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Evidence for multiple xenogenous origins of plastids: comparison of *psbA*-genes with a xanthophyte sequence

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Summary. When only plastidic features are considered, it is difficult to distinguish between monophyletic and polyphyletic xenogenous origins of plastids. We suggest that a direct comparison of nuclear and plastidic sequence-similarity pattern will help to solve this problem. The D1 amino acid sequence of six major groups of photosynthetic eukaryotes and of the two groups of photosynthetic prokaryotes are now available, including the *psbA*-gene product from *Bumilleriopsis filiformis*, which is the first molecular sequence reported for a xanthophycean alga. Evidence is provided for an independent and polyphyletic origin of plastids from five out of the six major taxa of photosynthetic eukaryotes. This conclusion is reached by comparing a plastid-based pattern of D1 similarity with a nucleus-based similarity pattern published recently. Furthermore, the availability of D1 sequences from five eukaryotic algae led to a re-evaluation of the taxonomic position of *Prochlorothrix*.

Key words: Endosymbiosis – *psbA* – Xanthophyceae – *Prochlorothrix*, *Bumilleriopsis* – Eukaryotic algae

Introduction

Molecular data have played an instrumental role in the discussion of whether plastids are of autogenous or xenogenous origin (Giovanni et al. 1988). It is known that not only molecular sequences (Gray and Doolittle 1982; Giovanni et al. 1988) but also gene organization and mode of expression, as well as other features of plastids, closely resemble those of eubacteria (Margulis 1981; Golden et al. 1986; Cavalier-Smith 1987) and are fundamentally different from those of the nucleus. Consequently, the xenogenous origin of plastids is no longer a matter for discussion.

The classification of photosynthetic eukaryotes is disputed. The differences in pigment composition and ultrastructure of the plastid, as well as features of the nucleus and flagella, led to the separation of at least four, but more often seven to ten, major groups. For instance, the superkingdom of eukaryotes may be divided into nine kingdoms. Within these, five kingdoms include photosynthetic organisms (Cavalier-Smith 1981; Gray 1989). Considering the vast differences between these five kingdoms, the general proposal of a multiple polyphyletic origin of plastids (Margulis 1981; Raven 1970; Whatley and Whatley 1981) seems understandable, but is nevertheless actively disputed (Cavalier-Smith 1982, 1987; Kowallik 1989). Cavalier-Smith (1982), for instance, emphatically suggests a monophyletic origin of all plastids, interpreting the differences by subsequent loss or convergent evolution of characters, whereas the nucleotide sequences of plastidic genes coding for ribulose-1,5-bisphosphate carboxylase/oxygenase in *Cyanophora* (Valentin and Zetsche 1990a), *Antithamnion* (Kostrzewa et al. 1990) and *Cyanidium* (Valentin and Zetsche 1990b) have been discussed in a polyphyletic model.

To contribute to this vivid discussion, suitable sequence data from as many photosynthetic eukaryotes as possible must be gathered from both plastid DNA and nuclear DNA. We suggest that the comparison of nuclear with plastidic sequence-similarity pattern will help to distinguish more clearly between monophyletic and polyphyletic origins of plastids. Such a comparison has not yet been performed. Accordingly, we decided to compare nuclear sequence-similarity patterns which have been published recently (Perasso et al. 1989; Sogin et al. 1989) with that of the *psbA* gene, coding for the D1-protein of photosystem II (herbicide-binding protein). This is a most suitable probe for such evaluations since it is localized on the plastome of all eukaryotic algae and plants so far examined, is ubiquitous for oxygen-evolving photosystems, highly conserved and comparatively long (360 amino acids), all of which is essential when deeply branched groups of protists have to be resolved.

