Short Communication

POLYPHYLETIC ORIGINS OF CHLOROPHYTE CHLOROPLASTS

HIROSHI OYAIZU,* SATOSHI MATSUMOTO, HIROSHI INOUE,¹
AND HIROSHI TAKEDA²

Faculty of Agriculture, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

¹*Department of Biology, Faculty of Science, Toyama University, Toyama 930, Japan*

²*Department of Biology, College of General Education, Niigata University,
Niigata 950-21, Japan*

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Evolution of organisms including microbes cannot be discussed without molecular phylogenetic informations. Especially small subunit ribosomal RNA (ssu rRNA) is a good molecule for phylogenetic inference (9,26). Eukaryotes with chloroplasts appear on many branches together with organisms without chloroplasts in molecular phylogenetic trees of cytoplasmic ssu rRNA, and some of them (e.g. *Euglenophytes*) form very deep lines of descent (1,3,5,10,11,20). Therefore, if chloroplasts had a single origin, it should be postulated that the non-photosynthetic eukaryotes lost their chloroplasts in their independent evolution. However, some of unicellular algae like cryptomonads demonstrate anomalous cellular structures (nucleomorph and chloroplast with four membranes) which imply the endosymbiosis between two kinds of eukaryotes (15,17). Consequently another hypothesis that chloroplasts evolved from multiple origins is proposed to be more attractive (2). A molecular phylogenetic study about cryptomonad algae ensured that the cryptomonads were really chimeras of two phylogenetically distinct unicellular eukaryotes (3). As a consequence of the accumulated molecular phylogenetic (1,3–5,10,11,20) and electron microscopic (15,17) analyses, the polyphyletic hypothesis involving two step endosymbioses (the first between a cyanobacterium and phagotrophic eukaryote and the second between the resulting photosynthetic eukaryote and a different phagotrophic eukaryote) seems to be likely one. To assume second endosymbioses is acceptable, however, it is quite difficult to corroborate how many endosymbioses had occurred for the emergence

* Address reprint requests to: Dr. Hiroshi Oyaizu, Faculty of Agriculture, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113, Japan.

Fig. 1. Sequence alignments of chloroplast-encoded ssu rRNA for five species of *Chlorella*, *Chlamydomonas reinhardtii*, and *Zea mays*.

Only the nucleotides that differ from those of *Chlamydomonas reinhardtii* are shown, identities are denoted by periods, deletions by hyphens, and the nucleotides that could not be identified by n. The numbers on the sequences of *C. reinhardtii* denotes positions from the 5' end of *Escherichia coli* ssu rRNA. Sequences are aligned mostly according to the alignment of Neefs et al. (19). The positions (in *E. coli*) 69–99, 179–219, 841–845, 1004–1036, 1131–1141 and 1446–1456 are not aligned because those regions evolved rapidly and secondary structures are different among compared

of a special alga, especially when the alga lost its phagocytotic membranes surrounding chloroplasts.

During phylogenetic study of the chlorophytes we determined ssu rRNA sequences of chloroplasts for four strains of the genus *Chlorella* (data shown in Fig. 1). *Chlorella kessleri* 211-11h had an unusual insertion of 62 bases at a site corresponding to positions 747–748 of the *Escherichia coli* 16S rRNA (27). Although we did not examine whether the insert was an intron, it formed a clear stem and loop structure (secondary structure not shown), and did not show intron-like structure found in mitochondria and chloroplast genes (18). *Chlorella saccharophila* var. *ellipsoidea* 3.80 had an anomalous long stem and loop structure with 148 bases (secondary structure not shown) between positions 1132 and 1142 of the *E. coli* numbering system. Long stem and loop structures of this region were found in plant mitochondrial ssu RNAs (19). The stem and loop did not show intron-like structure (18).

Phylogenetic relation between the class *Chlorophyceae* and land plants was inferred by a distance matrix method by using neighbor-joining algorithm (22) (lower tree in Fig. 2) by comparison of phylogenetic trees between chloroplasts and cytoplasms. It is a good approach to discuss the origin of chloroplasts by comparison of phylogenetic trees between chloroplasts and cytoplasms. A phylogenetic tree was constructed from ssu rRNA of cytoplasm (upper tree in Fig. 2). The five *Chlorella* species were extremely diverged in both trees, and the divergence was larger than that found among land plants. According to the widely accepted hypothesis, the class *Chlorophyceae* and land plants arose from a single ancestor

