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Molecular, morphological and phylogenetic characterization of six chlorarachniophyte strains

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SUMMARY

We compare and contrast the morphological and molecular features of six chlorarachniophyte strains, and examine their evolutionary origins. Electron microscopical studies of nucleomorphs and chloroplasts, characterization of nucleomorph karyotypes, and phylogenetic analyses of small subunit ribosomal RNA (srRNA) genes derived from the nucleomorph and host cell genomes have been used to separate the six strains into three distinct groups. One group, dubbed the 'beast group', contains the strains *Chlorarachnion* sp. 242, *Chlorarachnion* sp. 621, *Chlorarachnion* sp. 1408 and *Chlorarachnion* sp. 1481. Members of the beast group have a novel flagellate form and are apparently picoplanktonic. The other two groups currently contain only one species each: *Chlorarachnion reptans* and *Lotharella* sp. 240. All chlorarachniophyte nucleomorphs examined house three small linear chromosomes each furnished with telomeres and srRNA genes.

Key words: *Chlorarachnion*, chlorarachniophyte, endosymbiosis, nucleomorph, pyrenoid, telomere, ribosomal RNA.

INTRODUCTION

Chlorarachniophytes are marine ameboflagellate unicells that harbor green algal endosymbionts within modified food vacuoles (Van de Peer *et al*. 1996). The endosymbionts are greatly reduced, having lost many subcellular structures such as mitochondria and cell walls (McFadden *et al*. 1994). A dramatic reduction of the endosymbiont's nucleus has produced a tiny nucleus-like structure called a nucleomorph that is housed within a vestige of cytoplasm. The only other significant structure remaining within each endosymbiont is a prominent green chloroplast.

The chloroplast manufactures carbohydrate and possibly other compounds for the host cell. The host cell stores carbohydrate reserves as a β-1,3 glucan within a cytoplasmic vesicle appressed to the chloroplast's bulbous pyrenoid (McFadden *et al*. 1997b). The protein content of pyrenoids implicates them in performing a central role in carbon fixation and metabolism (Yu *et al*. 1994; Suss *et al*. 1995; Delrio *et al*. 1996; Rawat *et al*. 1996; Morita *et al*. 1997) and in most chlorarachniophyte species the pyrenoid is penetrated by a finger-like projection(s) (Hibberd and Norris 1984; Ishida *et al*. 1996). These invaginations are lined by the chloroplast's double membrane envelope and contain some of the endosymbiont's cytoplasm. The morphology of the pyrenoid invaginations varies in a species-specific fashion (Ishida and Hara 1994; Ishida *et al*. 1996) and in *Chlorarachnion reptans* a single, enlarged projection houses the entire nucleomorph (Hibberd and Norris 1984).

Molecular studies of chlorarachniophyte nucleomorphs indicate their genomes are radically reduced (McFadden *et al*. 1994; Gilson and McFadden 1996). Nucleomorphs accommodate just three small linear chromosomes whose total genome size is less than 500 kb, making them among the smallest eukaryotic genomes discovered thus far (McFadden *et al*. 1994; Rensing *et al*. 1994; Gilson and McFadden 1995; Gilson *et al*. 1997; McFadden *et al*. 1997a). While the nucleomorph encodes genes that perform some genetic house-keeping and chloroplast-associated functions, it is apparent that many nucleomorph genes have either been lost or transferred to the host cell's nucleus (Gilson and McFadden 1996). With only 300 or so genes retained within the nucleomorph, it is apparent that most of the endosymbiont's needs are met by the host cell (Gilson and McFadden 1997; Gilson *et al*. 1997). Proteins synthesized by the host are probably targeted to the semi-autonomous endosymbiont via the host endomembrane system, but details are not known (Gilson *et al*. 1997).

The arrangement of genes upon the nucleomorph chromosomes of *Chlorarachnion* sp. 621, the only strain investigated to date, are particularly curious (Gilson and McFadden 1995; Gilson and McFadden 1996). Each chromosome is capped with an apparently identical 8.5 kb repeat that comprises a single ribosomal RNA (rRNA) gene cistron linked to a telomere consisting of $(TCTAGGG)_n$ motifs (Gilson and McFadden 1995). The genes nested between these termini are compactly arranged with very little spacer DNA (Gilson and McFadden 1996). Such are the reductive pressures placed

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upon this nucleomorph genome that the genome's spliceosomal-type introns are among the smallest found in any eukaryote (Gilson and McFadden 1996).

Phylogenetic analyses of nucleomorph small subunit rRNA (srRNA) genes indicate that the chlorarachniophyte endosymbiont was once a green alga (Cavalier-Smith *et al*. 1996; Van de Peer *et al*. 1996), while analyses of host srRNA and protein genes demonstrate they belong to the recently recognized Phylum Cercozoa, a collection of ameboid and flagellate heterotrophs (Bhattacharya *et al*. 1995; Cavalier-Smith 1995; Cavalier-Smith and Chao 1997; Keeling *et al*. 1998). This paper compares and contrasts srRNA nuclear and nucleomorph phylogenies of six strains of chlorarachniophyte. In addition, we describe the morphology and nucleomorph karyotypes of these strains.

MATERIALS AND METHODS

Cultures

Chlorarachniophyte strains *Chlorarachnion reptans* (CCMP 238), *Lotharella* sp. 240 (CCMP 240), *Chlorarachnion* sp. 242 (CCMP 242), *Chlorarachnion* sp. 621 (CCMP 621), *Chlorarachnion* sp. 1408 (CCMP 1408) and *Chlorarachnion* sp. 1481 (CCMP 1481) were obtained from the Culture Collection of Marine Phytoplankton, Bigelow Laboratory for Ocean Sciences and cultured in f/2 media with continuous lighting at 24°C. Motile cells of cultures *Chlorarachnion* sp. 242, *Chlorarachnion* sp. 621, *Chlorarachnion* sp. 1408 and *Chlorarachnion* sp. 1481 were grown in 1L conical flasks bubbled with filter-sterilized air. *Chlorarachnion reptans* and *Lotharella* sp. 240 predominantly form ameba and were grown in 500 mL plastic tissue culture flasks.

Electron microscopy

Ameboid chlorarachniophyte cells were collected by dislodging the cells from the bases of tissue culture flasks with a plastic scraper. Both ameboid and motile cells were concentrated by centrifugation (3000 *g*). After resuspension in 0.25 mol L^{-1} sucrose and 0.1 mol L^{-1} piperazine ethane sulfonic acid (PIPES) pH7.0, the algal cells were fixed by the addition of glutaraldehyde to a final concentration of 1% at 4°C for 1 h. After the sucrose was washed out with several washes of 0.1 mol L^{-1} PIPES buffer that contained decreasing concentrations of sucrose, cells were post-fixed in 1% OsO_4 and 0.1 mol L^{-1} PIPES at 4°C overnight. The cells were washed twice in 0.1 mol L^{-1} PIPES and embedded in 1% agarose. The agarose blocks were then dehydrated with ethanol and infiltrated with Spurr's resin (Spurr 1969). The resin was polymerized at 70°C overnight. Cell sections were stained for 20 min in saturated aqueous uranyl acetate followed by 5 min in Reynold's lead citrate (Reynold 1963). Electron microscopy was performed with a Siemens 102 (Siemens, Erlangen, Germany), a JOEL 1200ex (JOEL Co. Ltd, Tokyo, Japan) or a CM 120 Bio-Twin (Philips, The Netherlands) transmission electron microscope.

