The Plant Cell, Vol. 7, 1899–1911, November 1995

Fate of Parasite and Host Organelle DNA during Cellular Transformation of Red Algae by Their Parasites

Lynda J. Goff[a,1] and Annette W. Coleman[b]

[a] Department of Biology, University of California, Santa Cruz, California 95064

[b] Department of Molecular and Cellular Biology, Brown University, Providence, Rhode Island 02912

The transfer of a nucleus into a cytoplasm of a genetically foreign cell and its subsequent multiplication in the cytoplasm of this cell characterize most parasitic red algal species and their interactions with specific red algal hosts. Nuclei enter the host's cytoplasm upon cell fusion of parasite and host cell; here, they replicate, are spread to contiguous host cells, and ultimately are packaged into spores that reinfect other host thalli. In this study, we examined whether the proplastids and mitochondria that occur in these red algal adelphoparasites are acquired from their host or whether they are unique to the parasite and are brought into the host along with the parasite nucleus. To establish their origins and fates, plastid and mitochondrial restriction fragment length polymorphisms (RFLPs) of parasite cells were compared with those of their host plastid and mitochondrial DNA in three host and parasite pairs. For plastids, no RFLP differences were found between hosts and parasites, supporting an earlier conclusion, based on microscopic studies, that the proplastids of parasites are acquired from their hosts. For mitochondria, characteristic RFLP differences were detected between host and parasite for two of the pairs of species but not for the third. Evidence of the evolutionary difference between hosts and their parasites was shown by RFLP differences between nuclear ribosomal repeat regions.

INTRODUCTION

A high proportion of parasitic genera characterizes the red algae. More than 15% of all known red algal genera occur only as obligate parasites of other red algae (Goff, 1982). They are referred to as parasitic because they are small and morphologically reduced, highly host specific, and colorless; as a consequence, they are dependent on their host as a source of photosynthates (Setchell, 1918; Callow et al., 1979; Goff, 1979, 1982; Kremer 1983). Approximately 80% of all red algal parasites are adelphoparasites; these parasites occur in association with closely related red algal hosts (same tribe or family). The remaining taxa, the alloparasites, are found in association with taxonomically unrelated red algal hosts.

While investigating how cells of parasitic red algae interact with cells of their specific red algal hosts, we and others (Peyrière, 1977; Goff and Coleman, 1984, 1985; Wetherbee et al., 1984) have observed that upon contact of a parasite cell with a host cell, the parasite cell cuts off a small, nucleated cell termed a conjunctor cell. This cell fuses with the adjacent host cell. Fusion of the conjunctor cells with the host cell then leaves the parasite cell connected to the host cell by the glycoprotein pit plug (Figure 1).

Following this developmental process by using quantitative epipfluorescence microscopy (Goff and Coleman, 1984, 1985), we determined that the nuclei of the conjunctor cell formed by the alloparasite *Choreocolax* (*Leachiella*) are transferred into the host cytoplasm. In *Choreocolax*, the parasite nuclei do not undergo DNA synthesis in the host's cytoplasm, and they do not divide. However, their presence is associated with host cellular responses that result in the success of the parasitism.

In more recent studies of adelphoparasites and their closely related hosts (Goff and Coleman, 1987; Goff, 1991; Goff and Zuccarello, 1994), we have observed that parasite nuclei also are transferred to host cells during host infection. Infection begins upon the attachment of parasite spores to their specific host where they germinate and produce an infection peg that fuses with an epidermal or subepidermal host cell (Figures 2A and 2B). This process delivers a parasite nucleus into the underlying host cell, and this nucleus undergoes DNA synthesis and karyokinesis within the host's cytoplasm (Figure 2C). The replicated parasite nuclei are transferred from the initial heterokaryotic cell to adjacent host cells via the direct fusion of "infected" heterokaryotic host cells with adjacent uninfected host cells or via conjunctor cells (Figure 2D). The concomitant induction of host cell division surrounding the region of infection (Figure 2D) results in the formation of a gall of host tissue that is eventually "transformed" into cells containing parasite nuclei.

