae*

Plastid *rrn16* from the three clades of nonphotosynthetic green algae investigated here all show an increased AT content relative to their closest known photosynthetic relative. An "A/T drift" was reported for the holoparasite plastid *rrn16* (Nickrent et al. 1997a) and was suggested to be related to adaptation to highly specialized trophic modes. Interestingly, although the nuclear 18S rDNA sequences of nonasterid holoparsites are more AT-rich than their counterparts in green dicots (Nickrent and Starr 1994; Duff and Nickrent 1997), in *Polytoma* and *Prototheca,* nuclear 18S rDNA sequences do not show an increase in percentage AT content (data not shown).

Furthermore, a proportional correlation between the AT content and the number of transversions has been noted in the AT-rich plastid *rrn16* of holoparasite angiosperms (Nickrent et al. 1997a); this correlation was also observed in noncoding regions of chloroplast genomes in photosynthetic plants and is thought to be due to the fact that the number of transversions increases when the 5′- and 3′-flanking nucleotides are A's or T's (e.g., Morton 1995). It is noteworthy that the plastid *rrn16* sequences in the *P. uvella* clade show the same trend: the higher the AT content and nucleotide substitution level, the higher the number of transversions (and the lower the TS/TV ratio). However, this correlation is not observed in the *Prototheca* clade (Tables 4 and 5), where, with the increase in AT content and number of substitution per site, the TS/TV ratios increase (due to a higher increase in transitions relative to transversions). Interestingly, in both lineages, the increase in AT content is paralleled by a relative increase in G-to-A vs G-to-T transitions and G-to-T vs C-to-A transversions, regardless of the TS/TV trend in the clade (Table 5).

Most of the nonphotosynthetic green algae analyzed here (the exception is *P. oviforme*) exhibit high substitution rates in their plastid *rrn16* (as suggested by the relative differences in branch lengths in Fig. 1B). The same trend has been reported previously for plastid *rrn16* in holoparasitic angiosperms (Nickrent et al. 1997a). The fact that the *P. oviforme* plastid *rrn16* sequence shows less rate acceleration than the homologue in its photosynthetic relative is also not without precedent: *rps2* sequences of some holoparasites have also been reported to

show no evidence of acceleration, while those in some hemiparasite (photosynthetic) relatives experienced accelerated rates of evolution (dePamphilis et al. 1997).

Accumulation of indels is a general phenomenon among plastid 16S rRNA genes in both *Polytoma* and *Prototheca* groups, and 1-bp indels are acquired first. However, the type of indel that is the most prominent in the evolution of plastid *rrn16* differs between the two groups. No deletions larger than 1 bp, but many insertions larger than 1 bp, are found in *Polytoma* plastid *rrn16.* In contrast, plastid *rrn16* sequences in *Prototheca* feature deletions as large as 24 bp but did not acquire any insertions that exceed 5 bp. Interestingly, insertions larger than 100 bp are also missing in the plastid *rrn16* of holoparasitic angiosperms, although deletions as extensive as entire helices are common.

Most of the structural variations in *Prototheca* plastid *rrn16* (i.e., insertions and deletions no larger than 24 bp) are located on apical helices/loops, specifically in those that also show variation in length and sequence in holoparasitic counterparts (e.g., helices 43 and 46). In contrast, the location of most of the structural changes is rather different between *Polytoma* and holoparasite plastid 16S rRNA sequences. While most of the indels in holoparasite plastid *rrn16* are located on apical helices or loops (Nickrent et al. 1997a), in *Polytoma* plastid *rrn16,* only one of the four insertions exceeding 5 bp, namely, the ca. 1.2-kb insertion in *P. oviforme,* is located on the loop of an apical helix. The other three insertions larger than 5 bp in *Polytoma* plastid *rrn16* are located on internal helices.