Pulsed-field gel electrophoresis and Southern analyses

Chromosomal DNA for pulsed-field gel electrophoresis was prepared as per Eschbach *et al*. (1991) with the following modifications. Pelleted cells were resuspended in buffer containing 10 mmol L^{-1} Tris-HCl pH8.0, 100 mmol L^{-1} EDTA, 200 mmol L^{-1} NaCl and 0.5% low gelling temperature agarose at 37°C. The molten cell mixture was poured into a plug mold prechilled to 4°C. When set, the agarose/cell plugs were digested in 10 mmol L^{-1} TrisHCl pH8.0, 400 mmol L^{-1} EDTA, 1% N-lauryl sarkosyl and 1 mgm L^{-1} Pronase E (P-5417, Sigma Chemical Co., St Louis, MO, USA) for 48 h at 50°C. Digested plugs were washed in 10 mmol L^{-1} TrisHCl pH8.0 and 400 mmol L^{-1} EDTA. The concentration of cells in plug preparations were 4.3×10^8 cells mL^{-1} for *Chlorarachnion* sp. 621 and 1.83×10^8 cells mL^{-1} for the other strains. Pulsed field gel electrophoresis was performed in 1% agarose gels loaded into a contour-clamped homogeneous electric field (CHEF) DRIII apparatus (BioRad) at 14°C containing 0.5 × TBE buffer. Electrophoresis conditions were 20 s pulse-time for 16 h followed by 10 s pulse-time for 16 h. Both pulse times were performed at 175 V with a 120° electrode angle.

Pulsed-field gels were capillary blotted onto Zeta-probe (BioRad, Richmond, CA, USA) under alkaline conditions. Telomere (Gilson and McFadden 1995) and srRNA probes (Gilson and McFadden 1996) were made by labeling cloned DNA fragments with [α^{32}P]-dCTP using a random primer DNA labeling kit (Megaprime Kit, Amersham, Buckinghamshire, UK). Hybridization experiments were performed at high stringency (48°C) in a buffer containing 50% formamide, 7% sodium dodecyl sulfate (SDS) and 0.25 mmol L^{-1} Na_2HPO_4 pH7.2. Membranes were washed at high stringency. After probing with the srRNA gene the blot was stripped (95°C, 0.1X standard saline citrate, 0.5% SDS, 20 min) before probing with the telomeric probe.

srRNA gene isolation and phylogenetic analyses

The nucleomorph srRNA gene from *Chlorarachnion* sp. 621 (Genbank U58510) was isolated from a genomic clone of a rRNA gene cistron (Gilson and McFadden 1995; Gilson and McFadden 1996). The host nuclear srRNA gene (Genbank AF054832) was amplified by PCR from *Chlorarachnion* sp. 621 genomic DNA with

universal srRNA gene primers. Genomic DNA isolation, PCR conditions and primer sequences have been described previously (McFadden *et al*. 1994).

From a pre-aligned database of srRNA gene sequences (http://rrna.uia.ac.be/rrna/ssuform.html Eukarya) (Van de Peer *et al*. 1998) two subsets comprising green algal/chlorarachniophyte nucleomorph and ameboid/chlorarachniophyte host cell sequences were extracted. Within each of these separate alignments common gaps were removed using the sequence editing program SeqPup (http://iubio.bio.indiana.edu/ IUBio-Software+Data/seqpup/). *Chlorarachnion* sp. 621 nucleomorph and host cell sequences were aligned by eye to their corresponding prealigned groups. Distance and maximum likelihood analyses were carried out using all positions with the Dnadist and Dnaml programs within the Phylip 3.57c package (Felsenstein 1989). Parsimony analysis was performed with PAUP 3.1.1 (Swofford 1993). For distance and parsimony analyses, bootstraps were performed with 100 subreplicates.

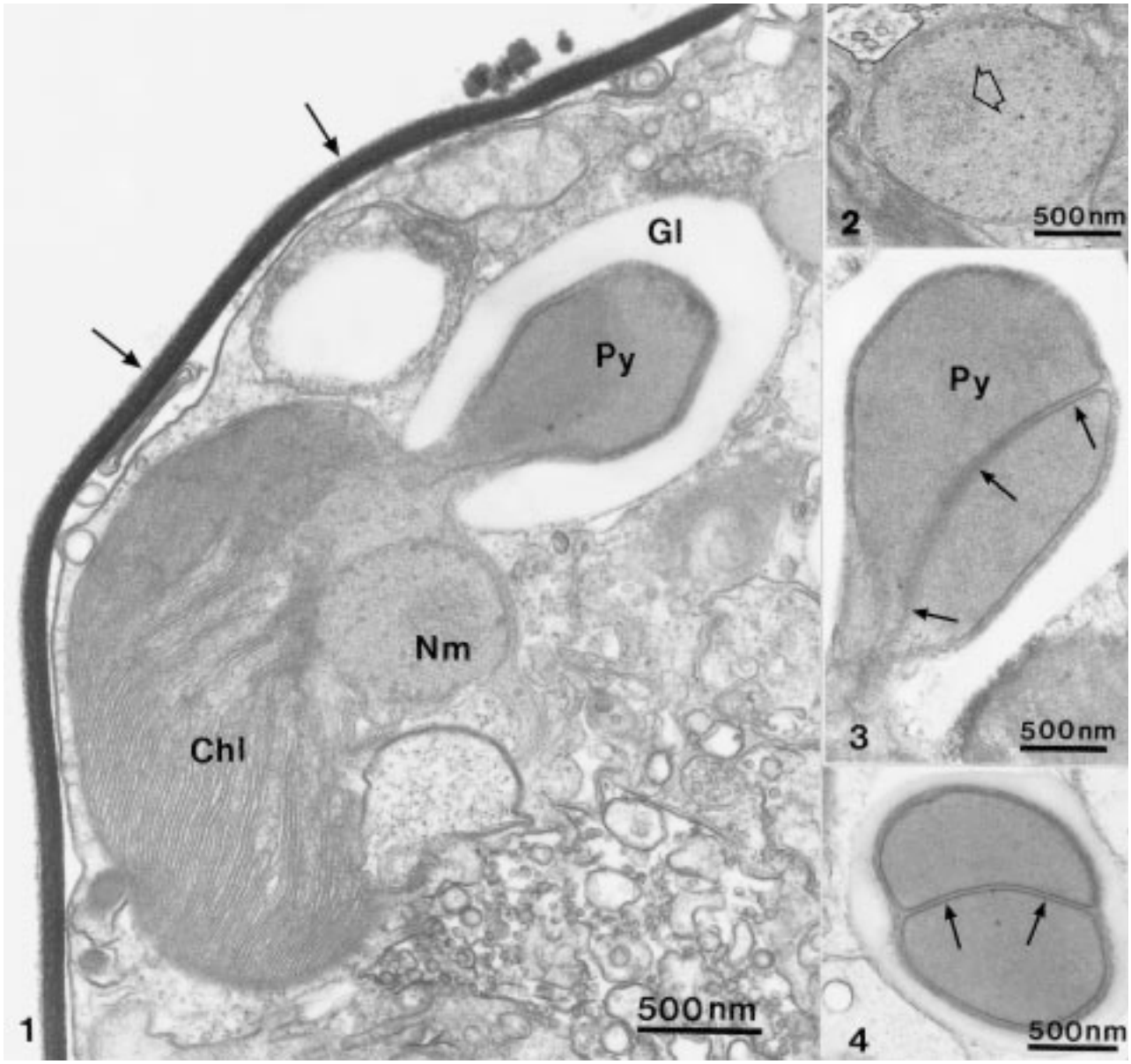


Fig. 1. Longitudinal section of immotile stage of *Lotharella* sp. 240. The nucleomorph (Nm) is situated adjacent to the pyrenoid (Py) which protrudes from the chloroplast (Chl). The lamellate spore wall (arrows) is also visible. **Fig. 2.** High magnification of nucleomorph from *Lotharella* sp. 240 showing the double membrane and nucleolus (open arrow) and electron-dense globules around the nucleomorph periphery. **Fig. 3.** Longitudinal section of a pyrenoid (Py) from *Lotharella* sp. 240 showing the longitudinal slit (arrows) invaded by the plastid membranes and endosymbiont cytoplasm. **Fig. 4.** Transverse section of pyrenoid showing the slit created by the invasion of endosymbiont cytoplasm bounded by the plastid membranes (arrows).