Not only nuclei but plastids, mitochondria, and other cellular inclusions are transferred from parasite to host cells during these cellular fusion events. However, because parasite cells are derived from heterokaryotic host cells (host plus parasite nuclei and host cytoplasm), we asked whether the plastids and mitochondria that are transferred along with parasite nuclei

[1] To whom correspondence should be addressed.

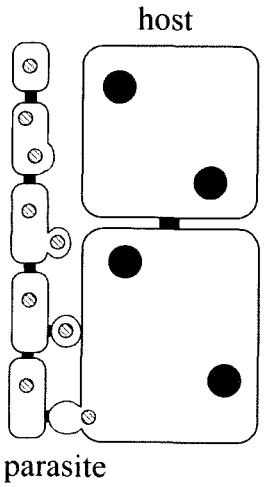

Figure 1. Process of Secondary Pit Connection between Cells of Parasitic Red Algae and Their Host.

Parasite nuclei are striped, light circles and host nuclei are black circles.

are those of the host or whether the parasite maintains its own genetically unique plastid and mitochondria during the process of host cellular transformation.

In this study, we compared plastid and mitochondrial DNA of three adelphoparasites and their respective hosts to determine whether these parasites each have unique plastid and mitochondrial genomes or whether they simply acquire these organelles from their host. These analyses support the conclusions based on cellular observations (Goff and Zuccarello, 1994) that proplastids observed in these adelphoparasites are acquired from their host during the process of host cellular transformation. In contrast, parasite cells and heterokaryotic host cells of two of the parasites examined contain mitochondrial and nuclear genomes that differ from those of their hosts. The close association of parasite mitochondria with parasite nuclei (Goff and Zuccarello, 1994) suggests that parasite mitochondria are transferred into the host cell in concert with parasite nuclear transfer; here, they rapidly divide within the heterokaryotic cell. Ultimately, the parasite nuclear and mitochondrial genomes and host-derived plastid DNA are packaged into parasite reproductive cells that infect additional hosts.

RESULTS

Plastid, mitochondrial, and nuclear DNAs of each of three adelphoparasites, *Plocamiocolax pulvinata*, *Gracilariophila oryzoides* and *Gardneriella tuberifera*, were compared with those of their respective hosts, *Plocamium cartilagineum*, *Gracilariopsis lemaneiformis*, and *Sarcodiotheca gaudichaudii*. These three adelphoparasites are members of different red algal orders (Plocamiales, Gracilariales, and Gigartinales) and were chosen to provide taxonomic diversity.

Total DNA was isolated from each of the parasites and each of their hosts and was fractioned into two major bands on Hoechst 33258–cesium chloride gradients. The lower fraction in the gradients contained primarily nuclear DNA, whereas the upper fraction contained primarily plastid DNA and, as deter-

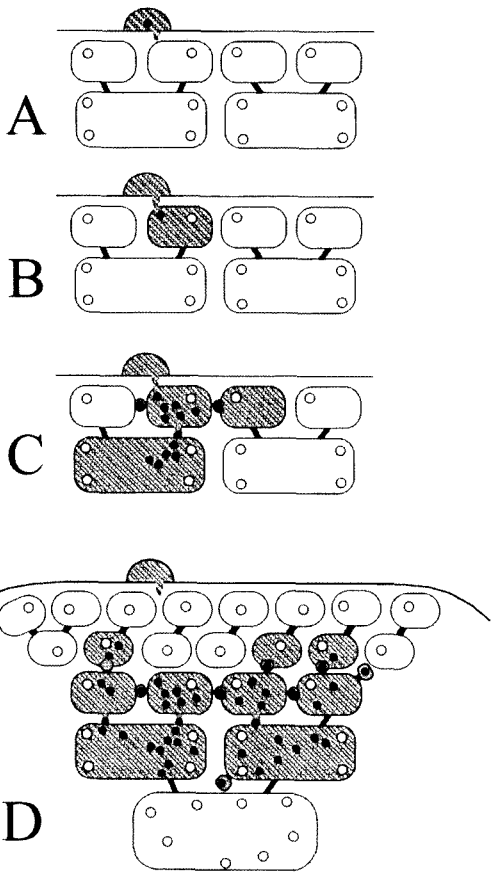

Figure 2. Host Infection and Cellular Transformation by Adelphoparasites.

