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Comparative Analysis of Chloroplast Genomes: Functional Annotation, Genome-Based Phylogeny, and Deduced Evolutionary Patterns

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All protein sequences from 19 complete chloroplast genomes (cpDNA) have been studied using a new computational method able to analyze functional correlations among series of protein sequences contained in complete proteomes. First, all open reading frames (ORFs) from the cpDNAs, comprising a total of 2266 protein sequences, were compared against the 3168 proteins from *Synechocystis* PCC6803 complete genome to find functionally related orthologous proteins. Additionally, all cpDNA genomes were pairwise compared to find orthologous groups not present in cyanobacteria. Annotations in the cluster of orthologous proteins database and Cyanobase were used as reference for the functional assignments. Following this protocol, new functional assignments were made for ORFs of unknown function and for *ycfs* (hypothetical chloroplast frames), which still lack a functional assignment. Using this information, a matrix of functional relationships was derived from profiles of the presence and/or absence of orthologous proteins; the matrix included 1837 proteins in 277 orthologous clusters. A factor analysis study of this matrix, followed by cluster analysis, allowed us to obtain accurate phylogenetic reconstructions and the detection of genes probably involved in speciation as phylogenetic correlates. Finally, by grouping common evolutionary patterns, we show that it is possible to determine functionally linked protein networks. This has allowed us to suggest putative associations for some unknown ORFs.

The so-called postgenomic era is linked to the knowledge of complete genomes for many organisms. In this context, the design and the testing of new mathematical and computational tools able to assign function to gene products and compare complete genomes are becoming crucial. The use of computational tools to infer, analyze, and compare both structure and function of the complete predicted proteome is being considered an essential new instrument for the progress of biological research (for reviews, see Andrade and Sander 1997; Bork et al. 1998; Eisenberg et al. 2000; Pellegrini 2001). Still, the field of bioinformatics is in its infancy. For example, the fraction of hypothetical proteins or open reading frames (ORFs) in complete genomes remains remarkably high. Thus, the most recently sequenced first complete plant genome, from *Arabidopsis thaliana* (*Arabidopsis* genome 2000), presents 25,498 identified genes, out of which ~30% correspond to hypothetical proteins or proteins of unknown function. Similarly, and in spite of the impressive accumulation of genome information over the last several years, the availability of tools for comparative genome analysis to establish the implications of the differences in gene content between species from a biomolecular perspective is virtually absent.

The chloroplast is an essential organelle in plants. It performs photosynthesis and therefore is required for the photoautotrophic plant growth that moves our biosphere. The generally accepted endosymbiotic hypothesis states that

chloroplasts have arisen from an internalized cyanobacterial ancestor (Cavalier-Smith 2000). Chloroplasts have maintained an independent genome that encodes an important part of the proteins required for their photosynthetic activity and different housekeeping functions. The chloroplast genome (cpDNA) consists of homogeneous circular double-stranded DNA molecules of 110–200 kb size, containing between 30 to 50 different RNA genes and a number of protein-coding genes, which ranges from about 100 in land plants and green algae to 150–200 in nongreen algae (Sugiura 1995). These protein-coding genes can be roughly classified into two main groups: genes enrolled in the expression and translation machinery of the chloroplast and genes related to bioenergetics and photosynthetic function. The largest known chloroplast genome corresponds to the red alga *Porphyra* and has 70–80 additional genes, one-third of which are related to biosynthesis of amino acids and other essential biomolecules. A feature of chloroplast genomes from most plants is the presence of two large inverted repeats (IRs) of 6–76 kb that divide the cpDNA in one large and one small single-copy region (called LSC and SSC, respectively; Sugiura 1995).

The nonrecombinant, uniparentally inherited nature of organelle genomes makes them potentially useful tools for evolutionary studies. However, in practice, detecting useful polymorphism at the population level is often difficult due to the low level of substitutions (slow substitution rates) in plant chloroplast genomes. Attempts to reconstruct plastid evolution with traditional biomolecular approaches (i.e., sequence-based analyses of RNAs or of protein-encoding genes) have proven particularly difficult (Martin et al. 1998; Sugiura et al.

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1998; Adachi et al. 2000). Thus, in a recent study, Martin and coworkers attempted to build phylogenetic trees and to obtain evolutionary information by comparing 45 common chloroplast proteins, pasted together in a unique macroprotein with 9957 (Adachi et al. 2000) and 11,039 (Martin et al. 1998) amino acid sites. The investigators encountered some difficulties to statistically discriminate among the several possible phylogenetic trees obtained. Therefore, alternative and independent types of evidence that might provide new information about ancient plastid history are required. Some new alternatives for phylogenetic assignments involve examination of the arrangement or order of genes in genomes by gene-cluster analysis history (Stoebe and Kowallik 1999). However, such analysis are usually carried out with some particular sets of related proteins or group of genes and, therefore, they tend to reflect the partial phylogeny of these genes rather than that of the whole organisms or genomes.

The knowledge of complete genomes opens up the possibility of developing innovative tools for phylogenetic reconstruction and evolutionary analyses. A promising approach is presented here, based on the quantitative analysis of clusters of orthologous proteins (COGs) and applied to chloroplast history. Chloroplast genomes are, as a result of the considerable number available and small size, excellent model systems in computational genomics studies. A number of important questions from the bioinformatics perspective can be addressed by experimenting with them. For example, how accurate can phylogenetic reconstructions be made by using the complete genome information? Or is it possible to uncover evolutionary forces by comparing complete genomes? Finally, can phylogenetic profiles, derived from the absence/presence of a given gene in the set of genomes under study, be used to determine functional associations? In this study we try to address these questions. First, we apply a new program developed in our laboratory for genome annotation, and compare its performance with the well-known approach of using PSI-BLAST. Then, a comparative genomics study is carried out using techniques borrowed from multivariate analysis.

RESULTS AND DISCUSSION

Functional Annotations of cpDNA Proteins

Quality of Annotations

All protein sequences from 19 complete chloroplast genomes (cpDNA) were compared against the 3168 proteins from *Synechocystis* PCC6803 complete genome. The number of proteins in a chloroplast proteome ranges from 66 in the smallest genome (*Euglena gracilis*) to 209 proteins in the largest one (*Porphyra purpurea*). Taking all genomes, the total number of chloroplast proteins compared was 2266. As described in Methods, we compared genomes pairwise, taking all proteins in one genome and comparing them with all proteins in the other, trying to find the most likely ortholog pair. The degree of functional matching was evaluated with the μ -score, a measure of the structurally implied similarity between two sequences (see Methods). Figure 1 presents the values of μ -scores obtained by pairwise comparison of all the chloroplast proteins against the following: (1) the set of *Synechocystis* proteins annotated in the COGs database (<http://www.ncbi.nlm.nih.gov/COG/>), which included 2113 proteins; (2) the complete proteome of *Synechocystis*, which included 3168 proteins and was taken from the CyanoBase database (<http://www.kazusa.or.jp/cyano/>); and (3) the set of *Saccharomyces* proteins constituted by its database of COGs, which included 2175 proteins.

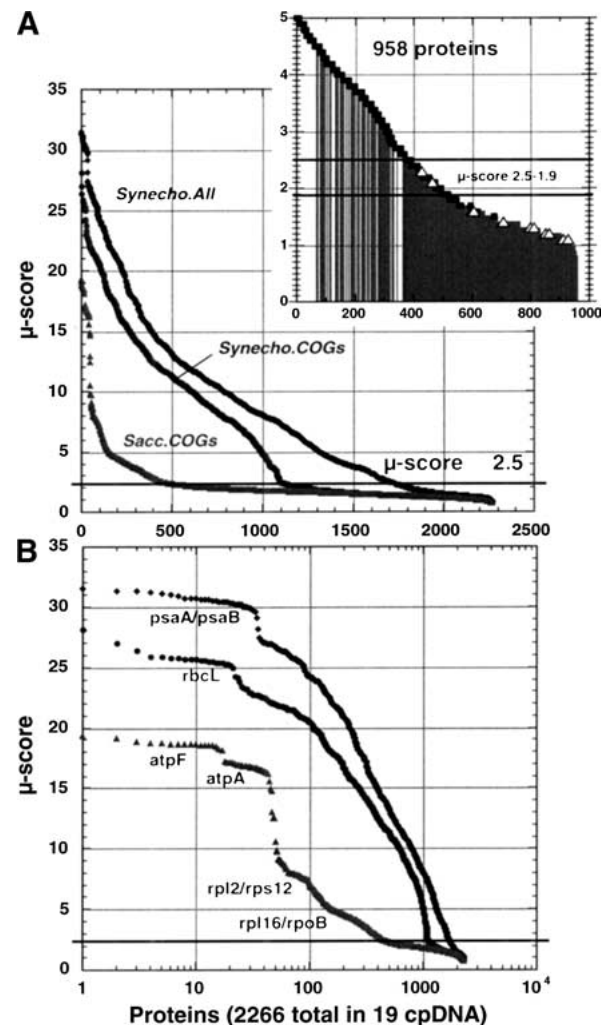


Figure 1 Plot of the μ -scores obtained by pairwise comparison of all proteins of each cpDNA (a total of 2266 proteins) against the complete predicted proteome of *Synechocystis* (3168 proteins), against the *Synechocystis* clusters of orthologous proteins (COGs; 2113 proteins) and against *Saccharomyces cerevisiae* (2175 proteins). The figures are plotted in linear (top graph) and in log scale (bottom graph). Inset indicates the correct pairs of proteins (squares) and the incorrect pairs (open triangles) for the data corresponding to comparison with the complete *Synechocystis* proteome below μ -score 5; gray lines indicate cases in which both proteins are hypothetical.

kazusa.or.jp/cyano/); and (3) the set of *Saccharomyces* proteins constituted by its database of COGs, which included 2175 proteins.