RESULTS AND DISCUSSION

Nucleomorph morphology

In each of the chlorarachniophyte strains examined, we observed a nucleomorph (Figs 1,2,5–7,9–11). The nucleomorphs are located between the inner and outer pairs of chloroplast envelopes and are themselves bounded by a double membrane. Within all the nucleomorphs is a matrix of granular material (Figs 1,2,5–7, 9–11). Additionally, there are small electron-dense globules (usually around the nucleomorph perimeter) and a slightly more electron-dense region (Figs 1,2, 5–7,9–11) that is equivalent to the nucleolus of a standard nucleus (McFadden *et al*. 1994). The electron dense globules have been observed in all strains but their presence is not consistent and their composition is unknown. The nucleomorphs are roughly spherical in all strains except *Chlorarachnion reptans*, in which the nucleomorph is more wedge-shaped (see below). The position of the nucleomorph has previously been reported to vary among different species and strains (Ishida and Hara 1994; McFadden *et al*. 1994; Ishida *et al*. 1996) and our observations concur with these reports. In *Lotharella* sp. 240 (CCMP 240), *Chlorarachnion* sp. 242 (CCMP 242), *Chlorarachnion* sp. 621 (CCMP 621), *Chlorarachnion* sp. 1408 (CCMP 1408) and *Chlorarachnion* sp. 1481 (CCMP 1481), we observe the nucleomorph lying adjacent to the pyrenoid stalk (e.g. Figs 1, 5), whereas in *Chlorarachnion reptans* (CCMP 238) the nucleomorph is invariably located within a cleft of the pyrenoid

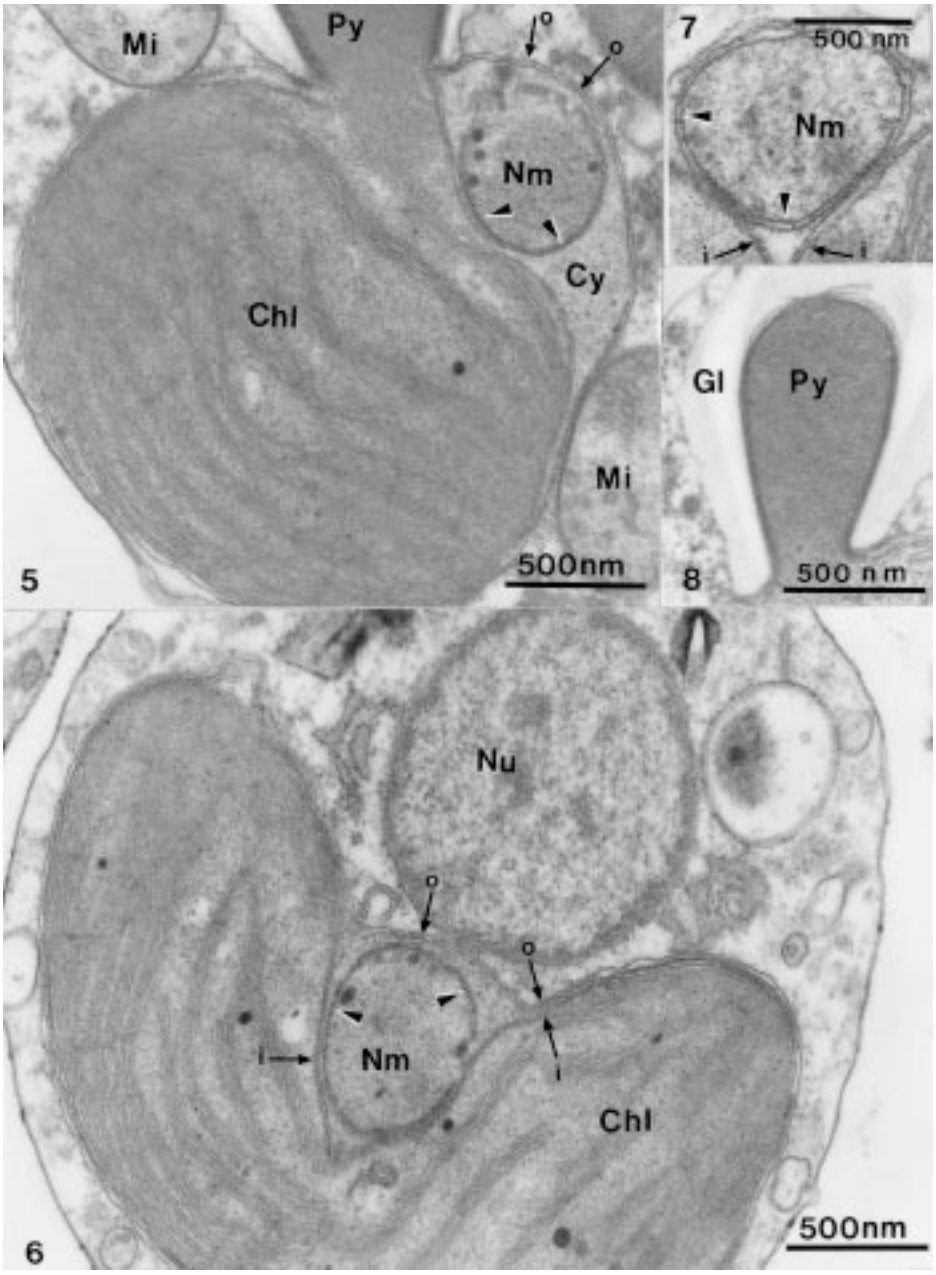


Fig. 5. Longitudinal section of flagellate of *Chlorarachnion* sp. 621. The nucleomorph (Nm), which is surrounded by two membranes (arrowheads), is situated adjacent to the pyrenoid (Py) between the inner and outer pairs (arrows with 'o') of membranes surrounding the plastid. Mitochondria (Mi) with tubular cristae are also visible in the host cytoplasm. **Fig. 6.** Longitudinal section of flagellate of *Chlorarachnion* sp. 1408. The nucleomorph (Nm) is situated between the inner (arrows with 'i') and outer pairs (arrows with 'o') of membranes surrounding the chloroplast. The nucleus of the host cell is also visible. **Fig. 7.** High magnification of nucleomorph from *Chlorarachnion* sp. 621 showing the double bounding membrane (arrowheads) and the adjacent inner membranes of the chloroplast (arrows with 'i'). **Fig. 8.** Longitudinal section of pyrenoid from *Chlorarachnion* sp. 621 showing a shallow longitudinal groove at its tip and the surrounding cap of β1–3 glucan (Gl) in a host cytoplasmic vacuole.

(Figs 9–11). As has been shown previously (Hibberd and Norris 1984), the pyrenoid of *C. reptans* is almost cleaved in two by a wedge of cytoplasm that harbors the nucleomorph whose shape closely matches the pyrenoid cavity in which it resides (Figs 10,11). The pyrenoid of *Lotharella* sp. 240 possesses a longitudinal slit reminiscent of that observed in *C. reptans* but the projection that divides the pyrenoid of *Lotharella* sp. 240 is far slimmer than *C. reptans* and does not contain a nucleomorph (Figs 3,4). Rather, the nucleomorph of *Lotharella* sp. 240 resides at the base of the pyrenoid adjacent to the longitudinal slit. No pronounced pyrenoid slit is observed in the other strains (*Chlorarachnion* sp. 242, *Chlorarachnion* sp. 621, *Chlorarachnion* sp. 1408 and *Chlorarachnion* sp. 1481), although a shallow groove is sometimes seen at the tip of the pyrenoid (Fig. 8). This groove contains endosymbiont cytoplasm and is perhaps a highly reduced or incipient version of the slit seen in