Black circles represent parasite nuclei, and white circles represent host nuclei. Shaded cells represent either the infecting parasite spore **(A)** or transformed host cells containing both parasite and host nuclei **(B)** to **(D)**.

(A) A parasite spore (gray) attached to the host surface penetrates into the host via an infection peg.

(B) Fusion of the infection peg with an underlying epidermal host cell results in the transfer of a parasite nucleus (black) into a host. Gray cells represent the heterokaryotic (host plus parasite nuclei) cell to which the parasite spore has fused and transferred a parasite (black) nucleus.

(C) The parasite nuclei replicate and are spread from the infected host cell to adjacent host cells either upon dissolution of host–host pit connections or via secondary pit connection formation. The host cells shaded in gray are heterokaryotic.

(D) Host cells surrounding the heterokaryotic tissue proliferate to form a tumorlike mass of host cells that are subsequently "transformed" into parasite cells upon the transfer of nuclei and other organelles from adjacent heterokaryotic cells.

mined in this study, mitochondrial DNA. In all species, the mitochondrial DNA banded slightly above the plastid DNA; however, with the exception of *Plocamiocolax*, it generally could not be separated cleanly from the underlying plastid DNA fraction.

In the case of *Gardneriella*, where excessive carbohydrates prevented gradient separation of nuclear and plastid–mitochondrial DNA, total DNA from the parasite was compared with purified plastid–mitochondrial DNA or nuclear DNA from its host. In *Plocamiocolax* and *Gracilariophila*, purified plastid–mitochondrial DNA and purified nuclear DNA were compared with similar host fractions. In none of the three parasites was it possible to separate parasite tissue cleanly from host tissue. Consequently, all parasite DNA fractions contained some host DNA, whereas host DNA contained no parasite DNA because this was isolated from uninfected host tissue.

Plastid DNA Comparisons

To determine whether the plastid DNA of parasite cells is genetically unique to the parasite or whether this genome is acquired from the host during cellular transformation, DNA restriction fragments from parasites and their hosts were compared. In the case of the parasite *Plocamiocolax pulvinata* and its host *Plocamium cartilagineum*, restriction fragment length polymorphism (RFLP) patterns of DNA from the upper fraction of Hoechst 33258–cesium chloride gradients are nearly identical (Figure 3A; compare lanes 1, 2, and 3, lanes 5, 6, and 7, and lanes 9, 10, and 11). Only a few restriction fragments are unique to the parasite (Figure 3A, white dots), and the stoichiometry of their staining suggests that they are not part of the plastid genome. These fragments are most conspicuous in restriction digests of the DNA fraction isolated from just above that of the plastid-enriched fraction in the Hoechst gradients (Figure 3A, lanes 4, 8, and 12); as described later, these fragments are part of the mitochondrial genome.

Gel blots of restricted DNA from the Hoechst gradient fraction enriched in plastid DNA were hybridized with heterologous and homologous plastid DNA probes to determine whether these probes would hybridize with fragments of the same size in both the parasite and host. These analyses were performed to eliminate the possibility that another genome, presumably unique to the parasite, might occur in the DNA isolated from the parasite but at a concentration too low relative to contaminant host plastid DNA to be visualized on an ethidium bromide–stained gel. Initially, the plastid ribosomal 16S gene (p16 from *Chlamydomonas* [probe from E. Harris, Duke University, Durham, NC]) was used to probe a blot of restricted *Plocamiocolax* (parasite) and *Plocamium* (host) DNA (Figure 3B). This probe hybridized to identical fragments in both the parasite and host (Figure 3A, white arrows, and Table 1), and it also hybridized more weakly to some of the additional unique bands in the parasite DNA (Figure 3B, double arrows). Another heterologous probe encoding the large subunit of ribulose bisphosphate carboxylase (rbcL; p67 from *Chlamydomonas* [probe from E. Harris]) hybridized to the same-sized fragments