organisms. Those regions were eliminated for comparison, and those sequences are enclosed with rectangles in this figure. *Chlorella kessleri* 211-11h, *Chlorella saccharophila* var. *ellipsoidea* 3.80, *Chlorella saccharophila* var. *saccharophila* 211-1d, and *Chlorella vulgaris* 211-1e were obtained from Sammlung von Algenkulturen, Pflanzenphysiologisches Institut der Universität Göttingen (Germany), and their ssu rRNA sequences were determined by the following method. The strains were cultured by the method reported by Takeda (25). Total DNAs were extracted from ca. 0.5 g wet cells by grinding with alumina and quartz sands, and purified by repeated phenol extraction. The ssu rRNA coding region of DNA was amplified with Taq DNA polymerase (Cetus, Inc., U.S.A.) by using two primers, 5' AGTTTGATCCTGGCTC 3' (which is the same sequence to the positions from 10 to 25 (*E. coli* numbering) of chloroplast ssu rRNA) and 5' AAGGAGGTGATCCAGCC 3' (which is complementary to the positions from 1541 to 1525 (*E. coli* numbering) of chloroplast ssu rRNA). The amplified DNA was purified by the agarose gel electrophoresis by using glass beads (Easytrap Kit, Takara Shuzo, Inc., Japan). The amplified and purified DNA was sequenced by Sequenase Kit (United Biochemical, Inc., U.S.A.) with ³⁵S dATP with 16 different primers (28). When double-strand DNAs were sequenced with Sequenase, the DNA polymerase reaction usually stopped at several locations of the sequence, and we used the Mn buffer (24) for avoiding such problem. The sequences of 5' end of ssu rRNA were determined by the reverse transcriptase method (14). The sequences of *Zea mays* and *Chlamydomonas reinhardtii* were cited from data bases (19).

Fig. 2. Phylogenetic trees for cytoplasmic ssu rRNA (upper tree) and for chloroplast ssu rRNA (lower tree).

The trees are drawn by neighbor-joining method (22) based on the genetic differences estimated by K_{nuc} value (13). *Euglena gracilis* has been used as a root organism for both trees. The topology of the reconstructed phylogenetic trees were evaluated by bootstrap sampling (100 replications) method (7), and the confidence limits are shown on each branch.

with chloroplasts containing chlorophyll *a* and *b* and surrounded by two membranes. If this hypothesis is true, the tree depicted by cytoplasm ssu rRNA should basically be similar to that depicted by chloroplast ssu rRNA. However, the cytoplasm ssu rRNA tree was significantly different from the chloroplast ssu rRNA tree. Bootstrap analysis (7) (100 replicates) for the cytoplasmic tree demonstrated 100% confidence for the branching between *Chlamydomonas*-*Chlorella* and land

plants, and the same analysis for the chloroplast tree demonstrated quite reliable confidence (96%) for the branching between *Chlamydomonas* and *Chlorella*-land plants. Huss and Sogin (12) reported the same topology for the cytoplasm tree, and Douglas and Turner (4) and Hendriks et al. (11) reported the same topology for a chloroplast tree among *Chlorella*, *Chlamydomonas*, and land plants based on ssu rRNA sequences.

A possible reason for the discrepancy is that the molecular evolution of the genes in cellular organelles was affected significantly by unknown factors thus leading to a dramatic distortion of the phylogenetic tree. Such kind of uncertainty of phylogenetic trees is found when trees are drawn among either very closely related organisms (21,23) or organisms of which evolutionary speeds are quite different from each other (6,26). However, those kind of indications were not found in the chloroplast phylogenetic tree. *Chlamydomonas*, *Chlorella*, and land plants are phylogenetically far distinct taxa, and evolutionary speed of the three taxa seems to be similar, as judged from the fact that all branches in the chloroplast tree (Fig. 2) had a similar length as measured from the reference root organism (*Euglena gracilis*).

The other possible illustration is that the origins of chloroplasts would be different between *Chlamydomonas* and *Chlorella* (and land plants). In this case the following two alternatives would account for chloroplast-evolution of the class *Chlorophyceae* and land plants. One is that the ancestors of the *Chlorophyceae* and land plants would be formed relatively recently (probably 400 to 600 million years ago just before the emergence of land plants (16)) by the endosymbioses of several kinds of green algae to protists or fungi. In this alternative, the invasion of green algae to the hosts occurred after the diversification of host organisms proceeded to *Chlamydomonas*, *Chlorella*, and land plants. In this scenario the ancestors of land plants and *Chlorella* would be invaded by the same or similar kind of green algae, and that of *Chlamydomonas* by the different kind of green algae. The other alternative is that the common ancestor of *Chlamydomonas*, *Chlorella* and land plants possessed the same chloroplast and later after the diversification to *Chlamydomonas* and *Chlorella* the chloroplasts of *Chlamydomonas* or those of land plants and *Chlorella* were replaced by the other algal endosymbionts which gave rise to contemporary lineages.

The above-mentioned hypotheses are somehow extravagant, and it may be reasonable to recognize the chloroplast tree as an artifact of tree construction. However, a large evolutionary gap was also found between *Chlamydomonas* and land plants in mitochondrial ssu rRNAs (8), and relatively recent endosymbiotic event by some α proteobacteria has been proposed for the evolution of plant mitochondria (8). Probably, many complex evolutionary processes took place in engendering green algae and land plants, and the presented results should be combined with results of past and future studies on this highly fascinating topic.

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