In Figure 1A the values of μ -scores obtained are plotted versus the number of proteins, and in Figure 1B the same data are represented using a log scale for the number of proteins. From these plots it is possible to estimate the optimal cutoff value μ_c (see Methods). Previously (Fabrega et al. 2001) it was found that a cutoff of about 2.5 was reliable to separate pairs with the same function from those with different functions, and such cutoff value is marked with a line in Figure 1. In the curves (Fig. 1A), especially in the comparison against *Synechocystis* COGs, a clear inflexion point at around μ -score 2.5 can be observed. The inflexion point is more evident when genomes are matched against the COGs database because, in

our interpretation, this database includes protein families with function assignments across phylogenetically distant species, and consequently it is difficult to provide an accurate functional class assignment for largely diverged sequences. On the other hand, since CyanoBase is specialized in cyanobacteria, it seems that it has been possible to detect during the database compilation highly diverged sequences, that is, functional assignments for diverged sequences are more reliable. The observed 2.5 inflexion point marks our threshold for functional assignment. Above μ -score of 2.5 all assignments were correct when compared against *Synechocystis* COGs. It is also interesting to see that the comparison against proteins of an eukaryote such as *Saccharomyces* finds, as expected, a much smaller number of significant matches, but it also presents the indicated inflexion point around μ -score 2.5. The log plot in Figure 1B highlights proteins with a high degree of functional conservation. For example, it can be observed that a large proportion of the highest μ -scores (>30) were obtained for two polypeptides corresponding to photosystem I (i.e., *psaA* and *psaB*). An analogous step is observed in the comparison with *Synechocystis* COGs, in which the highest points (of μ -scores >25) correspond mainly to ribulose biphosphate carboxylase (i.e., rubisco) large subunit (*rbcL*). In the comparison with *Saccharomyces* COGs, the highest values (μ -score between 15 and 20) correspond mainly to the contribution of two ATP synthase subunits (*atpF* and *atpA*). These data indicate that in chloroplast genomes there is a very strong conservation of those protein complexes essential for bioenergetics. This is not surprising, considering that chloroplasts are the organelles responsible for the energy supply in plant cells. But our data seem also to indicate that these conserved subunits of the multimeric protein complexes PSI, rubisco, and ATPase may constitute an ancient core for these protein complexes because they correspond to the most conserved genes. The comparison with *Saccharomyces* COGs also shows two other smaller steps around μ -scores 8 and 4, which mainly correspond to ribosomal proteins (*rpl2*, *rps12*, *rpl16*) and to RNA polymerase subunit (*rpoB*). These marks indicate that together with photosynthetic-energy production the other essential function in chloroplasts is the translation machinery. A previous study of ribosomal and *atp* gene clusters in chloroplasts has also shown a strong conservation in genes *atpA*, *atpF*, *rpl2*, and *rpoB* (Stoebe and Kowallik 1999).

The Figure 1A inset presents in more detail the region in the μ -score between 1 and 5 for the curve corresponding to the comparison of all the cpDNA proteins versus the complete *Synechocystis* proteome. The inset shows in solid squares the correct matches, in open triangles the detected mismatches, and with gray lines unresolved matches (i.e., those in the pairwise comparison in which at least one protein is hypothetical or has unknown function). It can be seen that up to 2.5, all the known matches are correct. The number of possible false-positives for μ -scores ≥ 2.5 was checked by looking at the identity of function of well-known and well-annotated proteins pairs. Among the first 100 known *Synechocystis* proteins, all assignments were correct. Therefore, the cutoff of 2.5 seems a good, conservative threshold for functional assignment in this case. Below 2.5, most pairs do not have assigned function (gray lines). There is an intermediate zone between 2.5 and 1.9 in which the method still maintains a significant proportion of correct matches. This region includes 131 matches, out of which 50 are correct, 2 are erroneous, 15 correspond to new assignments, and 64 correspond to hypothetical proteins in both sides. The new assignments identi-

fied in this region are included in Table 3 (see below). Below 1.9 a significant amount of mismatches start to appear.

Fraction of Annotations in Complete Genomes

The number of proteins with μ -scores above the cutoff ($\mu_c = 2.5$) was 1097 when *Synechocystis* COGs database was used and 1696 when *Synechocystis* CyanoBase was used. A good linear correlation was found between proteome size and number of assignments to COGs. Table 1 includes this information and some other derived data. In the first three columns we show the size of each cpDNA, the size considering only ORFs (i.e., counting only the nucleotides corresponding to ORFs), and the percentage of nonencoding DNA (No cod DNA, calculated by subtracting columns 1 and 2 and then dividing by total DNA). The next four columns show the total number of proteins in each cpDNA (Prot Total) and the assignments based on *Synechocystis* COGs (Syn COGs), on all *Synechocystis* proteome (Syn CyanoB), and on the comparison with all other cpDNAs (in other cpDNA). This last pairwise comparison between cpDNAs was done using only proteins not assigned to any *Synechocystis* orthologs. The final number of cpDNA proteins assigned was 1837 out of 2266, which is 81% of the ORFs in the cpDNAs. Of these proteins, 1696 correspond to *Synechocystis* orthologies and 141 to orthologous groups specific to the plant chloroplasts. The remaining 429 proteins were left unassigned.

There is a linear correlation between the predicted proteome size and number of assignments, which, however, presents exceptions in three genomes: *Chlorella vulgaris* (ChlVul), *Nephroselmis olivacea* (NepOli), and *Pinus thunbergii* (PinThu). These contain the major fraction of unassigned proteins: 102 in *C. vulgaris*, 57 in *N. olivacea*, and 89 in *P. thunbergii* (that represent 58% of the total, 247/429). The average number of proteins left unassigned in the other 16 cpDNA is ~ 11 proteins per genome, which gives a percentage of assigned proteins of 89.8% in these 16 cpDNAs. By contrast, the average number of assignment proteins in the mentioned 3 species is only 49.5%. The most up-to-date annotation of *Chlorella* and *Pinus* shows many tRNA genes (*trn*) in the encoding region (see <http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/framik?gi=13217&db=Genome>), including 69 *trn* in *Chlorella*-encoding region and 46 *trn* in *Pinus*. If these *trn* genes are removed from the proteomes, the number of unassigned ORFs is 33 and 43 for *Chlorella* and *Pinus*, respectively (Table 1). After this adjustment, the size of their proteomes is now 106 and 114 proteins, respectively. These numbers are in better agreement with the trends in proteome size observed for chloroplast genomes in green algae and land plants (see Table 1). Still, we have reasons to suggest that many of the other unassigned ORFs (perhaps ~ 20 per genome) in these three genomes also correspond to RNA genes or perhaps pseudogenes: (1) No sequence homology was detected internally among the members of the group of 247 ORFs; (2) most of these ORFs are too short (for example, *Chlorella* has 60 ORFs of 40–59 codons, which would not give polypeptides longer than 20 amino acids); and (3) the inverted repeat (IR) is not present, or it is very reduced, in *Chlorella* and *Pinus*. The IR is a portion of cpDNA well conserved in angiospermae (Goulding et al. 1996), which mainly includes RNA genes. Complete clarification of this issue is out of the scope of this paper.

The proportion of nonencoding DNA correlates in some extent with the presence of introns and the maturase gene (*matK*) in the cpDNAs (see Table 1). On the basis of these genomic characteristics, green algae seem to define an evolu-

Table 1. cpDNA Size, Percentage of Assignments, and Presence of Introns, *matK*, and IR

Species	Total DNA cpDNA bp	No. nucleotides in ORFs	No. cod DNA (%)	Prot Total	In Syn COGs	In Syn CyanoB	In other cpDNA	No. assigned		No. introns	Presence of <i>matK</i>	Presence of IR
								ORFs	(%)			
Nongreen algae												
CyaPar	135,599	97,656	28.0	149	83	135	5	9	6.0	1	No	Yes
GulThe	121,524	97,233	20.0	147	86	132	8	7	4.8		No	Yes
OdoSin	119,704	91,098	23.9	140	75	118	3	19	13.6	0	No	Yes
CyaCal	164,921	142,182	13.8	197	115	172	6	19	9.6		No	
PorPur	191,028	153,396	19.7	209	120	194	9	6	2.9	0	No	
Euglenophyta												
EugGra	143,172	50,964	64.4	66	32	52	4	10	15.1	155	Yes	
Green algae												
ChiVul	150,613	91,074	39.5	(174)	45	71	2	(101)	(58.0)	3	No	Not present
MesVir	118,360	77,412	34.6	105	60	95	2	8	7.6	0	No	Yes
NepOli	200,799	127,470	36.5	155	64	96	2	57	36.8	0	No	Yes
Land plants												
PinThu	119,707	76,605	36.0	(160)	37	63	8	(89)	(55.6)		Yes	Very small
MarPol	121,024	72,528	40.1	89	50	78	6	5	5.6	≈20	Yes	Yes
SpiOle	150,725	75,108	50.2	98	49	76	4	18	18.4	≈20	Yes	Yes
AraTha	154,478	85,044	44.9	88	50	78	9	1	1.1	≈20	Yes	Yes
OenEla	159,443	88,197	44.7	119	50	77	12	30	25.2		Yes	Yes
NicTab	155,939	80,343	48.5	101	50	74	16	11	10.9	≈20	Yes	Yes
OrySat	134,525	67,434	49.9	108	53	78	23	7	6.5	≈20	Yes	Yes
ZeaMay	140,387	68,772	51.0	111	50	75	17	19	17.1	≈20	Yes	Yes
Other												
EpiVir	70,028	30,348	56.7	24	17	20	4	0	0.0		Yes	Yes
ToxGon	34,996	18,747	46.4	26	11	12	1	13	50.0			
TOTAL				2266	1097	1696	141	429				

Data about the genomes that include the size of each cpDNA, the percentage of encoding DNA (counted as the size of ORFs), the number of proteins assigned for each species, and the presence of introns, maturase (*matK*) and inverted repeats (IR) in the cpDNAs.

tionary area marking a transition between unicellular non-green algae (closer to cyanobacteria) and higher plant chloroplast genomes. The percentage of nonencoding DNA steadily increases along with the increase in biological complexity (from unicellular aquatic algae to pluricellular terrestrial plants): It is 15%–30% in nongreen algae and rises to 35%–40% in green algae and 40%–50% in land plants. The presence of introns and maturase occurs in land plants and also marks a distinction from algae. An exception to this trend is the green flagellate *E. gracilis*, which has a high proportion of nonencoding DNA (68%) and a large number of introns (155; Hallick et al. 1993). This difference is reckoned by its phylogenetic classification because euglenoids are not plants but protist flagellates, that include both photoautotrophic species like *Euglena* and also many colorless heterotrophic species that do not have chloroplasts.

Discussion of New Annotations

We have been able to provide some new functional assignments for some *ycfs* (hypothetical chloroplast frames), which correspond to ORFs well conserved in cpDNAs but without a clear functional annotation (Rochaix 1999). Previous studies on cpDNA have indicated putative functions for several of them (Douglas and Penny 1999). Our study confirms all functions attributed to *ycfs* as reported by Douglas and Penny (data not shown) and additionally provides assignments to some cpDNA ORFs of still unknown function. Table 2 presents a list of *Synechocystis* proteins that matched with *ycfs* at a significant μ -score. The identity number (prot.ID) of each protein is included. Table 2 also includes proteins annotated as ORFs or unknown but orthologous to a *ycf*. In some cases (see Table 2, rows 1, 3, 6, and 7) the unknown protein comes from the *Synechocystis* genome. In one case, the μ -score is below the threshold (μ -score = 2.2), but the function attributed to *ycf9* has been recently confirmed by experimental data indicating that encodes the photosystem II core subunit protein PsbZ (Swiatek et al. 2001). Other investigators have detected a down-regulation of photosynthetic activity when this gene was inactivated, consistent with an alteration of a photosystem II subunit (Baena-Gonzalez et al. 2001). We detect the presence of the *ycf9* gene in *Synechocystis* and in all

other cpDNA studied, excluding the nonphotosynthetic parasites *Epifaigus virginiana* and *Toxoplasma gondii*.