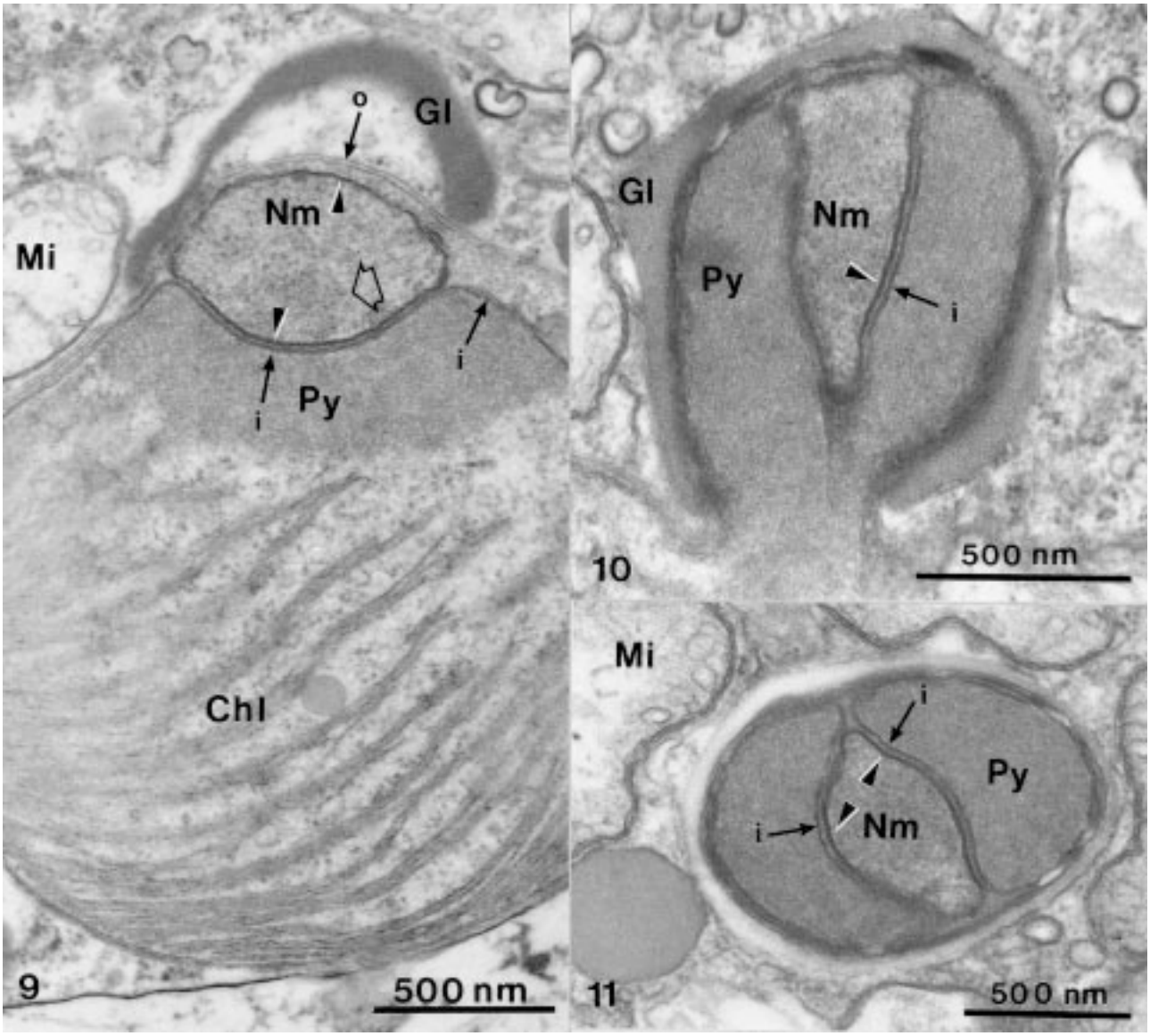
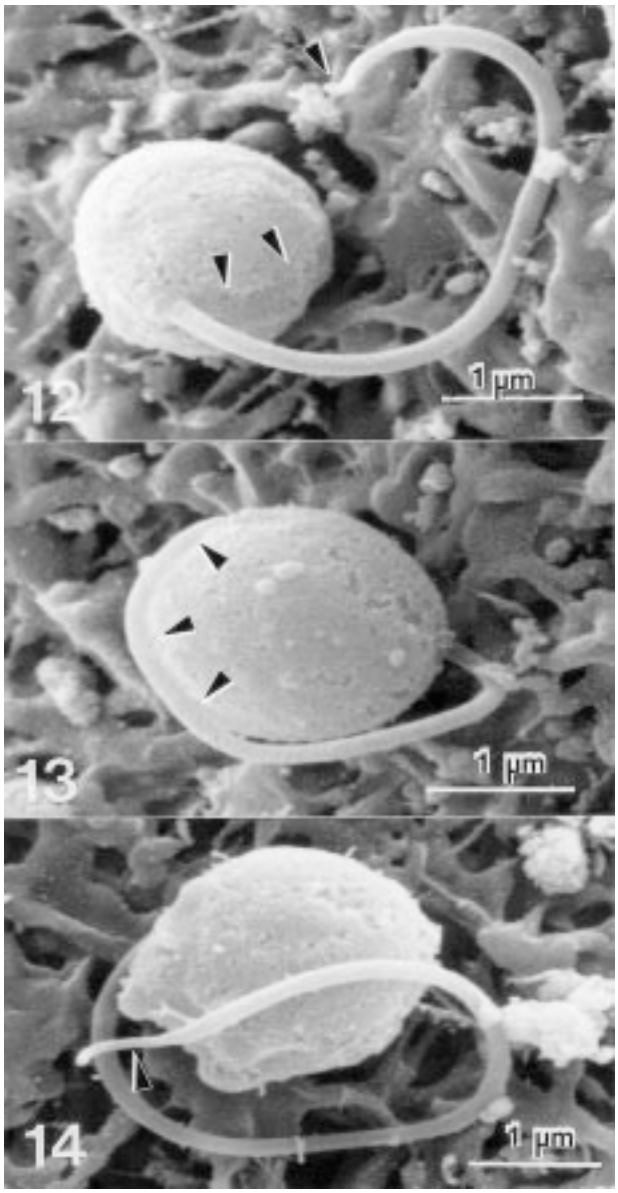


Fig. 9. Longitudinal section of ameba stage of *Chlorarachnion reptans*. The nucleomorph (Nm) is situated between the inner (arrows with 'i') and outer (arrows with 'o') pairs of membranes surrounding the chloroplast (Chl). The specialized region of the chloroplast comprising the pyrenoid (Py) is visible adjacent to the nucleomorph in this profile. Mitochondria (Mi) and cap of β1–3 glucan (Gl) in a host cytoplasmic vacuole surrounding the nucleomorph/pyrenoid complex are also visible. **Fig. 10.** Longitudinal section through *Chlorarachnion reptans* perpendicular to the section shown in Fig. 9. The bulbous pyrenoid (Py) with a wedge-shaped cleft containing the double-membrane-bound (arrowhead) nucleomorph (Nm) is visible. The β1–3 glucan (Gl) within the cytoplasmic storage vacuole that almost entirely encapsulates the pyrenoid is visible. **Fig. 11.** Transverse section through the pyrenoid (Py) of *Chlorarachnion reptans*. The nucleomorph (Nm) occupies the center of the pyrenoid which is split into two halves by the intrusion of the endosymbiont cytoplasm which is bounded by the inner pair (arrows with 'i') of chloroplast membranes. The nucleomorph has two bounding membranes (arrowheads) and is wedge shaped with two thin wings at each side.

C. reptans or *Lotharella* sp. 240. The nucleomorphs of *Chlorarachnion* sp. 242, *Chlorarachnion* sp. 621, *Chlorarachnion* sp. 1408 and *Chlorarachnion* sp. 1481 are always located at the base of the pyrenoid stalk (e.g. Figs 5,8). Exactly why the nucleomorph is located in the pyrenoid in *C. reptans* is not known. A similar phenomenon is observed in one lineage (Order Pyrenomonadales) of cryptomonads which are an unrelated group of algae that also have a nucleomorph (McFadden and Gilson 1995; Cavalier-Smith *et al*. 1996; McFadden *et al*. 1997a). It has been proposed that location of the nucleomorph within the pyrenoid may aid in segregation of daughter nucleomorphs during plastid division (McFadden 1993), but it is now clear that most nucleomorphs do not reside within the pyrenoid. Nevertheless, it is intriguing that among all the chlorarachniophytes observed thus far, there is a close spatial association between the nucleus and pyrenoid. Phylogenetic studies of cryptomonads (Cavalier-Smith *et al*. 1996) have indicated that embedment of the nucleomorph in a pyrenoid cleft is a derived feature (i.e. the ancestral cryptomonads had a free nucleomorph). We had hoped to define whether nucleomorph embedment is a primitive or derived feature in chlorarachniophytes but unfortunately the branching order in our trees is not sufficiently well resolved (Figs 16,17).