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Materials and methods

The eukaryotic alga *Bumilleriopsis filiformis* Vischer (Xanthophyceae, Heterokontophyta; Culture Collection of the University of Göttingen, Germany, no. 809-2) was grown as described by Böger et al. (1981). Cells were disrupted, proteins removed by phenol extraction, DNA was precipitated and *pt*DNA separated from *n*DNA by CsCl equilibrium density centrifugation, according to standard methods. *pt*DNA was digested with *Eco*RI, fragments were separated on agarose gels and blotted to nitrocellulose membranes. A 4.55 kb fragment, hybridizing to a biotinylated *psbA*-containing probe from *Phalaris paradoxa*, was excised from the gel, purified by glass milk adsorption (Geneclean, Bio 101 Inc, La Jolla, Calif., USA) and ligated to *Eco*RI-cleaved pBR322. For sequencing, a 2.3 kb *Eco*RV fragment of the cloned DNA was subcloned in Bluescript (Stratagene, La Jolla, Calif., USA). A family of overlapping deletions was produced by exonuclease III. Single-stranded DNA, generated by super-infection with the helper phage M13K07 (Pharmacia, Uppsala, Sweden), was sequenced (Sequenase, United States Biochemicals protocol, Cleveland, Ohio, USA, 1990). The similarity network was calculated on the basis of a weighted (Fitch and Margoliash 1967), as well as an unweighted, distance matrix by the FITCH modus of the PHYLIP phylogenetic program package (Joseph Felsenstein, Seattle, USA). Additionally, using the PROTPARS modus of PHYLIP, several most parsimonious analyses were performed by placing different weights to the seven-amino acid gap (see Fig. 3). A different similarity pattern, with unacceptably high average standard deviations, was obtained only, when a constant rate of molecular evolution was applied in tree construction (e.g., UPGMA-programs). Individual sequences are given in the following references: *Amaranthus* (Hirschberg and McIntosh 1983), *Secale* (Boyer and Mullet 1988), *Marchantia* (Ohyama 1986), *Chlamydomonas* (Erickson et al. 1984), *Euglena* (Karabin et al. 1984), *Cyanidium* (Maid et al. 1990), *Cyanophora* (Janssen et al. 1989), *Prochlorothrix* (Morden and Golden 1989a), *Synechococcus* 7002 (Gingerich et al. 1988), *Synechococcus* 7942 (Golden et al. 1986), *Freymella* (Mulligan et al. 1984), *Anabaena* (Curtis and Haselkorn 1984).

Results and discussion

Taxonomic position of eukaryotic algae

When *pt*DNA of *B. filiformis* (Xanthophyceae, Heterokontophyta) was digested with different restriction enzymes, only one fragment hybridized with a *psb*A-containing probe (Schönfeld et al. 1987) derived from *P. paradoxa* (Gramineae, Embryophyta). This indicates that only one copy exists per chloroplast genome, which was also reported for *Cyanophora* (Janssen et al. 1989), *Cyanidium* (Maid et al. 1990), *Euglena* (Karabin et al. 1984) and plant chloroplasts (Hirschberg and McIntosh 1983; Ohyama 1986; Zurawski et al. 1990). By contrast, in cyanobacteria (Curtis and Haselkorn 1984; Mulligan et al. 1984; Golden et al. 1986; Gingerich et al. 1988) and *Prochlorothrix* (Morden and Golden 1989a) several copies have been found. The sequence of the *Bumilleriopsis psbA* gene has been determined (Fig. 1) and the derived amino acid sequence compared with other D1 sequences in order to construct an unrooted similarity network (Fig. 2). Three obvious results emerge: (1) The cyanobacteria form a distinct cluster, including *Prochlorothrix*. (2) *Chlamydomonas* forms a cluster with the Embryophyta. It seems most likely that *Euglena* also belongs to this group. (3) The eukaryotic algae

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-106  AAAATTTTAAGAAGAGATATATTTAGGTTGCCTGGTATATAATAATATATAAC
            "-35"                                  "-10"
 -53  TGGCCAAGTGCCTGGGAAGAATAAACCTCAAAATTAAAAAACAAAACAAAATT
                      S.D.
ATG ACT GCA ACT TTA GAA AGA CGC GAA AGC ATT AGC TTA TGG GAA    45
Met Thr Ala Thr Leu Glu Arg Arg Glu Ser Ile Ser Leu Trp Glu

CGT TTC TGT TCT TGG ATT ACT AGC ACT GAA AAC CGT TTA TAC ATC    90
Arg Phe Cys Ser Trp Ile Thr Ser Thr Glu Asn Arg Leu Tyr Ile

GGT TGG TTT GGT GTA CTA ATG ATC CCT ACA TTA TTA ACT GCA ACA   135
Gly Trp Phe Gly Val Leu Met Ile Pro Thr Leu Leu Thr Ala Thr

ACT TGT TAC ATT ATT GCG TTC ATC GCT GCT CCT CCA GTA GAC ATC   180
Thr Cys Tyr Ile Ile Ala Phe Ile Ala Ala Pro Pro Val Asp Ile