Table 3 presents a list of chloroplast proteins, annotated as ORF or unknown, which are assigned to proteins of known function in the present study. Using the cutoff of 2.5, 11 ORFs were newly assigned: 7 to known proteins in *Synechocystis* and 4 to known proteins in other cpDNA. Table 3 also shows the μ -scores corresponding to each assignment. The identity number (prot.ID) and the number of residues of each protein (aa) are also included. PSI-BLAST (Altschul et al. 1997) searches were also conducted with all the protein sequences included in Table 3. PSI-BLAST detected similar orthologs for queries with μ -score above 2.5. One interesting result in Table 3 is the identification in *Euglena* of a gene corresponding to maturase (*matK*). As shown in Table 1, maturase separates land plants from the rest. *Euglena* cpDNA includes many introns (Hallick et al. 1993) and could not possibly survive without enzymes essential for RNA maturation.

Chloroplast genomes in most higher plants contain two giant ORFs designated *ycf1* and *ycf2*. The functional assignment of these genes has been the subject of some discussion. In tobacco, for example, *ycf1* potentially specifies a protein of 1901 amino acids, whereas the putative gene product of the *ycf2* reading frame is a protein of 2280 amino acids. It has been suggested that these long genes do not correspond to functional proteins; instead some investigators have suggested that they correspond to pseudogenes that have lost function. However, experiments with mutant alleles for targeted disruption and/or deletion of these two ORFs in tobacco plants (Drescher et al. 2000) have indicated that *ycf1* and *ycf2* encode products essential for cell survival. We assign *ycf2* to a cell division protein (*fstH*) and *ycf1* to an ABC-transporter, supporting the idea that these genes are indispensable for the plant. However, we do not include them in Table 2, as these functional assignments can also be detected by PSI-BLAST.

Factor Analysis of the Matrix of Orthologous Genes

Construction of the X-Matrix

During the construction of the X-matrix, it was observed that only five genes were present in all 20 genomes. These genes

Table 2. Functional Assignments for *ycfs* Hypothetical Chloroplast Frames

	μ -score	Prot. ID	<i>Synechocystis</i> Proteins		Chloroplasts Proteins		Organism	<i>ycf</i>	Gene assignment
			Prot. function	Gene	Prot. ID	Prot. function			
1	3.1	sml0004	hypothetical prot <i>ycf6</i> (29aa)	<i>petN</i>	6723740	cytb6/f subunit VIII (3.2kD)	<i>Oenothera elata</i>	<i>ycf6</i>	<i>petN</i>
2	2.2	sll1281	PSII subunit PsbZ (<i>ycf9</i>)	<i>psbZ</i>	1185234	ORF61 (<i>ycf9</i>)	<i>Odontella sinensis</i>	<i>ycf9</i>	<i>psbZ</i>
3	2.4	sll0047	hypothetical prot (103aa)	?	1016157	<i>ycf12</i>	<i>Cyanophora paradoxa</i>	<i>ycf12</i>	?
4	2.7	ssr1789	light inducible prot (CAB/ELIP/HILP family)	<i>hliA</i>	6466403	unknown	<i>Cyanidium caldarium</i>	<i>ycf17</i>	<i>hliA</i>
5	2.7	ssl0453	phycobilisome degradation prot NblA	<i>nblA</i>	2465760	unknown	<i>Cyanidium caldarium</i>	<i>ycf18</i>	<i>nblA</i>
6	2.5	sml0007	<i>ycf32</i> (39aa)	<i>psbY</i>	1185183	PSII subunit Y (ORF36)	<i>Odontella sinensis</i>	<i>ycf32</i>	—
7	2.4	sml0007	<i>ycf32</i> (39aa)	—	2465767	unknown	<i>Cyanidium caldarium</i>	<i>ycf32</i>	<i>psbY</i>

List of chloroplast *ycfs* that were assigned to specific proteins with known function.

Table 3. Proteins Annotated as ORF or unknown which Are Assigned to Proteins of Known Function

	μ -score	Synechocystis proteins				Chloroplasts proteins—ORF or unknown				PSI-BLAST	
		Prot. ID	Prot. function	Gene	aa	Prot. ID	Prot. function	Organism	aa	Hits	Prot. detected
1	17.10	6466376 ^a	rubisco expression prot	<i>cfxX/Q</i>	301	1276694	ORF301	<i>Porphyra purpurea</i>	301	>1000	CFXQ_CYAME
2	12.00	sl11536	molybdopterin biosynthesis prot MoeB	<i>moeB</i>	392	1276801	ORF382	<i>Porphyra purpurea</i>	382	585	moeB_MYCTU
3	9.60	sl11536	molybdopterin biosynthesis prot MoeB	<i>moeB</i>	392	6466397	chIN (incorrect)	<i>Cyanidium caldarium</i>	395	574	moeB_MYCTU
4	7.60	sl10621	thiol:disulfide interchange prot DsbD	<i>dsbD</i>	248	1276830	ORF240	<i>Porphyra purpurea</i>	240	58	cycZ_ECOLI
5	5.40	sl11035	uracil phosphoribosyl transferase BioY prot	<i>upp</i>	216	1276820	ORF198	<i>Porphyra purpurea</i>	198	88	UPP_STRSL
6	4.70	slr1365	ferrochelatase BioY prot	<i>bioY</i>	217	1016197	ORF188	<i>Cyanophora paradoxa</i>	188	26	BIOY_BACSH
7	3.90	slr0839	BioY prot	<i>hemH</i>	387	1276821	ORF48	<i>Porphyra purpurea</i>	48	47	HEMZ_SYNY3
8	3.60	slr1365	cytochrome b6/f subunit VI (3.5kD)	<i>bioY</i>	217	7259544	unknown	<i>Mesostigma viride</i>	223	2	bioY_SYNY3
9	2.50	11691 ^a	cytochrome b6/f subunit VI (3.5kD)	<i>petL</i>	31	5881712	ORF31	<i>Arabidopsis thaliana</i>	31	2	PETL_BETVU
10	2.50	12005 ^a	cytochrome b6/f subunit VI (3.5kD)	<i>petL</i>	31	1262631	ORF62b	<i>Pinus thunbergii</i>	62	2	PETL_BETVU
11	2.50	11809 ^a	maturase K	<i>matK</i>	509	295955	ORF506	<i>Euglena gracilis</i>	506	994	mat_PSEAL

List of ORFs from different cpDNAs for which functional annotation has been possible. The results from PSI-BLAST are also shown, indicating the number of significant hits obtained (column 11), and the ortholog assigned (column 12). Proteins in column 12 are labeled according to SWISSPROT nomenclature.

^aNot in Synechocystis.

(not included in the matrix) were *rpl2*, *rps2*, *rps3*, *rps4*, and *rps12*. All of them correspond to ribosomal proteins, and they form the most conserved core of the chloroplast ribosome probably essential for its translation activity. In the 277 groups of orthologous proteins that form the **X**-matrix (which are lines **101** type), 256 correspond to COGs present in *Synechocystis* and 21 to COGs that are specific to plant chloroplasts, not present in cyanobacteria. Functional annotation was taken from CyanoBase, with some small modifications. In the 277 COGs, a total of 73 correspond to hypothetical proteins (i.e., groups of proteins not having functional annotations in databases and present in at least two cpDNAs). The matrix will be available at <http://alice.usal.es/cpDNA20x277matrix> and can also be obtained by contacting the authors.

Loadings Matrix at the Optimal Dimensionality and Phylogenetic Analysis Derived

The **X**-matrix (a 20×277 binary matrix) was analyzed by multivariate analysis as described in Methods. These analyses allow us to obtain important quantitative information about the mutual relationships among genomes, as well as relationships among orthologous proteins. These relationships are mathematically expressed by the loadings (which provide information about the degree of similarity between the genomes) and by the dot product (DP) scores (see equation 6) and the factor scores (which provide information about the proteins and about the relationship between them). Table 4 shows the values of the loadings obtained for each genome. A second **X**-matrix including only 18 genomes was also analyzed. In this matrix all COGs corresponding to the nonphotosynthetic parasitic genomes (*E. virginiana* and *T. gondii*) were excluded. Exclusion of these two genomes was done for

the phylogenetic analysis (vide infra) to avoid possible noise introduced by nonphotosynthetic parasitic species. The values of the loadings for the second matrix are also presented in Table 4. A set of 22 chloroplast genes is lost in these excluded genomes: *atpA*, *atpB*, *atpE*, *atpF*, *atpH*, *petB*, *psaA*, *psaB*, *psbA*, *psbB*, *psbC*, *psbD*, *psbE*, *psbF*, *psbH*, *psbJ*, *psbK*, *psbL*, *psbT*, *rbcL*, *rpoC2*, and *ycf4*. They are mostly photosynthetic subunits that belong to ATPase and photosystem II. A set of 3 proteins is only lost in *Epifagus*: *rpl14*, *rpoB*, and *rpoC1*; 8 proteins, all ribosomal, are only lost in *Toxoplasma*: *rpl16*, *rpl20*, *rps7*, *rps8*, *rps11*, *rps14*, *rps18*, and *rps19*. The type of proteins lost in *Epifagus* and *Toxoplasma* genomes clearly reveals that these organisms have nonphotosynthetic plastids and that they have a very diminished ability for independent translation.

Table 4 presents the loadings corresponding to the most significant dimensions. The optimal number of dimensions was found to be four when 20 genomes were included (explaining 75.7% of the variance) and three for 18 genomes (explaining in this case 77.2% of the variance). Figure 2 shows the accumulated percentage of variance explained at each dimension. Selection of the optimal number of dimensions is based in eigenvalue analysis. To simplify, when the eigenvalue reaches 1, a major part of the variance included in the **X**-matrix has already been explained. As detailed in Table 4, the first dimension explains green plants (land plants, *streptophyta*, and green algae, *chlorophyta*) plus *euglenophyta* and separates them from the nongreen algae and cyanobacteria. The second dimension, on the other hand, explains the nongreen algae and separates them from green plants and cyanobacteria. The third dimension, finally, is a factor specific for cyanobacteria. These results are rather insensitive to the incorporation or not of the nonphotosynthetic genomes.