Overall, in both *Polytoma* and *Prototheca* plastid *rrn16* as well as in their holoparasitic counterparts, there is a proportional correlation among the number of substitutions, structural modifications, and AT content. However, the mutational processes associated with the evolution of these genes appear to be different in some respects. First, although increased substitution levels were achieved by both *Polytoma* and *Prototheca* as well as the holoparasite plastid *rrn16,* the type of substitution that prevails differs: with the increase in AT content, more transversions are noted in *Polytoma* and holoparasite angiosperms, while more transitions seem to be taking place in *Prototheca*. Second, although structural variations do accumulate in *Polytoma* and *Prototheca* (as well as holoparasitic angiosperms), the former is characterized by the presence of several insertions larger than 85 bp, while in the latter the indels do not exceed 24 bp.

Potential Evolutionary Forces and Mechanisms Involved in the Evolution of Plastid 16S rRNA Genes in Nonphotosynthetic Green Algae

Little is known about the factors that are responsible for the adoption of a nonphotosynthetic lifestyle in green plants. In nonasterid holoparasite angiosperms, it has been suggested that the loss of photosynthesis has followed the adoption of a parasitic lifestyle, and substantial evolutionary changes to the plastid genome have started before photosynthesis was irreversibly lost (i.e., during the hemiparasitic evolutionary history of these lineages) (dePamphilis et al. 1997). In this connection, it should be mentioned that many photosynthetic green algae, including *Chlamydomonas* and *Chlorella* are mixotrophic, i.e., they can grow both autotrophically and heterotrophically. Thus, the questions are, Is the loss of photosynthesis in green algae a consequence of long-term heterotrophic lifestyles, analogous to the situation noted among parasite angiosperms? and Alternatively, is the obligate heterotrophic lifestyle the consequence of unique "fatal" mutations that irreversibly affected the ability to photosynthesize in facultatively heterotrophic lineages? Based on the observations made in holoparasitic angiosperms, a long-term heterotrophic lifestyle that preceded the irreversible loss of photosynthesis should be reflected in increased substitution rates and AT content in the homologous plastid genes from the closest, but still photosynthetic, relative (i.e., the hemiparasites, in the case of parasitic angiosperms); in addition, nucleotide substitution levels might be increased in nuclear genes (Nickrent and Starr 1994).

Interestingly, in *P. uvella* clade, the branch lengths in Fig. 1B do not indicate an increased nucleotide substitution rate in the plastid *rrn16* sequence of the closest known photosynthetic relative, *C. applanata.* Similarly, plastid *tufA* is not accelerated in this lineage (unpublished data). Moreover, the nuclear rRNA genes are also not accelerated in this taxon (Fig. 1A). Therefore, it is likely that the last photosynthetic ancestor of this clade did not experience a long-term heterotrophic lifestyle during its evolutionary history and that most of the observed changes in plastid *rrn16* sequences in the *P. uvella* clade occurred subsequent to the irreversible loss of photosynthesis.

In contrast, in the *Prototheca* clade, the plastid *rrn16* sequence in the closest known photosynthetic relative, *C. prototheco*ides, is slightly accelerated relative to that in other *Chlorella* green relatives (see branch lengths in Fig. 1C), and its AT content is higher than that of the *C. vulgaris* homologue (51.2 vs 47.6% AT). Furthermore, as suggested by the branch lengths in Fig. 1A (and Huss et al. 1999), the nuclear 18S rDNA sequence in the *Prototheca* clade (including *C. protothecoides*), especially in *P. zopfii,* exhibits high rates of nucleotide substitution. Overall, these data are consistent with the possibility that the photosynthetic ancestor of the *Prototheca* clade has spent part of its recent evolutionary history under a heterotrophic regime, and thus the loss of photosynthetic abilities might have been a consequence of, rather than a cause for, the obligate heterotrophic lifestyle for these lineages, similar to the situation suggested for holoparasite angiosperms (dePamphilis et al. 1997). Arguing for

such a scenario is the fact that *C. protothecoides* is an auxotrophic and mesotrophic species (Huss et al. 1999), and *Prototheca* species are pathogens in a number of vertebrates (e.g., Janosi et al. 1999; Kremery 2000).