GAT GGT ATC CGT GAA CCA GTA GCT GGT TCA TTA CTT TAC GGA AAC   225
Asp Gly Ile Arg Glu Pro Val Ala Gly Ser Leu Leu Tyr Gly Asn

AAC ATC ATT TCT GGT GCT GTA ATT CCT TCT TCA AAC GCA ATC GGT   270
Asn Ile Ile Ser Gly Ala Val Ile Pro Ser Ser Asn Ala Ile Gly

GTA CAC TTC TAC CCT GTA TGG GAA GCT GCT TCA GTT GAT GAG TGG   315
Val His Phe Tyr Pro Val Trp Glu Ala Ala Ser Val Asp Glu Trp

TTA TAC AAC GGT GGT CCT TAC CAA TTA ATC GTA TTA CAC TTC TTA   360
Leu Tyr Asn Gly Gly Pro Tyr Gln Leu Ile Val Leu His Phe Leu

TTA GGT GTT GCT AGT TAC ATG GGT CGT GAA TGG GAA CTT AGC TAC   405
Leu Gly Val Ala Ser Tyr Met Gly Arg Glu Trp Glu Leu Ser Tyr

CGT TTA GGT ATG CGT CCT TGG ATC TTC GTA GCT TTC TCT GCT CCA   450
Arg Leu Gly Met Arg Pro Trp Ile Phe Val Ala Phe Ser Ala Pro

GTA GCA GCA GCT TCA GCT GTA TTC TTA GTA TAC CCA ATT GGT CAA   495
Val Ala Ala Ala Ser Ala Val Phe Leu Val Tyr Pro Ile Gly Gln

GGT TCA TTC TCT GAT GGT ATG CCA TTA GGT ATT TCT GGT ACT TTC   540
Gly Ser Phe Ser Asp Gly Met Pro Leu Gly Ile Ser Gly Thr Phe

AAC TTC ATG TTA GTA TTC CAA GCT GAA CAC AAC ATT TTA ATG CAC   585
Asn Phe Met Leu Val Phe Gln Ala Glu His Asn Ile Leu Met His

CCA TTC CAC ATG GCT GGT GTT GCT GGT GTA TTT GGT GGT TCA TTA   630
Pro Phe His Met Ala Gly Val Ala Gly Val Phe Gly Gly Ser Leu

TTC TCT GCT ATG CAC GGT TCT TTA GTA ACT TCA AGT TTA ATC CGT   675
Phe Ser Ala Met His Gly Ser Leu Val Thr Ser Ser Leu Ile Arg

GAA ACT AGT GAA GTA GAA TCT ACT AAC TAC GGT TAC AAA TTC GGT   720
Glu Thr Ser Glu Val Glu Ser Thr Asn Tyr Gly Tyr Lys Phe Gly

CAA GAA GAA GAA ACT TAC AAC ATC GTA GCT GCA CAC GGT TAC TTT   765
Gln Glu Glu Glu Thr Tyr Asn Ile Val Ala Ala His Gly Tyr Phe

GGT CGT TTA ATC TTC CAA TAC GCT TCA TTC AAC AAC TCT CGT GCT   810
Gly Arg Leu Ile Phe Gln Tyr Ala Ser Phe Asn Asn Ser Arg Ala

TTA CAC TTC TTC TTA GCT GCA TGG CCT GTA GTA GGT ATC TGG TTA   855
Leu His Phe Phe Leu Ala Ala Trp Pro Val Val Gly Ile Trp Leu

ACT GCT TTA GGC GTA ACG ACT ATG GCA TTC AAC TTA AAC GGT TTC   900
Thr Ala Leu Gly Val Thr Thr Met Ala Phe Asn Leu Asn Gly Phe

AAC TTC AAC CAA TCA GTA GTA GAC AGC CAA GGT CGT GTA ATC AAC   945
Asn Phe Asn Gln Ser Val Val Asp Ser Gln Gly Arg Val Ile Asn

ACT TGG GCA GAC ATC ATC AAC CGT GCT GAT TTA GGT ATG GAA GTA   990
Thr Trp Ala Asp Ile Ile Asn Arg Ala Asp Leu Gly Met Glu Val

ATG CAC GAA CGT AAC GCG CAC AAC TTC CCA TTA GAT TTA GCA GCT  1035
Met His Glu Arg Asn Ala His Asn Phe Pro Leu Asp Leu Ala Ala