Figs 12–14. Scanning electron micrographs of *Chlorarachnion* sp. 621. The single flagellum has a short hairpoint (arrowheads at top of 12 and 14) and is recurrent around almost the entire circumference of the cell lying in a shallow groove (arrowheads in middle of 12 and 13).

Nucleomorph karyotypes

srRNA genes

To investigate the karyotypic differences between chlorarachniophyte strains, the small chromosomes of chlorarachniophyte cells were separated by pulsed-field gel electrophoresis (Fig. 15A). In *Chlorarachnion* sp. 1408 (McFadden *et al*. 1994) and *Chlorarachnion* sp. 621 (McFadden *et al*. 1994; Gilson and McFadden 1995), three of these small chromosomes of sizes 145, 140 and 95 kb, reside in the nucleomorph. It is apparent that the morphologically similar strains of *Chlorarachnion* sp. 242 and *Chlorarachnion* sp. 1481 harbor similarly sized chromosomes (Fig. 15A). To determine if all of these similarly sized chromosomes reside in the nucleomorph, we used a nucleomorph-specific srRNA gene probe from *Chlorarachnion* sp. 621 in Southern blot analysis. Hybridization was performed at very high stringency to limit binding to nuclear chromosomes (solid arrow, Fig. 15A) and the results clearly demonstrate that in *Chlorarachnion* sp. 242 and *Chlorarachnion* sp. 1481 the chromosomes of sizes 145, 140 and 95 kb are all derived from the nucleomorph (Fig. 15B). We have therefore named all these chromosomes I, II and III, respectively (Fig. 15A). Since the *Chlorarachnion* sp. 621 lane (Fig. 15A) contains four times as much DNA as the other lanes, chromosome migration is retarded and they appear slightly larger in size.

Chlorarachnion reptans and *Lotharella* sp. 240 contain small chromosomes of markedly different sizes to the other chlorarachniophyte strains (Fig. 15A). As shown previously (Rensing *et al*. 1994), *C. reptans* possesses three small chromosomes that accommodate srRNA genes and possibly reside in the nucleomorph. Since transcripts of the *Chlorarachnion* sp. 621 nucleomorph srRNA gene have been shown to accumulate exclusively within the endosymbiont (McFadden *et al*. 1994) and a probe derived from this gene binds to the same three small *C. reptans* chromosomes at high stringency (Fig. 15B), the earlier supposition that these chromosomes are of nucleomorph provenance seems justified. Accordingly, these chromosomes are labeled as I, II and III (Fig. 15A) and their sizes are shown in

Fig. 17. Southern analysis also confirms that *Lotharella* sp. 240 nucleomorphs also contain three nucleomorph srRNA gene-bearing chromosomes (Figs 15A,B,17B). The nucleomorph chromosomes of *C. reptans* and *Lotharella* sp. 240 do not appear to bind the *Chlorarachnion* sp. 621 nucleomorph probe with the same affinity as the other chlorarachniophyte strains (Fig. 15B). This result can be rationalized by the fact the *Chlorarachnion* sp. 621 gene exhibits less sequence identity to the srRNA genes from *C. reptans* and *Lotharella* sp. 240 than to the other strains (see 'Phylogenetics').

It is interesting that both chromosomes III of *Chlorarachnion* sp. 621 and *C. reptans* encode a *hsp*70 gene (Rensing *et al*. 1994; Gilson and McFadden 1997a) and this raises the possibility that all nucleomorph chromosomes labeled with the same number will possess a similar complement of genes and will be homologous across much of their lengths. It should be noted, however, that in the nucleomorphs of cryptomonad species the *hsp*70 gene occurs on different sized nucleomorph chromosomes (Rensing *et al*. 1994) and it is not yet possible to identify equivalent nucleomorph chromosomes in chlorachniophytes until we have a better understanding of gene complement and synteny between the chromosomes. Such studies will also reveal what genetic factors contribute to the length variation between nucleomorph chromosomes (and genome sizes; Fig. 17) from different species.

Curiously, the nucleomorph srRNA gene probe also bound to a compressed band of large yeast chromosomes (solid arrow, Figs 15A,B) but not to the chlorarachniophyte nuclear chromosomes. We can only assume that there might be longer regions of sequence identity between yeast and the probe than between the probe and the nuclear genes or that yeast has many more copies of its srRNA genes.

Telomeres

While Southern analysis using an srRNA gene probe has identified at least three nucleomorph chromosomes in all the strains examined so far, it is possible that other nucleomorph chromosomes that do not encode srRNA genes await discovery. In seeking to elucidate the complete nucleomorph karyotype of *Chlorarachnion* sp. 621, we cloned the telomere of nucleomorph chromosome III and used it as nucleomorph-specific probe (Gilson and McFadden 1995). This approach was based on the tacit assumption that all chromosomes within a particular nucleus should carry identical telomeric