Using the loadings matrix corresponding to the 18 ge-

Table 4. Loadings Matrix Obtained by Factor Analysis of the Two **X**-Matrices

20 genome species	Dimensions ^b						18 genome species	Dimensions ^b							
1 <i>Synechocystis</i>	-0.02	0	0.281	0	-0.85	1	-0.11	0	<i>Synechocystis</i>	-0.05	0	0.244	0	-0.85	1
2 AraTha	0.949	1	0.124	0	0.071	0	-0.042	0	AraTha	0.951	1	0.124	0	0.043	0
3 ChlVul	0.753	1	0.330	—	-0.221	0	0.155	0	ChlVul	0.745	1	0.321	—	-0.299	0
4 CyaCal	0.012	0	0.712	1	-0.050	0	-0.050	0	CyaCal	0.010	0	0.704	1	-0.074	0
5 CyaPar	0.252	0	0.549	1	-0.244	0	0.060	0	CyaPar	0.243	0	0.540	1	-0.284	0
6 EpiVir	0.420	—	0.089	0	0.309	—	-0.248	0	—	—	—	—	—	—	—
7 EugGra	0.625	1	0.406	—	-0.001	0	0.271	0	EugGra	0.629	1	0.413	—	-0.069	0
8 GuiThe	0.190	0	0.840	1	0.023	0	0.071	0	GuiThe	0.194	0	0.851	1	0.016	0
9 MarPol	0.920	1	0.078	0	-0.104	0	0.000	0	MarPol	0.915	1	0.071	0	-0.146	0
10 MesVir	0.836	1	0.099	0	-0.289	0	0.086	0	MesVir	0.826	1	0.094	0	-0.316	0
11 NepOli	0.860	1	0.084	0	-0.264	0	0.105	0	NepOli	0.852	1	0.079	0	-0.301	0
12 NicTab	0.921	1	0.072	0	0.136	0	-0.032	0	NicTab	0.926	1	0.077	0	0.122	0
13 OdoSin	0.263	0	0.832	1	0.020	0	0.095	0	OdoSin	0.266	0	0.841	1	0.003	0
14 OenEla	0.939	1	0.129	0	0.094	0	-0.036	0	OenEla	0.942	1	0.129	0	0.066	0
15 OrySat	0.914	1	0.045	0	0.211	0	-0.013	0	OrySat	0.925	1	0.057	0	0.207	0
16 PinThu	0.830	1	0.327	0	0.026	0	0.010	0	PinThu	0.828	1	0.318	0	-0.046	0
17 PorPur	-0.067	0	0.791	1	-0.132	0	-0.065	0	PorPur	-0.073	0	0.781	1	-0.151	0
18 SpiOle	0.938	1	0.119	0	0.034	0	-0.044	0	SpiOle	0.938	1	0.116	0	0.003	0
19 ToxGon	0.030	0	0.057	0	0.067	0	0.932	1	—	—	—	—	—	—	—
20 ZeaMay	0.897	1	0.020	0	0.269	0	-0.008	0	ZeaMay	0.910	1	0.034	0	0.268	0

Loadings matrix for the genomes at the optimal dimensionality: four dimensions for the set of 20 genomes and three dimensions for the set of 18 genomes. Values vary from -1 to 1. The larger the absolute value of the loading, the larger the significance of that factor for the corresponding genome. Decimal numbers in bold indicate relevant loadings at each dimension. Integer numbers in bold are the result of converting the loading information into "fingerprints" (see text for details).

^aFour dimensions explain 75.7% variance.

^bThree dimensions explain 77.2% variance.

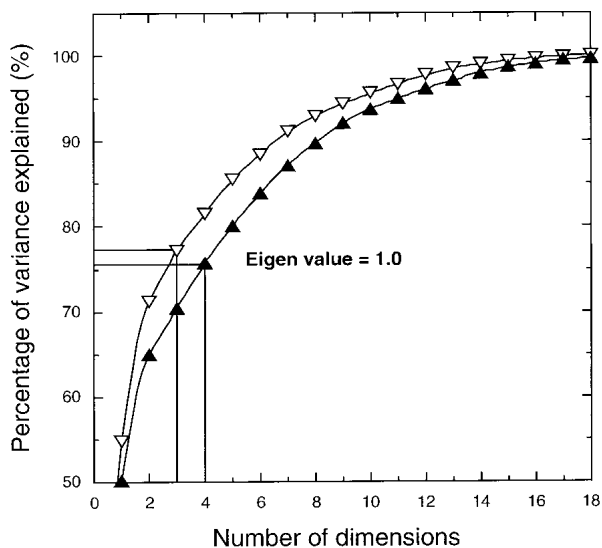


Figure 2 Accumulated percentage of variance explained at each dimensionality, as obtained by the factorial analyses of the X -matrices. The matrices include 277 groups of orthologous proteins from 20 genomes (solid triangles) or 18 genomes (open triangles).

nomes, a phylogenetic tree for the organisms was built. Figure 3A presents the tree in phylogram view. Figure 3C shows the same tree as a rectangular cladogram, including, close to each branch, the percentage of stability according to a bootstrapping analysis. Figure 3D shows the same tree in a radial view, including the names of the main phylogenetic groups corresponding to the species studied. The tree shows that the chloroplast of *Cyanophora paradoxa* is the closest to the ancient cyanobacteria type common ancestor, as it is included in the first short branch, the closest to *Synechocystis*. On this tree line a more distant second branch appears, including two sub-branches: one that corresponds to bacillariophyta (*Odontella sinensis*) and cryptophyta (*Guillardia theta*) and another that corresponds to rhodophyta (*Cyanidium caldarium* and *P. purpurea*). The other main branch, opposite to the previous ones in Figure 3D, includes first euglenophyta (*E. gracilis*) and then all the green plants, starting with chlorophyta (green algae) and following with all the streptophyta (land plants). The tree topology is consistent with the phylogenetic and evolutionary order currently assigned to the organellar genomes (Gray 1999). It also agrees with the phylogenetic classification of these genomes obtained by RNA analyses (Sugiura et al. 1998). The two endosymbiotic events known to occur during chloroplast evolution (Cavalier-Smith 2000) are indicated as small circles in Figure 3D, with 1° for the primary endosymbiosis and 2° for the secondary endosymbiosis. Secondary endosymbiosis has been described to occur in *Guillardia* (Douglas et al. 2001) and *Odontella* (Kowallik et al. 1995) and also in an independent way in *Euglena* (Hallick et al. 1993). The tree also locates well these genomes, in agreement with their endosymbiotic origin. Figure 3B presents another tree obtained with the raw 286 matrix obtained automatically before its manual editing (see Methods). This tree is very similar to the first one, with small variations in the relative location of the green algae and of *Oenothera*, and it confirms the ability and stability of the method to produce an adequate phylogenetic tree.

In recent years several molecular evolutionary studies have been conducted to clarify the evolutionary events in

chloroplasts (Martin et al. 1998; Tomitani et al. 1999; Turmel et al. 1999; Adachi et al. 2000; Lemieux et al. 2000; Moreira et al. 2000; Bauer et al. 2001; Provan et al. 2001). A phylogenetic tree for chloroplasts based on the analysis of the sequences of 45 common chloroplast proteins has been recently proposed (Martin et al. 1998; Adachi et al. 2000). The genomic tree presented here shows a remarkable agreement with the phylogenetic tree obtained by Martin and coworkers on the basis of molecular patterns (Martin et al. 1998; Adachi et al. 2000). The location of *Cyanophora* as the closest relative to the cyanobacterial origin, the location of euglenophytes separated from the rest of green plants, and the correct location of the three organisms involved in the secondary endosymbiotic event are some of the features illustrating such agreement.

It can be concluded that the loadings, as deduced from the matrix of orthologous proteins, can be used to explore and define rather accurately the phylogenetic relationships between cpDNA genomes. The approach is a reasonable extrapolation of the sequence-based methods to whole-genome-tree reconstruction. The initial matrix being diagonalized is basically a similarity matrix between genomes, encoding a reasonable description of genome distances. The use of the loadings matrix, rather than the original correlation matrix, for the clustering algorithm has the effect of giving higher weight to those combinations of genes changing together in a concerted way during evolutionary time. It therefore is equivalent to assuming that independent, uncorrelated gene acquisitions or deletions do not contribute to the phylogenetic tree. Although more studies are required, we argue that this approach, because it focuses on large-scale, correlated changes of the biochemical machinery of all the organisms under study, is adequate for phylogenetic analysis at the genome level.

Detection of Specific Genes as Phylogenetic Correlates

One of the most interesting features of a phylogenetic analysis at the genome level is that it not only allows one to obtain phylogenetic relationships between species, but it may also provide a way to identify the specific proteins or genes that can best explain the differences between species or groups of species, according to the derived phylogenetic tree. In Table 5 we list the proteins we find as the most specific or peculiar for the different groups of chloroplasts. The genes are in descending order according to their DP scores (see Methods for an explanation of this derivation). In this way, genes present at the top of each group in Table 5 are the most important to differentiate and define each branch of genomes in the tree. The profiles column marks with a **1** the presence of a certain gene in a specific genome. Each number (**1** or **0**) corresponds to 1 of the 20 species ordered from left to right as enumerated in Table 4, starting with *Synechocystis*.

Table 5 shows 12 genes that are most specific to *Cyanophora* cpDNA and only common with *Synechocystis*: *clpP*, *crE*, *groES*, *hemA*, *mntA*, *mntB*, *nadA*, and 5 hypothetical proteins. These genes could be considered as coding for the most conserved genes from the ancient chloroplast, among those that show variation. Among them, it is interesting the presence of *clpP*, a translocation-related protease, and *groES*, the small subunit of a main chaperonin. Both gene products are homologous to similar *Escherichia coli* prokaryotic proteins essential for proper protein translocation and folding. The large subunit of this chaperonin, *groEL*, is present in all nongreen algae but has disappeared in the rest of the higher plant genomes. The same occurs with *dnaK*, another chaperonin in-