The high substitution rates noted in the plastid *rrn16* of numerous holoparasites are proposed to be a consequence of increased levels of mutation in DNA replication and/or repair systems, which have not been eliminated due to the general "relaxed selectional environment of a nonphotosynthetic plastid" (Nickrent et al. 1997a). Furthermore, the extensive variation in evolutionary rates and patterns among leucoplast genes in holoparasitic angiosperms lineages is thought to be due to variations in mutation or repair rates (e.g., dePamphilis et al. 1997; Nickrent et al. 1998; Wolfe and dePamphilis 1998).

It is interesting that in the *P. uvella* clade, point mutations (both substitutions and 1-bp indels) and length mutations seem to be accumulating at different rates; this could reflect defective replication and/or repair mechanisms. Arguing for such defective mechanisms is the fact that the large insertions in this clade might have evolved through the expansion of insertions as small as 1 bp. The 228-bp insertion in *P. obtusum* and the ca. 1.6-kb insertion in *P. uvella* are situated at the same site where a single base insertion, namely, a T, is found in the less diverged *P. mirum rrn16* (Fig. 2A). Moreover, the short repeated sequences in both *P. uvella* and *P. obtusum* insertions contain a very similar sequence motif (TcC**GACGA** and TaC**GACGA,** respectively), and the central sequence, **GACG,** in these motifs also flanks the insertion site in all three *Polytoma* lineages, but not in *C. applanata* or *P. oviforme.* A very similar situation has been reported for an insertion in the plastid *rpoC2* of grasses: (i) the insertion contains a variable number of repeated sequences differing at few positions and separated by polypurine or polypyrimidine tracts, and (ii) the repeated unit is also present in the region flanking the insertion (Cummings et al. 1994). All these features are considered indicative of replication slippage (Levinson and Gutman 1987) and all characterize the plastid *rrn16* insertions in the *P. uvella* clade. In contrast, in the *Prototheca* clade, nucleotide substitutions and indels no larger than 24 bp seem to have been accumulating in parallel and at similar rates: with the increase in nucleotide substitution levels, the number and size of indels also increased. This pattern suggests that the evolution of plastid *rrn16* sequences in this clade was affected mainly by relaxed selection, with fewer additional mutational pressures.

Conclusion

Similar to the situation described previously for plastid 16S rRNA genes in nonphotosynthetic land plants, nucleotide substitution levels, structural variations, and percentage AT values are increased in nonphotosynthetic green algae compared to their closest photosynthetic relatives. However, the mutational processes appear to be different in many respects. First, with the increase in AT content, more transversions are noted in *Polytoma* and holoparasite angiosperms, while more transitions characterize the evolution of the 16S rDNA sequences in *Prototheca.* Second, although structural variations do accumulate in both *Polytoma* and *Prototheca* (as well as holoparasitic plastid 16S rRNAs), insertions as large as 1.6 kb seem to be more prevalent in the former, whereas significantly smaller indels (not exceeding 24 bp) characterize the plastid 16S rRNAs in the latter group. The differences in evolutionary rates and patterns within and between lineages might be due to mutations in replication/repair-related genes; slipped-strand mispairing is likely the mechanism responsible for the expansion of insertions in *Polytoma* plastid 16S rRNA.

Acknowledgments. I thank Dawne Vernon and C. William Birky, Jr., for providing total DNA from *Polytoma oviforme* and for allowing the use of their *Polytoma obtusum* plastid *rrn16* sequence prior to publication. I also thank the two anonymous reviewers for their greatly appreciated comments. The research was supported by startup funds to C. William Birky, Jr., and a Postdoctoral Fellowship from the Natural Sciences and Engineering Research Council (NSERC) of Canada to A.M.N.

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