GGT GAA GTT CTT CCT GTA GCT GTA TCT GCT CCA GCA GTA CAC GCG  1080
Gly Glu Val Leu Pro Val Ala Val Ser Ala Pro Ala Val His Ala

TAA  GGTTTAACAGAATCGTAATACTAAACTAGTTCTCGTTTGTTCTCCTTCTAGAAA  1037
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TAGAAGGAGAACAAAAAATAATTTCTTTTTCTGTCGATTCATTATCATTATTCTTATGT  1196
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Fig. 1. Nucleotide and deduced amino-acid sequence of the *psbA* gene from *B. filiformis*. Numbering starts at the first ATG in the open reading frame. Sequences that show homology to prokaryotic "−10" and "−35" promotor elements are *underlined*. A putative ribosome-binding site (Shine-Dalgano sequence) is indicated at position −37 to −39. The *vertical arrow* marks the start for transcription of the mRNA as determined by primer extension (data not shown). The *horizontal arrows* near the 3′ end of the transcript indicate an inverted repeat which is able to form a stem-loop structure. The cyanobacterial-like insertion of seven amino acids close to the carboxy terminus is shown in *bold type*

Cyanophora, *Bumilleriopsis* and *Cyanidium* cannot be assigned to either the plant or prokaryote cluster.

Taxonomic position of Prochlorothrix

In contrast to chlorophyll-*b*-containing cells, and similar to phycobiliprotein-containing cells, the predicted D1 amino acid sequence of *B. filiformis* consists of 360 amino acids (Fig. 1). The smaller size of the D1 protein from chlorophyll-*b*-containing cells results from a seven-amino acid gap close to the carboxy terminus of the protein (Fig. 3). This gap is found in *Euglena*, *Chlamydomonas*, plants and the prochlorobacterium *Prochlorothrix*, and has been taken as evidence for a distinct origin of green chloroplasts, being descendants of a *Prochlorothrix*-like ancestor (Morden and Golden 1989a, b). The D1 sequence, however, clearly clusters with the cyanobacterial sequences, even when the gap is weighted as seven individual mutational events (Fig. 2). This result is confirmed by confining the analysis to hydrophilic, highly variable, regions of the D1 protein (data not shown), by the number of *psbA* gene copies in prokaryotes and chloroplasts and, thirdly, by a 16S-rRNA analysis of *Prochlorothrix* (Turner et al. 1989). The topology of Fig. 2 was confirmed by the most parsimonious approach, when the gap of seven amino acids was treated as reflecting one or two evolutionary events. It appears unlikely that several mutational events produced the seven-amino acid gap, rather a single deletion may be assumed. In addition to the species available for previous studies, we were able to include three eukaryotic algae in our calculations. Although *Bumilleriopsis*, *Cyanidium* and *Cyanophora* do not have the seven-amino acid gap (Fig. 3), their D1 sequences are more similar to plants than the *Prochlorothrix* sequence. Therefore, the interpretation of *Prochlorothrix* being closely related to the ancestor of green chloroplasts needs to be reconsidered. This conclusion is in contrast to previous studies which exclusively used the disputed (Felsenstein 1983, 1988), most parsimonious, method of tree construction in which the seven-amino acid gap was weigthed as being the outcome of several independent mutations (Morden and Golden 1989a, b).

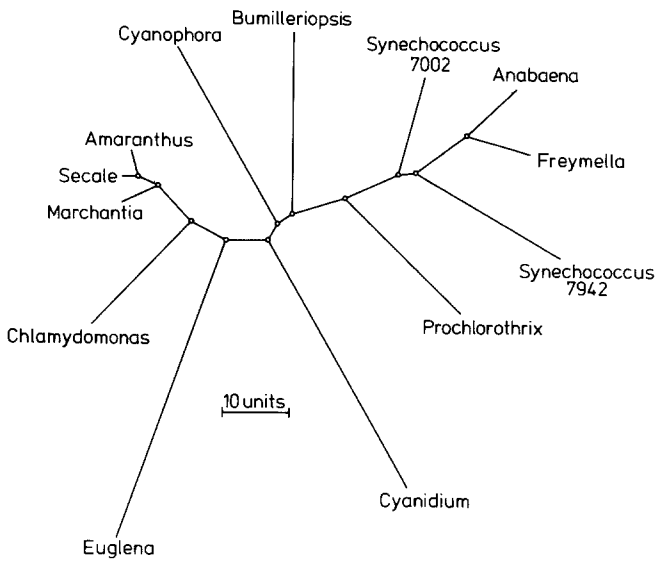

Fig. 2. Similarity pattern of D1 sequences, illustrating the relative positions of five eukaryotic alagae. The C-terminal gap of seven amino acids (see Fig. 3) was treated as if it were the result of seven independent mutations. The unrooted tree shown was the best of 255 examined (sum of squares, 0.6427; average standard deviation, 6.46%)

Multiple xenogenous origin of plastids?