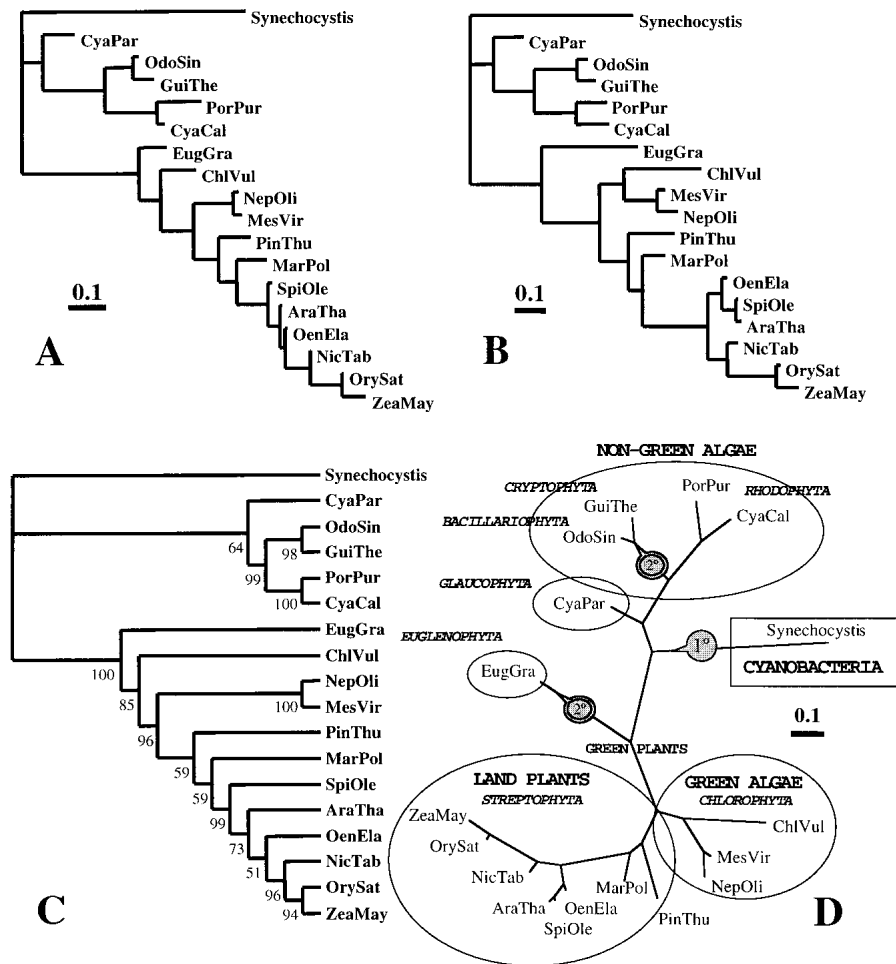


Figure 3 Phylogenetic tree derived from the analysis of the X-matrix of orthologs genes of 17 complete chloroplast genomes plus *Synechocystis* genome. (A) Tree in phylogram view. (B) A different tree obtained with the raw 286 X-matrix obtained automatically but before manual editing (see Methods). (C) The same tree as in A, but in a rectangular cladogram, including close to each branch the percentage of stability according to bootstrapping analysis. (D) The A tree in a radial view, including names of the main phylogenetic groups corresponding to the species studied.

involved in protein folding after translation, and with *secY*, which with *secA* form the thylakoid membrane translocase system, similar to the one found in prokaryotes. These observations indicate a trend of gene loss toward land plant chloroplasts that first affects to the translation, translocation, and folding machinery. The deletion of an important number of ribosomal proteins also is in keeping with this observation. The ribosomal proteins specific for nongreen algae are *rpl1*, *rpl3*, *rpl6*, *rpl11*, *rpl18*, *rps5*, *rps6*, *rps10*, *rps13*, and *rps17* (present also in *Cyanophora*) and *rpl4*, *rpl13*, *rpl24*, *rpl27*, *rpl29*, and *rpl31* (excluded in *Cyanophora*). This significant amount of ribosomal proteins only present in the cpDNAs more similar to *Synechocystis* may reveal an ancient part of the ribosomal architecture. In higher plants, many of these functions are taken on by genes encoded in the nuclear genome.

Two other interesting genes are only present in *Synechocystis* and *Cyanophora*. These are the ones that form the manganese transporter system: *mntA* and *mntB*. Manganese is essential for oxygenic photosynthesis. It is quite feasible that ancient chloroplasts were in origin closer to nonoxygenic

photosynthesis and that they acquired through evolution the ability for water splitting and oxygen production. Under these circumstances, it could have been useful to have manganese transporters to incorporate as much manganese as possible from the surrounding media, as this ion can by itself be an electron donor to photosystem II. Higher plants would have evolved toward a more sophisticated control and regulation of the manganese pool, avoiding having free manganese within the cells, perhaps due to its toxicity.

The most important phylogenetic correlate for nongreen algae chloroplasts is the rubisco expression protein (*cfxQ*; see Table 5), only present in genomes of nongreen algae (excluding *Cyanophora*). Thus, nongreen algae have incorporated through evolution a specific gene not present in cyanobacteria to control rubisco expression, an essential photosynthetic enzyme in charge of CO₂ fixation (Choquet and Vallon 2000). Rubisco is the most abundant protein in the chloroplast and its expression is crucial for the photosynthetic machinery. It has two main subunits, *rbcS* and *rbcL*, both of which are encoded by the chloroplast genome in nongreen algae. However, in higher plants, the cell nucleus encodes the small rubisco subunit (*rbcS*; Whitney and Andrews 2001) and the nucleus has also taken responsibility in the control of rubisco expression because *cfxQ* is also lost in higher plants.

Other significant genes in nongreen algae are some subunits of major protein complexes: *psaD* and *pasL*, which are not present in *Cyanophora*; *psaE* and *psaF*, all included in photosystem I complex; *psbW*, *psbV*, and *psbY*, from photosystem II; and *atpD* and *atpG* from ATPase. These subunits are not present in the cpDNAs of more evolved plants, and in this way they show some functions that may have been lost during evolution or which may have been replaced by better regulated subunits. This is, for example, the case of *psbV*, a subunit lost in the extrinsic part of the oxygen-evolving complex of higher plants. The plants have instead acquired two other polypeptides (*psbP* and *psbQ*) that stabilize the manganese cluster and are encoded by the nucleus of the plant cell (Shen et al. 1995).

The red algae constitute a subgroup inside the nongreen algae, but they have some genes that are unique to their cpDNAs. The most significant of them are genes related to the phycobilisome architecture and function: *apcA*, *apcB*, and *apcE*. Another significant type of genes characteristic of red algae, but not included in *Cyanophora* cpDNA, are genes involved in different biosynthetic pathways, such as the biosyn-

Table 5. Genes Selected as Phylogenetic Correlates Specific to Each Tree Branch

	DI score	Syn prot	Gene	Profile	Protein function
Branch CyaPar					
1	0.0400	slI0534	<i>clpP</i>	10001000000000000000	ATP-dep. translocation rel. protease
2	0.0400	slr0739	<i>crtE</i>	10001000000000000000	geranylgeranyl pyrophosphate synthase
3	0.0400	slr2075	<i>groES</i>	10001000000000000000	10kD chaperonin 1
4	0.0400	slr1808	<i>hemA</i>	10001000000000000000	transfer RNA-Gln reductase
5	0.0400	slI1599	<i>mntA</i>	10001000000000000000	Mn transporter
6	0.0400	slI1600	<i>mntB</i>	10001000000000000000	Mn transporter
7	0.0400	slI0622	<i>nadA</i>	10001000000000000000	quinolinate synthetase
8	0.0400	slr2034	<i>ycf</i>	10001000000000000000	hypothetical protein
9	0.0400	slr2073	<i>ycf</i>	10001000000000000000	hypothetical protein
10	0.0400	slr0181	<i>ycf</i>	10001000000000000000	hypothetical protein
11	0.0400	slr1552	<i>ycf</i>	10001000000000000000	hypothetical protein
12	0.0400	slI1702	<i>ycf</i>	10001000000000000000	hypothetical protein
Branch Nongreen algae (and CyaPar)					
1	0.1192	slI1325	<i>atpD</i>	10011001000010001000	ATP synthase d subunit
2	0.1192	slI1323	<i>atpG</i>	10011001000010001000	ATP synthase subunit b'
3	0.1192	slI0170	<i>dnaK</i>	10011001000010001000	DnaK protein chaperonin
4	0.1192	slr2076	<i>groEL</i>	10011001000010001000	60kD chaperonin 1
5	0.1192	slI0998	<i>lysR</i>	10011001000010001000	LysR transcriptional regulator
6	0.1192	sal0020	<i>petF</i>	10011001000010001000	ferredoxin
7	0.1192	ssr2831	<i>psaE</i>	10011001000010001000	photosystem I subunit IV
8	0.1192	slI0819	<i>psaF</i>	10011001000010001000	photosystem I subunit III
9	0.1192	slI1398	<i>psbW</i>	10011001000010001000	photosystem II 13 kD protein
10	0.1192	slI0258	<i>ptbV</i>	10011001000010001000	cytochrome c550
11	0.1192	sml0007	<i>psbY</i>	10011001000010001000	photosystem II PsbY protein
12	0.1192	slr0012	<i>rbcS</i>	10011001000010001000	ribulose biphosphate carboxylase small sub.
13	0.1192	slI1744	<i>rpl1</i>	10011001000010001000	50S ribosomal protein L1
14	0.1192	slI1799	<i>rpl3</i>	10011001000010001000	50S ribosomal protein L3
15	0.1192	slI1810	<i>rpl6</i>	10011001000010001000	50S ribosomal protein L6
16	0.1192	slI1743	<i>rpl11</i>	10011001000010001000	50S ribosomal protein L11
17	0.1192	slI1811	<i>rpl18</i>	10011001000010001000	50S ribosomal protein L18
18	0.1192	slI1812	<i>rps5</i>	10011001000010001000	30S ribosomal protein S5
19	0.1192	slI1767	<i>rps6</i>	10011001000010001000	30S ribosomal protein S6
20	0.1192	slI1102	<i>rps10</i>	10011001000010001000	30S ribosomal protein S10
21	0.1192	slI1816	<i>rps13</i>	10011001000010001000	30S ribosomal protein S13
22	0.1192	sal3437	<i>rps17</i>	10011001000010001000	30S ribosomal protein S17
23	0.1192	slI1814	<i>secY</i>	10011001000010001000	preprotein translocase subunit Y
24	0.1192	slr0075	<i>ycf16</i>	10011001000010001000	ABC transporter subunit
25	0.1192	slr0399	<i>ycf39</i>	10011001000010001000	hypothetical protein
Branch Red algae (and CyaPar)					
1	0.0712	slr2067	<i>apcA</i>	10011000000000001000	allophycocyanin a chain
2	0.0712	slr1986	<i>apcB</i>	10011000000000001000	allophycocyanin b chain
3	0.0712	slr0335	<i>apcE</i>	10011000000000001000	phycobilisome LCM core-membrane linker prot.
4	0.0712	slr0611	<i>sds</i>	10011000000000001000	solanesyl diphosphate synthase
5	0.0712	slr0055	<i>trpG</i>	10011000000000001000	anthranilate synthase component II
6	0.0712	slr2032	<i>ycf23</i>	10011000000000001000	hypothetical protein
Branch Nongreen algae (but no CyaPar)					
1	0.1460	—	<i>cfxQ</i>	00010001000010001000	rubisco expression protein
2	0.1339	—	<i>ycf</i>	00010001000000001000	hypothetical protein
3	0.1192	slr0228	<i>ftsH</i>	10010001000010011000	cell division protein
4	0.1192	slI0020	<i>clpC</i>	10010001000010001010	ATP-dep. translocation rel. protease, regulator
5	0.1306	sal2084	<i>acp</i>	10010001000010001000	acyl carrier protein
6	0.1306	slr0833	<i>dnaB</i>	10010001000010001000	replicative DNA helicase
7	0.1306	slr0737	<i>psaD</i>	10010001000010001000	photosystem I subunit II
8	0.1306	slr1655	<i>psaL</i>	10010001000010001000	photosystem I subunit XI
9	0.1306	slI1800	<i>rpl4</i>	10010001000010001000	50S ribosomal protein L4
10	0.1306	slI1821	<i>rpl13</i>	10010001000010001000	50S ribosomal protein L13
11	0.1306	slI1807	<i>rpl24</i>	10010001000010001000	50S ribosomal protein L24
12	0.1306	sar2799	<i>rpl27</i>	10010001000010001000	50S ribosomal protein L27
13	0.1306	sal3436	<i>rpl29</i>	10010001000010001000	50S ribosomal protein L29
14	0.1306	sal3445	<i>rpl31</i>	10010001000010001000	50S ribosomal protein L31
15	0.1306	slI0616	<i>secA</i>	10010001000010001000	preprotein translocase subunit A
16	0.1306	slI0194	<i>ycf43</i>	10010001000010001000	hypothetical protein
17	0.1306	slr2087	<i>ycf44</i>	10010001000010001000	hypothetical protein