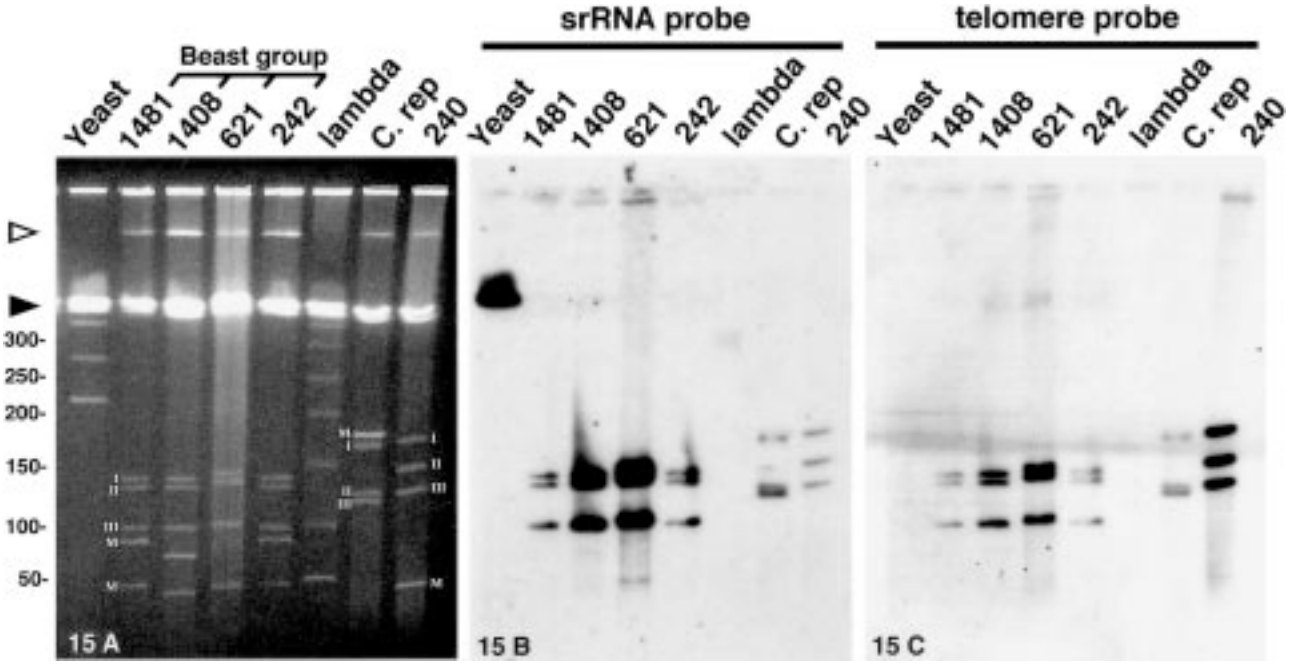


Fig. 15. Southern analyses of chlorarachniophyte nucleomorph chromosomes. (a) Pulsed-field agarose gel of chlorarachniophyte chromosomes stained with ethidium bromide. (b) Autoradiograph of a Southern blot of (a) probed with the srRNA gene from the nucleomorph of *Chlorarachnion* sp. 621. (c) Autoradiograph of a Southern blot of (a) probed with a telomere from a nucleomorph chromosome of *Chlorarachnion* sp. 621. The names of the species analysed are indicated above each lane and are abbreviated as follows: Yeast, *Saccharomyces cerevisiae*; 1481, *Chlorarachnion* sp. 1481; 1408, *Chlorarachnion* sp. 1408; 621, *Chlorarachnion* sp. 621; 242, *Chlorarachnion* sp. 242; lambda, Lambda bacteriophage; C. rep, *Chlorarachion reptans* and 240, *Lotharella* sp. 240. The sizes in kb of Lambda bacteriophage chromosomes are shown on the left of (a). The compressed nuclear chromosomes are shown by a solid arrow while the putative chloroplast chromosome is shown by a hollow arrow. Nucleomorph chromosomes I, II and III are abbreviated as I, II and III, while the presumed mitochondrial chromosomes are shown as M.

motifs at their termini. Indeed, Southern analysis with telomeric probes allowed us to confirm that the three chromosomes encoding nucleomorph srRNA genes were the only chromosomes residing within the nucleomorph of *Chlorarachnion* sp. 621 (Gilson and McFadden 1995). Moreover, host cell-nuclear chromosomes were found to carry different telomeric repeats $(TTAGGG)_n$ allowing us to discriminate between chromosomes from host and endosymbiont nuclei (Gilson and McFadden 1995). Southern analyses with the *Chlorarachnion* sp. 621 nucleomorph telomere probe labeled the same three chromosomes that encode nucleomorph srRNA in all the strains examined here (Fig. 15C). No other chromosomes labeled to any significant degree. Interestingly, *Lotharella* sp. 240 bound the telomeric probe with great affinity compared to the srRNA gene probe. Since approximately equivalent quantities of nucleomorph DNA are present in the various lanes (Fig. 15A), we conclude that nucleomorph chromosomes of *Lotharella* sp. 240 probably carry much larger telomeres than the other strains thereby presenting a greater target for the telomere probe. We previously showed that nucleomorph chromosomes of *Chlorarachnion* sp. 621 are furnished with between 25 and 45 copies of the telomere repeat motif (Gilson and McFadden 1995) and it will be interesting to obtain comparative data from *Lotharella* sp. 240 and determine if variability in telomere size contributes to differences in nucleomorph chromosome size.

Thus far, we have demonstrated that all nucleomorph chromosomes examined here carry both telomeres and srRNA genes. As mentioned above, the nucleomorph chromosomes of *Chlorarachnion* sp. 621 are capped with terminal inverted repeats comprising a telomere linked to a single rRNA gene cistron (Gilson and McFadden 1995). It will be of great interest to discover if the ends of the nucleomorph chromosomes of the other chlorarachniophyte stains are arranged in a similar fashion.

Other chromosomes

Chlorarachnion sp. 621 host cells possess mitochondria that harbor a 36 kb linear chromosome which also exists as a 72 kb dimeric form (Gilson *et al*. 1995). These chromosomes, stained with ethidium bromide, often fluoresce more brightly under ultraviolet light than nucleomorph chromosomes due to their higher copy number (Gilson *et al*. 1995). Ethidium bromide staining of pulsed-field gels of chlorarachniophyte cells reveals that *Chlorarachnion* sp. 1481 and *Chlorarachnion* sp. 242 also possess similar sized, brightly staining chromosomes (labeled as M, Fig. 15A). *Chlorarachnion* sp. 1408 appears to carry slightly smaller mitochondrial chromosomes (the smallest two chromosomes, Fig. 15A), while in *Lotharella* sp. 240 a similarly sized molecule probably also represents the mitochondrial genome (M, Fig. 15A). In *C. reptans*, no such small band is evident but a brightly staining band of 180 kb is observed (M, Fig. 15A). Probing of *C. reptans* chromosomal DNA with a cytochrome oxidase subunit 1 gene (*cox*I) has identified this chromosome as the mitochondrial genome (data not shown) indicating that the mitochondrial genome size is highly variable in chlorarachniophytes.

In addition to the nucleomorph, nuclear and mitochondrial chromosomes identified above, we observe brightly staining bands (hollow arrow, Fig. 15A) between the wells and the compressed band of nuclear chromosomes (solid arrow, Fig. 15A). The origin of these molecules is unknown but they may be circular chloroplast chromosomes that migrate slowly through pulsed field gels compared to similarly sized linear molecules (Oldenburg and Bendich 1996). Further verification that these molecules might be of chloroplast provenance is provided by the absence of such bands in the yeast and lambda bacteriophage lanes. With the exception of gene sequences for *tuf*A (Ishida *et al*. 1997), rRNA and *rbc*L (McFadden *et al*. 1995) determined from PCR products, nothing is known about chloroplast DNA in chlorarachniophytes.

Phylogenetics

Relationships between chlorarachniophyte endosymbionts

To clarify the evolutionary relationship between the various chlorarachniophyte strains we conducted phylogenetic analyses of the nucleomorph srRNA gene sequences. We chose 20 green algal species representing members of the Chlorophyceae, Prasinophyceae, Ulvophyceae and Charophyceae to serve as an outgroup to the nucleomorphs. All chlorarachniophytes are united as a monophyletic lineage strongly suggesting that they arose from a single secondary endosymbiotic event. They are divided into three lineages: *Chlorarachnion reptans* (represented by strains isolated from Mexico and Tunisia), *Lotharella* sp. 240, and a group including *Chlorarachnion* sp. 621,*Chlorarachnion* sp. 242 and *Chlorarachnion* sp. 1408. All analyses (distance, parsimony and maximum likelihood) unite *Chlorarachnion* sp. 621, *Chlorarachnion* sp. 242 and *Chlorarachnion* sp. 1408 as a robust, monophyletic clade with 100% bootstrap replicate support (Fig. 16). Unfortunately, we are unable to place *Chlorarachnion* sp. 1481 on the phylogenetic tree because no srRNA gene sequence is available.

It is not apparent from our trees whether *C. reptans* is more closely related to the *Chlorarachnion* sp. 621,*Chlorarachnion* sp. 1481 and *Chlorarachnion* sp. 1408 group or to *Lotharella* sp. 240 (Fig. 16). Distance and parsimony bootstrap analyses weakly support the latter grouping but maximum likelihood supports the former (data not shown) and we have represented the phylogenetic tree as an unresolved trichotomy (Fig. 17).