Although the presence or absence of the seven-amino acid gap, together with pigment composition, could be used as an argument for at least two independent endosymbiotic events leading to either chloroplasts or phycobiliprotein-containing plastids, this evidence is by no means conclusive. The following evolutionary scenario is given to illustrate that the data available can just as well be interpreted in terms of a monophyletic origin of plastids.

It may be speculated that only one ancient endocytobiotic event happened (Cavalier-Smith 1982, 1987), involving a cyanobacteria-like photosynthetic prokaryote which could synthesize chlorophyll *a*, *b* and *c*. As was shown by Wilhelm (1988), chloroplasts of the green alga *Mantoniella* are indeed able to synthesize functional chlorophyll *a*, *b* and *c*. This finding requires a reinvestiga-

Bumilleriopsis	**L D L A** A G E V L P V A V S A P A V H A
Cyanophora	**L D L A** S G E V M P V A L T A P S I N A
Cyanidium	**L D L A** S E V S L P V A L N K V E I N G
Anabaena	**L D L A** A G E V A P V A L T A P A I N G
Prochlorothrix	**L D L A** A V K - - - - - - - A P S I I G
Euglena	**L D L A**
Chlamydomonas	**L D L A** S T N - - - - - - - S S S N N -
Marchantia	**L D L A** A V E - - - - - - - A P A V N G
Spinacia	**L D L A** A I E - - - - - - - A P S T N G

Fig. 3. Comparison of amino-acid sequences at the C-terminus of the D1 polypeptide from different genera. The deduced amino acids from residue 341 to the termination codon are shown for each sequence, and those conserved among all sequences analyzed are printed in *bold type*. The *arrow* marks the cleavage site at which nine amino acids are removed from the C-terminus of the D1 pre-protein of *Spinacia* during maturation

tion of evolutionary models in which the type of chlorophyll present has been used as a phylogenetic marker. Immediately after endocytobiosis, one line of descendants, eventually leading to green chloroplasts, lost seven (or more, see *Euglena*) amino acids as well as the ability to synthesize phycobiliproteins and chlorophyll *c*. After these speculative events, but before the rather complex process of gene transfer between the endocytobiont and the host genome started, the endocytobiont escaped from some cells, giving rise to the prochlorobacteria. The second major evolutionary line retained the ability to synthesize phycobiliproteins and the full *psbA* sequence, but lost chlorophyll *b* and *c*, leading to the chloroplasts of the Rhodophyta and *Cyanophora*. Early in evolution, a sister group of this branch lost its ability to synthesize phycobiliproteins and chlorophyll *b*, but retained chlorophyll *c* and eventually led to the Heterokontophyta (Chromophyta).

Whether or not such a scenario is accepted, this discussion demonstrates that an unequivocal interpretation of plastidic features in terms of a single or multiple xenogenous appearance of plastids is, to say the least, difficult (see also Gray 1989; Cavalier-Smith 1987; Perasso et al. 1989; Scherer et al. 1985; Reith and Cattolico 1986). As Gray (1989) stated recently, "there is at present no clear-cut molecular evidence" for a polyphyletic origin of plastids. Largely unknown mutational events must be postulated to establish an endocytobiosis. Apart from complex processes of directed gene transfer including regulatory elements, a functional endocytobiosis would require the addition of transit peptides to many hundreds of genes to be transferred to the nucleus, a chain of events which, according to Cavalier-Smith (1987), makes it rather improbable that plastids evolved more than once. Therefore, if no other compelling data were available, the assumption of a monophyletic origin of plastids implies the least difficulties.