Table 5. (Continued)

	DI score	Syn prot	Gene	Profile	Protein function
	Branch <i>Red algae</i> (but no <i>CyaPar</i>)				
1	0.0822	sll0728	<i>accA</i>	10010000000000001000	acetyl-CoA carboxylase alpha subunit
2	0.0822	slr0435	<i>accB</i>	10010000000000001000	biotin carboxyl carrier of acetyl-CoA carboxy
3	0.0822	slr1898	<i>argB</i>	10010000000000001000	N-acetylglutamate kinase
4	0.0822	sll1214	<i>AT103</i>	10010000000000001000	phytochrome-regulated gene
5	0.0822	sll1498	<i>carA</i>	10010000000000001000	carbamoyl-phosphate synthetase subunit A
6	0.0822	slr2051	<i>cpcG</i>	10010000000000001000	phycobilisome rod-core linker polypeptide
7	0.0822	sll0698	<i>dfr</i>	10010000000000001000	drug sensory protein A
8	0.0822	sal0707	<i>glnB</i>	10010000000000001000	nitrogen regulatory protein P-II
9	0.0822	sll1499	<i>gltB</i>	10010000000000001000	ferredoxin-dependent glutamate synthase
10	0.0822	slr0974	<i>infC</i>	10010000000000001000	initiation factor IF-3
11	0.0822	sll1536	<i>moeB</i>	10010000000000001000	molybdopterin biosynthesis protein
12	0.0822	sll1423	<i>ntcA</i>	10010000000000001000	global nitrogen regulator
13	0.0822	slr1934	<i>pdhA</i>	10010000000000001000	pyruvate dehydrogenase E1 component α sub
14	0.0822	sll1796	<i>petJ</i>	10010000000000001000	cytochrome c553
15	0.0822	slr0966	<i>trpA</i>	10010000000000001000	tryptophan synthase α chain
16	0.0822	slr0623	<i>trxA</i>	10010000000000001000	thioredoxin
17	0.0822	slr1780	<i>ycf</i>	10010000000000001000	hypothetical protein
18	0.0822	sll1737	<i>ycf</i>	10010000000000001000	hypothetical protein
19	0.0822	sll0558	<i>ycf</i>	10010000000000001000	hypothetical protein
20	0.0822	sll1879	<i>ycf</i>	10010000000000001000	hypothetical protein
	Branch <i>EugGra</i>				
1	0.0731	—	<i>ycf</i>	00000010000000000010	hypothetical protein
2	0.0731	slr0913	<i>ycf</i>	10000010000000000000	hypothetical protein
3	0.0731	slr0914	<i>ycf</i>	10000010000000000000	hypothetical protein
	Branch <i>Green algae</i>				
1	0.1373	sll1833	<i>fts1</i>	10000000011000000000	penicillin-binding protein
2	0.1373	slr1972	<i>ycf</i>	10000000011000000000	hypothetical protein
3	0.1333	slr1455	<i>cysA</i>	10100000111000000000	sulfate transport system permease protein
4	0.1333	slr1453	<i>cysT</i>	10100000111000000000	sulfate transport system permease protein
5	0.1064	sll0289	<i>minD</i>	10100001011000000000	septum site-determining protein
6	0.1054	slr0772	<i>chlB</i>	10101000111000011000	protochlorophyllide reductase subunit B
7	0.1054	slr0749	<i>chlL</i>	10101000111000011000	light-indep. protochlorophyllide reductase Fe
8	0.0899	slr0750	<i>chlN</i>	10101000011000011000	protochlorophyllide reductase subunit N
9	0.0979	sll1463	<i>ftsH</i>	10100000101000000000	cell division protein
	Branch <i>Land plants</i>				
1	0.1017	—	<i>petL</i>	01000000101101110101	cytochrome b6-f subunit VI
2	0.1006	—	<i>ycf2</i>	01000100000101000001	hypothetical protein
3	0.0965	—	<i>matK</i>	01000110100101110101	maturase K
4	0.0813	—	<i>ycf1</i>	01100100111101010100	hypothetical protein
5	0.0970	slr1281	<i>ndhJ</i>	11000000110101100101	NADH dehydrogenase subunit J
6	0.0921	sll0519	<i>ndhA</i>	11000000111101100101	NADH dehydrogenase subunit 1
7	0.0921	sll0223	<i>ndhB</i>	11000000111101100101	NADH dehydrogenase subunit 2
8	0.0921	slr1279	<i>ndhC</i>	11000000111101100101	NADH dehydrogenase subunit 3
9	0.0921	slr0331	<i>ndhD</i>	11000000111101100101	NADH dehydrogenase subunit 4
10	0.0921	sll0522	<i>ndhE</i>	11000000111101100101	NADH dehydrogenase subunit 4L
11	0.0921	slr0844	<i>ndhF</i>	11000000111101100101	NADH dehydrogenase subunit 5
12	0.0921	sll0521	<i>ndhG</i>	11000000111101100101	NADH dehydrogenase subunit 6
13	0.0921	slr0261	<i>ndhH</i>	11000000111101100101	NADH dehydrogenase subunit 7
14	0.0921	sll0520	<i>ndhI</i>	11000000111101100101	NADH dehydrogenase subunit I
15	0.0921	slr1280	<i>ndhK</i>	11000000111101100101	NADH dehydrogenase subunit K
16	0.0878	sml0003	<i>psbM</i>	11101000101101100101	photosystem II M protein
	EpiVir				
1	0.0169	—	<i>ycf2</i>	01000100000101000001	hypothetical protein
2	0.0119	—	<i>ycf1</i>	01100100111101010100	hypothetical protein
3	0.0119	—	<i>matK</i>	01000110100101110101	maturase K
4	0.0119	ssl3441	<i>infA</i>	10100100111000110101	initiation factor IF-1

Genes acting as phylogenetic correlates, as derived from the factor analysis and DP scoring.

thesis of fatty acids *accA* and *accB* and synthesis of amino acids *argB*, *glnB*, *gltB*, and *trpA*.

The genes best identified as phylogenetic correlates within green algae cpDNAs are several genes involved in the synthesis of chlorophyll—*chlB*, *chlL*, and *chlN*—and two genes involved in sulfate absorption—*cysA* and *cysT* (see Table 5).

Many of the green algae are unicellular organisms that need to take nutrients from the aquatic environment. Sulfate is one of these important nutrients. Most of the absorption and nutritional functions are coded by nuclear genes in higher multicellular plants. In fact, in land plants the root cells are specialized to carry out such functions.

The following phylogenetic correlates (see Table 5) were identified in the case of land plant chloroplast genomes: maturase (*matK*), NADH dehydrogenase (*ndhA, B, C, D, E, F, G, H, I, J, and K*), one subunit of cytochrome b6/f (*petL*), and another subunit from photosystem II (*psbM*). The presence of maturase is a distinct feature in higher land plants directly related with the appearance of introns (see Table 1; Wolfe et al. 1991). The requirement of a correct translation for genes with introns in plant chloroplasts may have forced incorporation of maturase into these cpDNAs. On the other hand, appearance of new specific subunits to multienzymatic protein complexes, like cytochrome b6/f and photosystem II, indicate that the selected genes (*petL* and *psbM*) are incorporated later than the other common subunits to the plant cpDNAs. They correspond to subunits carrying some new regulatory function. The *petL* subunit is only present in land plants and contrasts with two other cytochrome b6/f subunits that are

only present in nongreen algae: *petF* and *petM*. Recent work has shown that *petM* is involved in cytochrome b6/f regulation in cyanobacteria (Schneider et al. 2001). Finally, the presence of NADH dehydrogenase in land plants, and also in cyanobacteria but not in any algae, may be related to the control of redox equilibrium. Oxyphotosynthetic organisms avoid photoinhibition and inconvenient oxidations through different mechanisms (Aro et al. 1993; De Las Rivas et al. 1993), but although in vivo algae have many potential mechanisms to avoid photoinhibition, static land plants may need better controlled mechanisms that involve the incorporation of specific molecular systems. Maintenance in the land plant chloroplasts of an alternative redox enzyme like NADH dehydrogenase, also present in the cyanobacteria, will most probably be related with such protection and balancing mechanisms (Allen and Pfanschmidt 2000). The *ndh* chloroplastic genes have certain homology to genes encoding subunits of the

Table 6. Gene Clusters Deduced from the X-Matrix, for a Selected Set of Complexes/Functional Units

A. Percentage of each complex accumulated in each one of the nine clusters										
Complexes	Cluster No. No. of Prot.	1 51	2 55	3 58	4 12	5 17	6 6	7 15	8 9	9 54
PSI	12	33.33	8.33	8.33	0	0	0	0	8.33	41.67
PSII	18	16.67	0	5.56	0	5.56	0	0	0	72.22
ATPase	8	25	0	0	0	0	0	0	0	75
Cytb6f	6	16.67	0	0	0	0	0	16.67	0	66.67
NADHase	11	0	0	0	0	100	0	0	0	0
Phyb	9	11.11	11.11	77.78	0	0	0	0	0	0
RibProt	43	46.51	4.65	2.33	0	2.33	0	0	9.3	34.88
RNAApol	4	0	0	0	0	0	0	0	0	100
CellDiv	5	20	40	0	40	0	0	0	0	0
HypoProt	73	8.22	30.14	24.66	2.74	1.37	6.85	17.81	1.37	6.85
B. Weight (in percentage) of each complex within each of the clusters										
Complexes	Cluster No. No. of Prot.	1 51	2 55	3 58	4 12	5 17	6 6	7 15	8 9	9 54
PSI	12	7.84	1.82	1.72	0	0	0	0	11.11	9.26
PSII	18	5.88	0	1.72	0	5.88	0	0	0	24.07
ATPase	8	3.92	0	0	0	0	0	0	0	11.11
Cytb6f	6	1.96	0	0	0	0	0	6.67	0	7.41
NADHase	11	0	0	0	0	64.71	0	0	0	0
Phyb	9	1.96	1.82	12.07	0	0	0	0	0	0
RibProt	43	39.22	3.64	1.72	0	5.88	0	0	44.44	27.78
RNApol	4	0	0	0	0	0	0	0	0	7.41
CellDiv	5	1.96	3.64	0	16.67	0	0	0	0	0
HypoProt	73	11.76	40	31.03	16.67	5.88	83.33	86.67	11.11	9.26
C. Recovery of original complexes in the clusters and Purity inside the clusters										
Organisms best represented in each cluster										
Cluster No.	Complexes	-1n(P-value) >3	Recovery %	Purity %	HypoProt %	<i>Synecho.</i>	Nongreen algae	Red algae	Green algae	Land plants
1	RiPr	4.09	46.51	39.22	8.22	×	×			
3	Phyb	3.12	77.78	12.07	24.66			×		
4	CellDiv	(2.9)	40	16.67	2.74				×	
5	NADHase	11.01	100	64.71	1.37	×				×
9	PSII	4.72	72.22	24.07	6.85	×	×	×	×	×
Total	All clusters	>3	73.05	36.45						

Cluster analysis of genes as deduced from the scores matrix. The optimal number of clusters was found to be equal to nine. Tables include data about nine well-known chloroplast complexes (see Methods) and the hypothetical proteins. (A) Percentage of each complex accumulated in each one of the nine clusters obtained. (B) Percentage of weight of each complex within each one of the clusters. (C) The most relevant functional units as detected with the parameter of the statistical significance (P -value $< 10^{-3}$). The P -value was derived assuming a background Poisson distribution (J.J. Lozano and A.R. Ortiz, in prep.). %R is the percentage of recovery of original complexes in the clusters. %P is the purity inside the clusters. %H is the percentage of functionally unknown proteins. Groups of genomes maximally represented in each cluster are marked by 'x's on the right of the table.