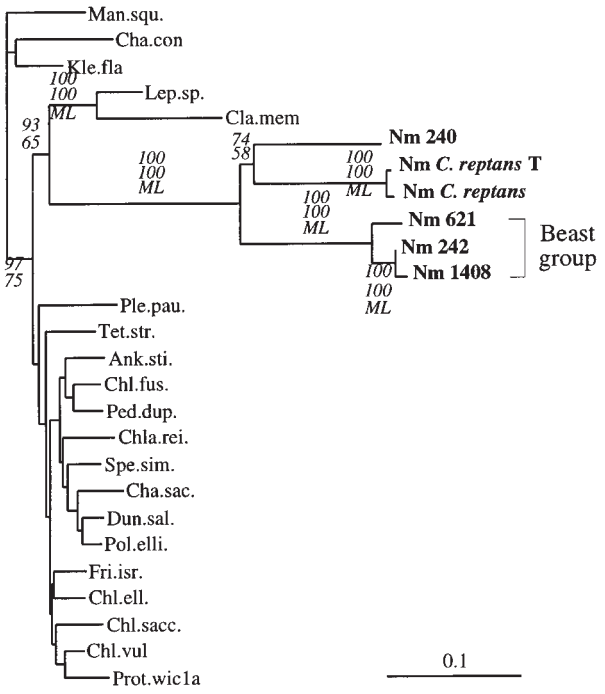


Fig. 16. Distance tree of green algal and nucleomorph srRNA gene sequences. The bootstrap values of important nodes are shown with the uppermost value being for distance analysis and the lowest value for parsimony analyses. Those nodes that are supported by maximum likelihood analysis are shown as 'ML'. Species abbreviations, full names, Genbank accession numbers and green algal family are as follows: Cha.con, *Chara connivens* U18493 (Charophyta/Embryophyta); Kle.fla, *Klebsormidium flaccidum* X75520 (Charophyta/Embryophyta); Man.squ, *Mantoniella squamata* X73999 (Prasinophyceae); Lep.sp., *Leptosira* species U18510 (Chlorophyceae); Cla.mem, *Cladophoropsis membranacea* Z35322 (Ulvophyceae); Fri.isr, *Friedmannia israeliensis* M62995 (Microthamniales); Ple.pau, *Pleurastrum paucicellulare* Z47997 (Chlorophyceae); Chl.fuc, *Chlorella fusca* X74002 (Chlorophyceae); Ank.sti, *Ankistrodesmus stipitatus* X56100 (Chlorophyceae); Spe.sim, *Spermatozopsis similis* X65557 (Chlorophyceae); Dun.sal, *Dunaliella salina* M84320 (Chlorophyceae); Ped.dup *Pediastrum duplex* M62997 (Chlorophyceae); Pol.elli, *Polytoma ellipticum* U22933 (Chlorophyceae); Cha.sac, *Characium saccatum* M84319 (Chlorophyceae); Chla.rei, *Chlamydomonas reinhardtii* M32703 (Chlorophyceae); Chl.vul *Chlorella vulgaris* X13688 (Chlorophyceae); Prot.wic1a, *Prototheca wickerhamii* X56099 (Chlorophyceae); Chl.sacc, *Chlorella saccharophila* X63505 (Chlorophyceae); Chl.ell and *Chlorella ellipsoidea* X63520 (Chlorophyceae). The chlorarachniophyte nucleomorph sequences used in this analysis were: Nm *C. reptans*, *Chlorarachnion reptans* U03275; Nm *C. reptans* T, *Chlorarachnion reptans* (Tunisia) X70808; Nm 621, *Chlorarachnion* sp. 621 (*Pedinomonmas minutissima*) U58510; Nm 242, *Chlorarachnion* sp. 242 U03478; Nm 1408, *Chlorarachnion* sp. 1408 U02040; Nm 240, *Lotharella* sp. 240 AF54889.

What was the chlorarachniophyte endosymbiont?

Comparisons of the trees generated by the different phylogenetic algorithms proved inconclusive as to which green algae the chlorarachniophyte endosymbionts are most closely related to. Both distance and parsimony bootstrap analyses offered moderate support (93% and 65%, respectively) for a relationship between nucleomorphs and a group containing a *Leptosira* sp. (Chlorophyceae) and *Cladophoropsis membranacea* (Ulvophyceae) (Fig. 16). This association was not supported by maximum likelihood analysis that instead grouped the nucleomorphs with *Pleurastrum paucicellulare* (Pleurastophyceae) (data not shown). The long branch length separating the nucleomorphs from the other species (Fig. 16) indicates that nucleomorph gene sequences are highly divergent and are probably evolving rapidly. This feature has confounded phylogenetic analysis of nucleomorphs in the past (Cavalier-Smith *et al*. 1994) but has, to a degree, recently been overcome with better phylogenetic algorithms (Cavalier-Smith *et al*. 1996; Van de Peer *et al*. 1996). Overall, however, our trees do not indicate with any certainty which green algal species was a close relative of the chlorarachniophyte endosymbiont except that it was probably not a prasinophyte or a charophyte. Recent phylogenetic analysis of the translation elongation factor Tu suggests a close relationship between the chlorarachniophyte endosymbiont and the Ulvophyceae (Ishida *et al*. 1997). As unicellular ulvophytes are known and as it is these cell forms that were likely to have been captured by the chlorarachniophyte host, the ulvophyte/chlorarachniophyte endosymbiont link deserves further attention.

Relationships between chlorarachniophyte host cells

Because analyses of nucleomorph srRNA genes did not resolve all the phylogenetic relationships between the chlorarachniophyte species examined here, we also analyzed the nuclear srRNA gene sequences of the host cells. The nuclear srRNA gene from *Chlorarachnion* sp. 621 was aligned to a selection of prealigned nuclear srRNA genes sequences (Van de Peer *et al*. 1998) comprising chlorarachniophyte host cells and the filose ameba *Paulinella chromatophora* and *Euglypha rotunda* (Bhattacharya *et al*. 1995). Distance and parsimony analyses supported a relationship between the *C. reptans* strains and the group comprising *Chlorarachnion* sp. 621, *Chlorarachnion* sp. 242 and *Chlorarachion* sp. 1408 to the exclusion of *Lotharella* sp. 240 (Fig. 18). Maximum likelihood analysis also substantiated this relationship. Nuclear

srRNA genes may prove more useful in resolving the phylogenetic relationships among the chlorarachniophytes than their nucleomorph homologes because they are much less divergent.

Morphology and evolution

The beast group

In addition to the phylogenetic evidence that supports the grouping of *Chlorarachnion* sp. 621,*Chlorarachnion* sp. 242 and *Chlorarachnion* sp. 1408, three other lines of evidence are congruent with this association: (i) morphological similarity of their nucleomorph/pyrenoid complexes; (ii) morphological similarity of their flagellate form; and (iii) their similar nucleomorph and mitochondrial karyotypes. Unfortunately, no rRNA sequence data is available for *Chlorarachnion* sp. 1481 but as it shares a semblance of both karyotype and general cell morphology (as viewed under the light microscope) to *Chlorarachnion* sp. 621, *Chlorarachnion* sp. 242 and *Chlorarachnion* sp. 1408, it almost certainly belongs to this lineage. We are temporarily assigning these strains to *Chlorarachnion* but it is already clear from these preliminary data that they do not fit within the generic description for *Chlorarachnion* (Ishida 1994; Ishida and Hara 1994; Ishida *et al*. 1996) and will eventually require a new genus. Meanwhile, we will refer to these organisms as the 'beast' group of species after the clone synonym 'beast' that Bob Guillard used for *Chlorarachnion* sp. 242 in the Provasoali-Guillard National Center for Culture of Marine Phytoplankton (http://ccmp.bigelow.org/index.html).

Host cell characteristics and distribution of the beast group

One strain in the new Beast lineage, *Chlorarachnion* sp. 621 has only ever been observed by us as small flagellates (Figs 12–14) and never as amebae. *Chlorarachnion* sp. 242, *Chlorarachnion* sp. 1408 and *Chlorarachnion* sp. 1481 switch between solitary amebae and small flagellates that we are able to maintain in this motile form for many months by frequent subculturing. *Lotharella* sp. 240 and *Chlorarachnion reptans,* in contrast, exist primarily as amebae and flagellate forms occur infrequently in culture (Hibberd and Norris 1984; Ishida *et al*. 1996).

It is interesting that all the small flagellate strains (beast group) were collected from the open ocean (North Atlantic and Sargasso Sea; see Provasoali-Guillard National Center for Culture of Marine Phytoplankton) tempting us to speculate that they may comprise a lineage of chlorarachniophytes that can be planktonic. Several other strains in the Provasoali-Guillard National Center for Culture of Marine Phytoplankton, which were also collected from the open ocean (CCMP 1242, CCMP 1258, CCMP 1259) probably also belong to this group on the basis of our light microscopic observations. Until now, all chlorarachniophytes have been reported to be benthic, often living in the ameboid form attached to the substrate or among sandgrains (Geitler 1930; Norris 1967; Hibberd and Norris 1984; Hibberd 1990; Ishida 1994; Ishida and Hara 1994; Ishida *et al*. 1996) and the possibility that the beast group represents a planktonic lineage has important repercussions.