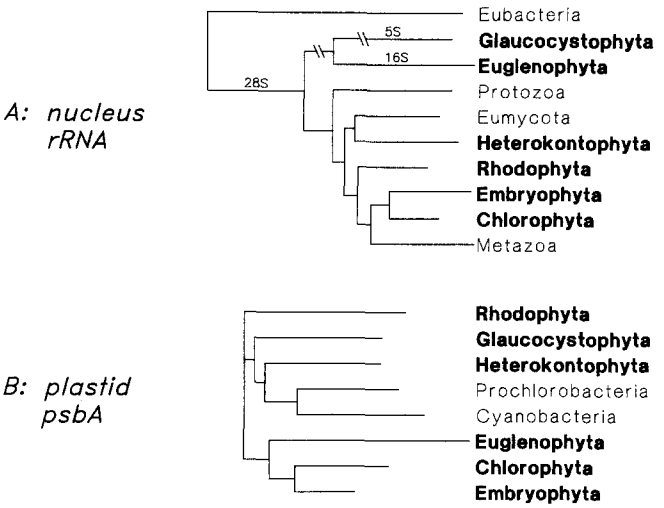

Fig. 4 A, B. Similarity pattern based on nuclear rRNA (**A**) and plastidic D1 (**B**). Photosynthetic eukaryotes are shown in *bold type*. Panel (A) was compiled based on data from Perasso et al. (1989). Sogin et al. (1989) and Maxwell et al. (1986). The photosynthetic eukaryotes investigated were *Cyanophora* (Glaucocystophyta), *Euglena* (Euglenophyta), *Ochromonas, Synura, Vacuolaria* (Heterokontophyta), *Porphyridium* (Rhodophyta), *Nicotiana, Zea, Oryza* (Embryophyta) and *Pyramimonas, Chlorogonium* (Chlorophyta). As indicated, most of the tree is based on 28S-rRNA sequences, while the branch leading to the Euglenophyta and Glaucocystophyta was adapted from 16S-rRNA and 5S-rRNA data, respectively. For better comparison, panel (B) is rearranged from Fig. 2 but still represents the unrooted tree (for details see legend of Fig. 2). The most appropriate "rooting" is indicated, using *Cyanidium* as an outgroup

Nuclear versus plastidic similarity pattern

Recently, nuclear sequence data for a variety of protists were published (Perasso et al. 1989; Sogin et al. 1989). These data, together with the availability of D1 sequences for the same protist groups, for the first time allowed a comparison of similarity patterns of nuclear DNA and plastidic DNA as presented in Fig. 4. Provided plastids were of a single xenogenous origin, with subsequent diversification, the nucleus and the plastid, present in the same cell, would share an identical evolutionary history. Therefore, the similarity pattern (not necessarily the branch lengths, because evolutionary rates apparently vary greatly, Scherer 1989, 1990) of plastidic and nuclear sequences should be congruent. If, in contrast, plastids were of multiple xenogenous origin, the evolutionary history of nuclei and plastids would have been different, which, most likely, would be reflected by a divergence of the corresponding similarity patterns of sequences.

Evidently, the tree produced from nuclear rRNA (Fig. 4A) does not match that produced from plastidic D1 sequences (Fig. 4B), except for the cluster of Chlorophyta and Embryophyta where the hypothesis of a monophyletic origin of chloroplasts is compatible with the data. Our interpretation of Fig. 4 would assume multiple xenogenous origins of plastids from Euglenophyta, Glaucocystophyta, Heterokontophyta, Rhodophyta, and Chlorophyta plus Embryophyta. This interpretation of the data relies on the following assumptions: (1) The major groups under discussion (see Fig. 4) are monophyletic. This assumption is necessary because the same species were not always available for the construction of nuclear and plastidic trees. For instance, in the construction of the nuclear tree, *Pyramimonas* and *Chlorogonium* (Chlorophyta) were used, but the D1 sequence is only available for *Chlamydomonas*; (2) The basic branching behavior of the trees is robust enough to yield similar branching patterns when different algorithms of tree construction are applied, (3) Similarities found between either nuclear or plastidic sequences are not due to convergence, i.e., unknown selection pressure did not cause different branching patterns in nuclei and plastids. While the second assumption was tested (see materials and methods), the first and the third (as is unavoidable for historical theories) remain disputable.

In summary, we propose that a comparison of both nuclear and plastidic sequence data of the same taxa is an excellent approach to decide whether plastids can be interpreted as being of multiple xenogenous origin and that the data on *psbA* genes are in favour of a polyphyletic origin of plastids. Future comparison of other plastidic

genes, together with additional information on nuclear genes, for different photosynthetic prokaryotes will help to test our conclusion.

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