NADH ubiquinone oxidoreductase (complex I) of mitochondria and eubacteria and they could be involved in a proposed chloroplast respiratory chain (Burrows et al. 1998).

Finally, the phylogenetic correlates for *E. gracilis* and *E. virginiana* cpDNAs are also included in Table 5. For *Euglena* not much can be said because all correspond to hypothetical proteins. *E. virginiana*, on the other hand, is a parasitic flowering plant that lacks most of the photosynthetic genes, but it is remarkable that it conserves, as phylogenetic correlates, three of the genes that best define land plants in chloroplast genomes: *ycf1*, *ycf2*, and *matK*. This probably indicates that *Epifagus* cpDNA was once a real chloroplast genome that has suffered a strong positive selection in its parasitic ecosystem (Wolfe et al. 1992).

Clustering of Phylogenetic Profiles

The clustering method automatically classifies genes in the score matrix within nine groups. The main results from the clustering analysis can be found in Table 6, which presents the proportion of a subset of selected complexes or functional units in each cluster. Complexes selected to test the ability of the clustering procedure are indicated in Methods. Figure 4 presents the three-dimensional plots of the relative position in a three-dimensional projection of the original euclidian

space spanned by the 20 genomes (Fig. 4A,B), along with the position of the nine gene clusters (Fig. 4C,D). As can be seen in Table 6A, the method locates in cluster number 9 a hypothetical photosynthetic chloroplast core, composed by a major number of subunits from ATPase, photosystem II (PSII), and cytochrome b6f (Cytb6f) and by important populations of photosystem I (PSI) and ribosomal proteins (RibProt). The other clusters include proteins or polypeptides characteristic of specific groups of organisms. For example, cluster number 1 contains a significant proportion of photosystem I subunits (33.33%) and ribosomal proteins (46.51%), specific of nongreen algae; cluster no.; 3 includes a major proportion of phycobilisome (Phyb) proteins (77.78%) representing mainly red algae; and cluster number 5 includes all NADH dehydrogenase (NADHase) proteins (100%) representing land plants. The rest of the clusters are less significantly assigned to one group of organisms. The method also clearly identifies in which functional units a specific loss of proteins or subunits occur along the evolution. Thus, in cluster number 1 it can be observed that two main complexes (PSI and ribosome) suffer a strong reduction of genes when passing from nongreen algae to green plants (see Table 6C).

Finally, in these analyses the quality of the functional recovery and purity of the clusters has been measured. The

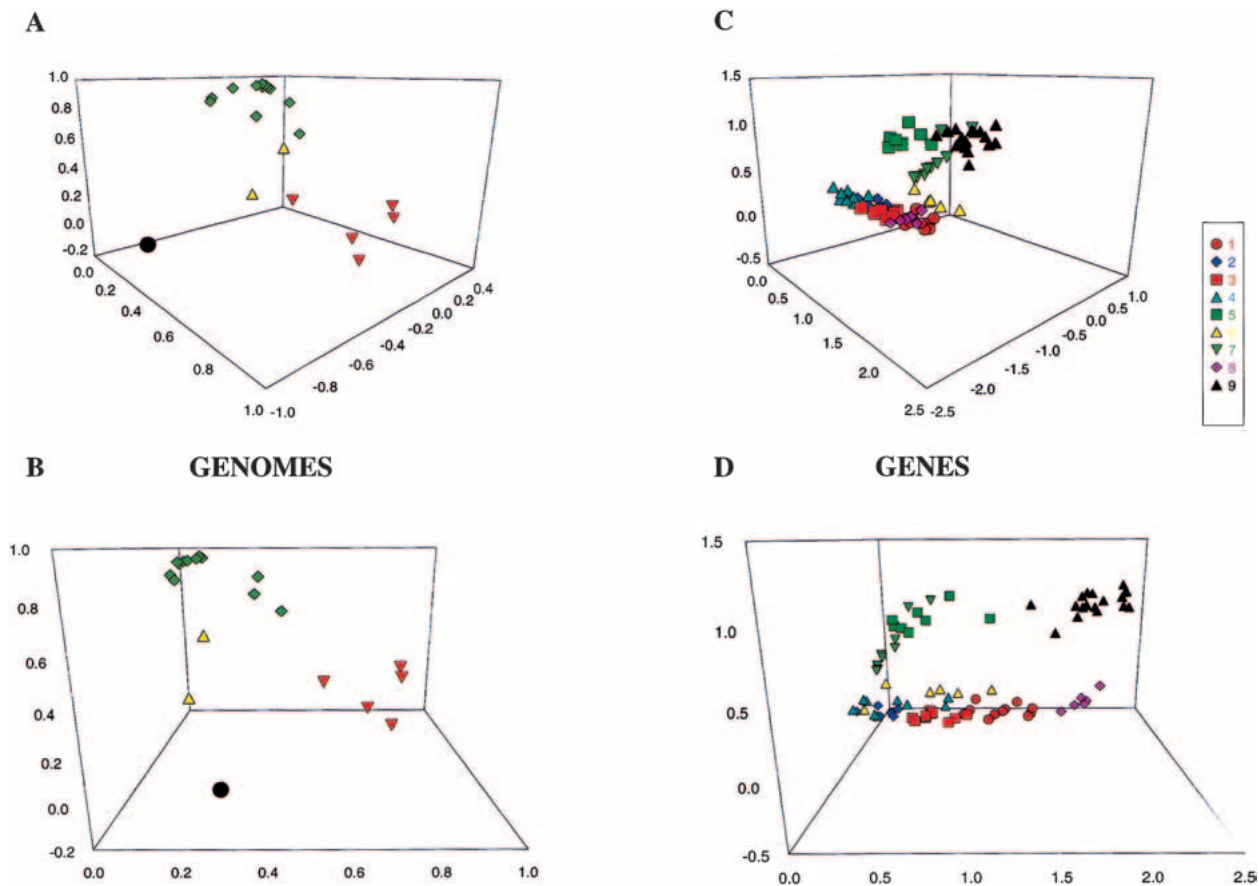


Figure 4 (A) and (B) Projections of the 20 analyzed genomes on the first three factors. Unicellular nongreen algae are shown in red triangles. Green algae and superior plants are in green diamonds. Parasite chloroplasts are in yellow triangles. Cyanobacteria is in solid circles. (C) and (D) Projections of the nine clusters of 20 analyzed genome orthologous groups. The same approximate view of A (in C) and B (in D) is used for comparison. Each cluster is shown with a color-symbol pair: 1, red circles; 2, blue diamonds; 3, red squares; 4, light blue triangles; 5, green squares; 6, yellow triangles; 7, green inverted triangles; 8, purple diamonds; 9, black triangles.

method allows a recovery of 73.0% of the original functional associations in the clusters for the statistically significant associations (those with P -value $< 10^{-3}$). The percentage of proteins belonging to one specific functional unit is 36.4% within the nine clusters produced. The specificity level seems to be high enough to allow prediction of tentative functional associations for hypothetical proteins. For example, the following proteins in nongreen algae present exactly the same set of scores in the \mathbf{X} -matrix: 30S ribosomal protein S20 (*rps20*), 50S ribosomal protein L34 (*rpl34*), 50S ribosomal protein L35 (*rpl35*), and two *ycf* proteins (*ycf33* and *ycf35*). On the basis of our results, it is tempting to speculate that *ycf33* and *ycf35* are ribosomal or translation-related proteins.

Conclusions

A set of recently developed methods for function annotation and genome comparison has been applied to a series of 19 chloroplast genomes. Genome annotation using these methods has proved to be very reliable, providing high confidence functional assignments for an average of 81% of the proteins in chloroplast genomes. Multivariate analysis of a binary data matrix derived from these genomes has allowed us to derive rather accurate phylogenetic relationships between them at the genome level. One of the most interesting features of such analysis is the possibility of detecting genes acting as phylogenetic correlates, genes critical to the formation of the observed tree topology. These genes are, from a mathematical perspective, responsible of the tree topology and, on the basis of the quality of the tree, possibly related to speciation from a biological viewpoint. Therefore, this type of analysis has the potential to help uncover the evolutionary forces shaping the organisms and their adaptative responses through the modification of their biochemical systems. In the case of chloroplasts, we have found in our analysis that these genes acting as phylogenetic actually form part of important components of the chloroplast biochemical machinery.

It is important to emphasize that the phylogenetic correlates should not be identified with genes that lead the way in evolution. A more plausible mechanism is that gain/loss of function would follow adjustments to new environments, which, by imposing a selective pressure, can select subpopulations generated by a random process. However, by studying them it may be possible to infer what general environmental selective pressure could operate in the different lineages. From our analysis, one of the driving forces in the evolution of green algae and plant chloroplasts appear to be the acquisition of molecular systems providing higher levels of regulation, probably with increasing levels of involvement by the genome from the host cell. This increased level of regulation appears to be reflected at two levels: First, regulatory units appear to be added to the energy generating complexes, along with specific molecular systems to control photooxidative stress. Second, at the same time an eukaryote-like genome organization of the chloroplast genome is developed, changing the translation and translocation machinery and incorporating intron-processing enzymes such as maturase, presumably for all to have a higher level of synchronization between the gene expression of the photosynthetic apparatus and the host genes. It is obvious that a better understanding of these processes, in addition to the insight gained in basic biological processes, can have enormous impact in genetic engineering and biotechnology. We have shown that comparative genomics is a powerful tool toward that goal.