The flagellates of beast cells are around 2 μm in diameter and they could be classified as picoplankton. These putatively planktonic chlorarachniophytes could prove to be relatively abundant. It seems that in the past they have been misidentified as very small green algal flagellates. For instance, *Chlorarachnion* sp. 621 was originally identified as *Pedinomonas minutissima* Skuja, a similar-sized uniflagellate green alga (Ettl and Manton 1964; Ettl 1972; Pickett-Heaps and Ott 1974), but early molecular evidence revealed it to be a chlorarachniophyte. Phylogenetic studies of ribosomal RNA by Kantz *et al*. (1990) were initially perplexing, as

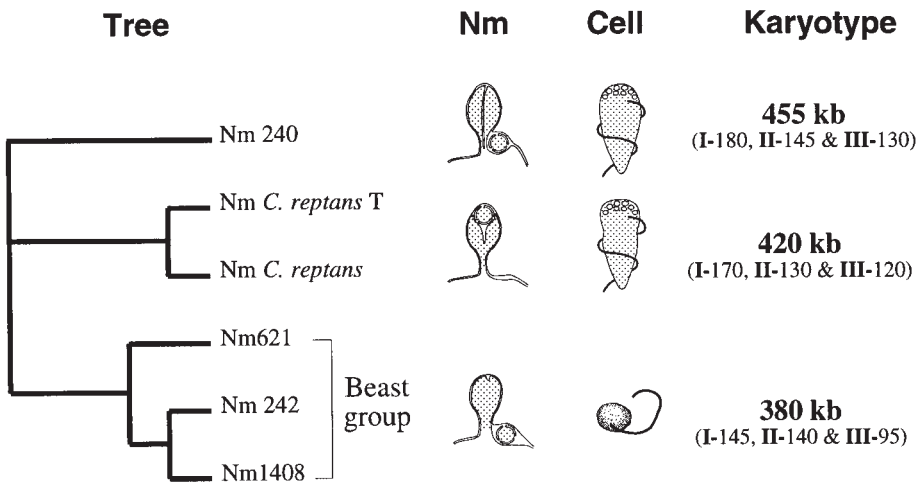


Fig. 17. A partially resolved tree of chlorarachniophyte nucleomorph srRNA gene sequences on which have been superimposed morphological and karyotypic characters.

their sequence from what they thought was *P. minutissima* (CCMP VA3= CCMP 621) was positioned anomalously at the base of their green algal trees and not with the other *Pedinomonas* species. Recently, Daugbjerg *et al*. (1995) discovered that the alga studied by Kantz *et al*. (1990) was not a green alga but a chlorarachniophyte, and our morphological and molecular data confirm this misidentification. The nuclear srRNA sequence for *Chlorarachnion* sp. 621 reported here is almost identical to the partial, reverse transcriptase sequences fragments reported by Kantz *et al*. (1990).

Relationships of the beast group to the other chlorarachniophyte strains

Unfortunately, the morphology of the nucleomorph/pyrenoid complex is as inconclusive as the endosymbionts' srRNA gene data in establishing the evolutionary relationship between the beast group and *Lotharella* sp. 240 and *C. reptans.* All three species possess cytoplasmic invaginations of the pyrenoid but their nucleomorphs occupy different positions within the endosymbiont (Fig. 17). Perhaps the only feature that unites the *C. reptans* and *Lotharella* sp. 240 group and excludes the beast group is the gross morphology of their motile cell stages. Both *C. reptans* and *Lotharella* sp. 240 produce fusiform cells with small refractile bodies at their apices and a single flagellum wrapping around the cell (Hibberd and Norris 1984; Ishida 1994). The beast group, however, produces small, spherical motile cells (Figs 12–14) that do not contain any obvious refractile bodies (data not shown). Unfortunately, as the ancestral condition is unknown, the morphology of the flagellates is not a useful character in resolving relationships between the three lineages at present.

Other chlorarachniophyte species formally described to date include *Gymnochlora stellata* and *Lotharella globosa* (Ishida *et al*. 1996) and *Cryptochlora perforans* (Calderon-Saenz and Schnetter 1987, 1989). Without electron microscopical or molecular evidence it is not clear that *Cryptochlora perforans* is really a chlorarachniophyte, but *Gymnochlora stellata* and *Lotharella globosa* undoubtedly are, and when srRNA sequences become available for the nucleomorphs of these species it will be interesting to see where they branch on the nucleomorph tree and if they can further clarify the relationships between the strains examined here.

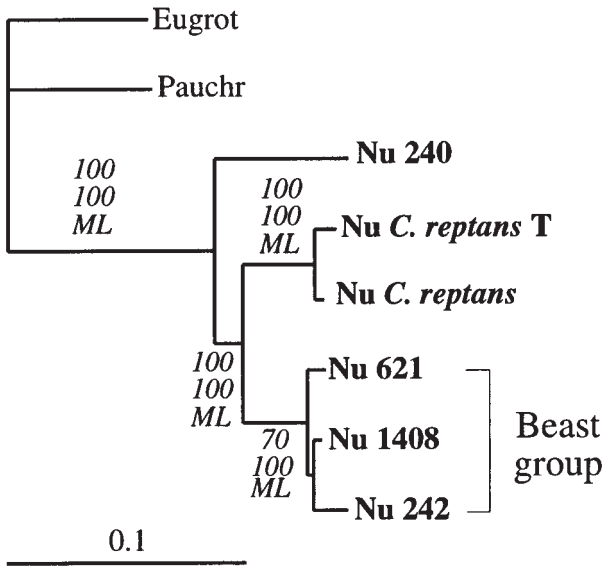


Fig. 18. Distance phylogenetic tree constructed from the srRNA gene sequences of the host cell and filose ameba. The boostrap values for particular nodes are shown as described in Fig. 16. The abbreviations of the species analyzed and their accession numbers are listed as follows: Pauchr, *Paulinella chromatophora* X81811; Eugrot, *Euglypha rotunda* X77692; Nu *C. reptans*, *Chlorarachnion reptans* U03275; Nu *C. reptans* T, *Chlorarachnion reptans* (Tunisia) X70809; Nu 621, *Chlorarachnion* sp. 621 (*Pedinomonas minutissima*) AF054832; Nu 242, *Chlorarachnion* sp. 242 U03479; Nu *Chlorarachnion* sp. 1408 U02075; Nu 240, *Lotharella* sp. 240 AF054890.

CONCLUSIONS

We have shown that a group of chlorarachniophyte strains comprising *Chlorarachnion* sp. 621, *Chlorarachnion* sp. 242, *Chlorarachnion* sp. 1408 and *Chlorarachnion* sp. 1481 form a closely related lineage distinct from *C. reptans* and *Lotharella* sp. 240. Members of this so-called beast group await formal description but are characterized by containing nucleomorphs that are basally located next to the bulbous pyrenoid. The nucleomorph and mitochondrial karyotypes of all members of the beast group are very similar and phylogenetic analyses of their srRNA genes further supports their close relationship. Additionally, these strains frequently exist as minute round flagellates that are markedly different to the flagellate forms produced by *C. reptans* and *Lotharella* sp. 240. The nearest relative of the beast group appears to be *C. reptans* as indicated by phylogenetics of the host cell's nuclear srRNA gene, but more conclusive analyses are desirable. Interestingly, all of the nucleomorphs of the chlorarachniophyte strains examined possess three linear chromosomes. Each chromosome encodes srRNA genes and is apparently capped with identical telomeric motifs. We, therefore, propose that all nucleomorph chromosomes may be capped with inverted repeats containing telomeres and srRNA genes. The relative similarity in karyotypes and nucleomorph genome size among the chlorarachniophytes suggest that the post-endosymbiotic reductive process that converted a full eukaryotic nucleus into a nucleomorph occurred prior to the diversification leading to the known extant strains.

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