METHODS

Genome Data Set

At the time of conducting this work 17 cpDNA had been fully sequenced. They correspond to eight land plants (*Arabidopsis thaliana*, *Marchantia polymorpha*, *Nicotiana tabacum*, *Oenothera elata*, *Oryza sativa*, *P. thumbergii*, *Spinacia oleracea*, and *Zea mays*); three green algae (*C. vulgaris*, *Mesostigma viride*, and *N. olivacea*); one Euglenophyta (*E. gracilis*); two Rhodophyta or red algae (*C. caldarium* and *P. purpurea*); one Bacillariophyta (*O. sinensis*); one Cryptophyta (*G. theta*); and one Glaucocystophyceae (*C. paradoxa*). The complete proteomes of the plastid genomes from two nonphotosynthetic parasites were also available and were also included in this study: One is from the protozoan parasite *T. gondii* and the other from the parasitic flowering plant *E. virginiana*. These two genomes were included due to their functional similarities and evolutionary relationships to cpDNA. The *E. virginiana* chloroplast genome lacks the main photosynthetic genes (Wolfe et al. 1992). The *T. gondii* one cannot be considered a true chloroplast genome, but it can be considered a plastid genome of probable green algal origin (Kohler et al. 1997). All genome sequences were downloaded from http://www.ncbi.nlm.nih.gov/PMGifs/Genomes/plastids_tax.html.

Functional Annotations

Functional annotations of ORFs derived from the complete chloroplast genomes were carried out with a recently developed computer program for functional annotation (Fabrega et al. 2001). This program finds pairs or orthologs in two different genomes, **A** and **B**. To do so, each of the N sequences in genome **A** is scanned against all other M sequences in genome **B**. Pairs of sequences are aligned using the Needleman and Wunsch algorithm with zero end gaps with a normalized Gonnet matrix (Gonnet et al. 1992). After the scanning step, an orthology likelihood score (μ -score) for ORF i in genome **A** is defined as

$$\mu_i = \max\{\sigma_{i1}, \sigma_{i2}, \dots, \sigma_{iM}\} \quad (1)$$

In turn, each of the scores σ_{ij} are defined as the number of times the sequence similarity between sequences i and j exceeds the expected minimum value of the score consistent with a common fold: $s_c(n_i, m_j)$, where n_i is the length of sequence i and m_j is the length of sequence j , as derived from training sets of sequence-structure matches by Abagyan and Batalov (1997):

$$\sigma_{ij} = \frac{s_{ij}}{s_c(n_i, m_j)} \quad (2)$$

The cutoff value in the μ -score (μ_c) needed for the pair to have the same function is then obtained by finding the value in the μ -score providing optimal discrimination between proteins with the same and proteins with different functions in two given genomes. This was done as follows: A training set of known pairs of orthologs between both genomes is first derived. Then, the frequency distribution of true-positives and true-negatives as a function of the μ -score is computed. The μ -score value at which optimal discrimination is observed is finally chosen as the cutoff μ_c . Then, a protein i in genome **A** has an ortholog in genome **B** if $\mu_i > \mu_c$. Previous use of this technique in the comparison of the *Methanococcus jannaschii* genome with the genomes of *E. coli*, *Bacillus subtilis*, and *Haemophilus influenzae* gave a value for μ_c of -2.5 (Fabrega et al. 2001). Similar computations reported in this paper using the set of chloroplast genomes have shown that 2.5 is a safe cutoff to obtain all true-positives with a negligible amount of false-negatives, but it is possible to lower the threshold to -1.9 , in this case keeping a correctness level above 80%.

Construction of the X-Matrix

A matrix of orthologous chloroplast proteins was then constructed based on the above results of pairwise genome comparisons using a simple binary count for the presence (**1**) or absence (**0**) of a given ortholog. In this way, a matrix was built that had 20 columns (*Synechocystis* and 19 cpDNA species) and 277 lines, corresponding to 277 different orthologous groups (i.e., COGs). The matrix was built first by automatically including all the 1837 proteins with μ -score ≥ 2.5 (this gave a set of 286 lines) and secondly by manual correction of some lines to include proteins well annotated between μ -score 2.5 and 1.9 (this reduced the total set to 277 lines). The matrix designed was of the **101** type, meaning that each group of orthologous should have a member in at least two cpDNAs or in one cpDNA and in the *Synechocystis* genome. In this way, COGs present in only one species (lines type **100**) or COGs present in all species (lines type **111**) were discarded. COGs of the type **100** were not adequate to study pairwise relationships between genomes. Type **111** corresponds to functions present in all the cpDNAs and therefore did not include any differential information for the matrix. The **101**-type matrix seems to provide the best equilibrium between variability and conservation.

Factor Analysis of the X-Matrix

Factor analysis (FA; Reyment and Joreskog 1996) was used for the comparative genomics studies. FA seeks to find an underlying orthogonal factor model of an original **X**-matrix (in this case our **101** matrix) of the form

$$\mathbf{X} = \mathbf{L}\mathbf{F} + \mathbf{E} \quad (3)$$

where **L** is the loadings matrix, **F** the scores matrix, and **E** is the residual matrix. Principal components analysis (PCA) was used to obtain a solution of the factor model given by equation 3. First, the loadings were obtained by scaling the eigenvector matrix (**P**) obtained from PCA:

$$\mathbf{L} = \mathbf{P}\mathbf{\Lambda}^{1/2} \quad (4)$$

A lower dimensionality space was chosen from the optimal number of dimensions that explain most of the variance of the original **X**-matrix. After this, we proceeded to rotate the factors by means of a varimax rotation (Reyment and Joreskog 1996). Then the scores matrix was generated by an ordinary (unweighted) least squares procedure:

$$\mathbf{F} = \mathbf{\Lambda}^{-1/2} \mathbf{P}^*{}' \mathbf{X} \quad (5)$$

where **P*** are the rotated eigenvectors and the prime denotes the transpose.

Phylogenetic Reconstruction

Phylogenetic trees of the genomes studied were derived by clustering genomes in the loadings space with a neighbor-joining method (Saitou and Nei 1987). To generate the distance matrix needed in the neighbor-joining algorithm, we used the distribution of points representing the organisms in the space given by the loadings matrix at the optimal dimensionality found by FA (i.e., the dimensionality at which the eigenvalue of 1 is reached). In this space euclidean distances between each pair of genome loadings were calculated. Once the main tree was built, an estimation of confidence or reliability of each branch was obtained by means of a jackknife bootstrap analysis using 1000 replicates. Bootstrap values were computed by selecting random subsets of 75% of the genes per genome (Durbin et al. 1998), reanalyzing the new **X**-matrix by FA and recalculating the trees. Distribution of trees and frequency of each branch in the original tree were recorded using the CONSENSE program included in PHYLIP software package (Felsenstein 1996).

Detection of Phylogenetic Correlates and Gene Clustering

The FA results can be used to identify the specific COGs or groups of proteins contributing heavily to the specific character of different species, as inferred from the phylogenetic analysis. This is done here as follows: The values of the loadings at each dimension k are transformed to fingerprints by translating them to a binary form—**1** when the value is >0.5 and **0** otherwise, forming a vector. These patterns can be correlated with the patterns of presence/absence of the genes used to build up the original **X**-matrix, so that the specific genes that best define each dimension can be identified. For each variable (i.e., each COG in the **X**-matrix) i , with a profile in the **X**-matrix, and at each dimension k , we compute the phylogenetic correlates (i.e., the variables mainly responsible for discrimination in that dimension) as the 10% upper-ranking COGs obtained according to the following dot product (DP):

$$DP_{ki} = \vec{\mathbf{p}}_k \cdot \vec{\mathbf{x}}_i \quad (6)$$

Study of Functional Linkages

It has been suggested that functionally linked proteins tend to co-evolve, displaying patterns of correlation according to their presence or absence in a set of genomes. Consequently, this form of co-evolution could be detected using a bit-like representation of the genomes (Pellegrini et al. 1999), in a similar way to the one used here to create the **X**-matrix. We have tested the ability of our method to detect these functional associations using the scores derived from FA, as the projection into a low dimensional space should provide a better metric to establish these associations, and the chloroplast genomes are a good model system for such study.

We have clustered the elements of the scores matrix at the optimal dimensionality using two agglomerative methods sequentially. The procedure includes first the Ward algorithm (Ward 1963) to determine the set of centroids from the cloud of points and then the derivation of the members connected to each centroid by using a k -means algorithm (Johnson and Wichern 1992). As the optimal number of clusters is unknown, a stopping rule for determining the optimum number of clusters (in the interval of 5 to 50 clusters) must be employed. The figure of merit we have used in the stopping rule is the C -index (Milligan 1980), defined as

$$C_{\text{index}} = \frac{d_w - \min(d_w)}{\max(d_w) - \min(d_w)} \quad (7)$$

where d_w is the sum of within-cluster distances (a within-cluster is the squared euclidean distance between a case and its centroid). The cluster distribution yielding the smallest value of the C -index is considered optimal. Although we have not evaluated this algorithm extensively, it seems to provide satisfactory results.

Further, we have checked the significance of the observed clustering. For that, we have centered our study in the ability of the clustering procedure to classify well-defined macromolecular functional complexes present in chloroplasts. These complexes are as follows: photosystem I (which includes 12 polypeptides, genes *psa*-); photosystem II (18 polypeptides, genes *psb*-); ATPase (8 polypeptides, genes *atp*-); cytochrome *b6/f* complex (6 polypeptides, genes *pet*-); NADH dehydrogenase (11 polypeptides, genes *ndh*-); phycobilisoma (9 polypeptides, genes *apc*-, *cpc*-, and *nbl*); ribosome (43 ribosomal proteins, genes *rpl*- and *rps*-); RNA polymerase (4 polypeptides, genes *rpo*-); and cell division proteins (5 polypeptides, genes *fts*- and *min*-). Thus, we have used a test set of 116

polypeptides divided into nine different complexes or functional units, which include 42% of the full COGs matrix constructed and represent a model plant chloroplast genome that will have ~100 proteins. We evaluated the performance of the clustering procedure by monitoring two parameters: the recovery of a given complex in a given cluster, expressed as a percentage (%R), and the purity in a given functional complex of a specific cluster, expressed as a percentage (%P). Thus, the former informs about the ability of the clustering procedure to concentrate the elements of a functional complex in a cluster, whereas the latter informs about the specificity of the cluster toward a given complex. The two parameters are computed as

$$\%R = \frac{N_{KC}}{N_K} \cdot 100 \quad (8)$$

$$\%P = \frac{N_{KC}}{N_C} \cdot 100 \quad (9)$$

where N_{KC} are the number of COGs from macromolecular complex K observed in cluster C ; N_K is the number of COGs comprising complex K and N_C is the number of COGs in cluster C .

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