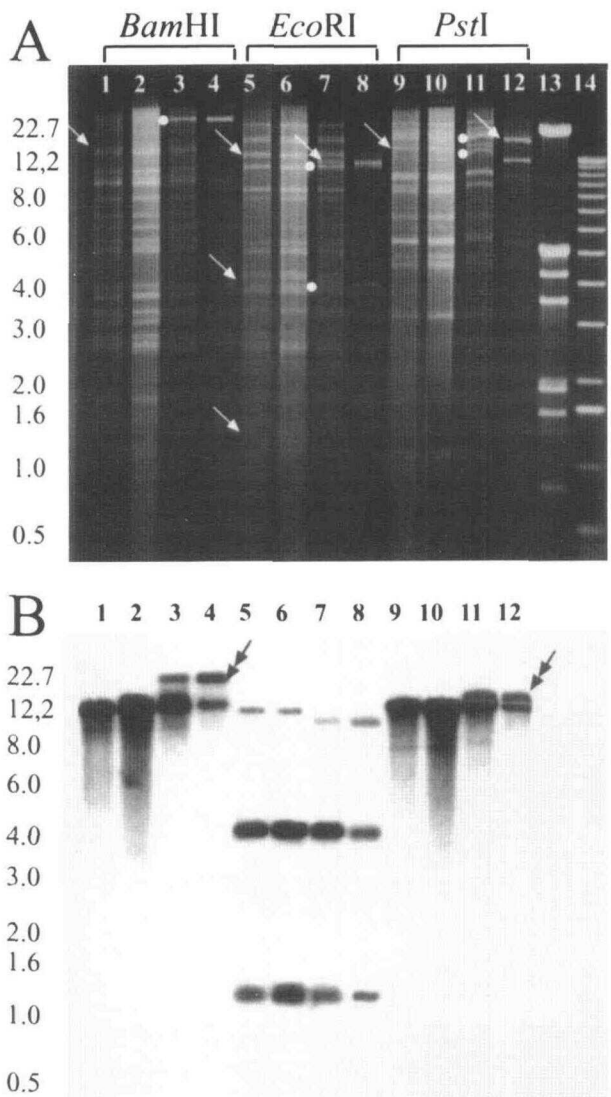

Figure 3. *Plocamiocolax* and *Plocamium* RFLP Probed with the p16 Plastid rDNA Probe (from *Chlamydomonas*).

All fractions except lanes 4, 8, and 12 are from a mixed plastid (primarily) and mitochondrial (secondarily) DNA fraction isolated from the top of a Hoechst 33258–cesium chloride gradient. Lanes 4, 8, and 12 are from a fraction that occurs just above the mixed plastid/mitochondrial fraction in *Plocamiocolax* and contains primarily mitochondrial DNA (plastid DNA is secondary). Lanes 1, 5, and 9 contain digested host (*Plocamium*) DNA collected in San Juan County, WA, and lanes 2, 6, and 10 contain digested host DNA from Santa Cruz County, CA. Lanes 3, 7, and 11 contain digested DNAs from the parasite (*Plocamiocolax*) collected in Santa Cruz County, CA.

(A) The white dots correspond to unique fragments in the parasite DNA that are determined to be mitochondrial. The white arrows correspond to the fragments recognized by the plastid ribosomal probe in **(B)**.

(B) The black double arrowheads indicate bands unique to the parasite that are recognized by this probe.

Lane 14 contains the fragment length markers in a 1-kb DNA ladder, and lane 13 contains λ DNA digested with EcoRI-HindIII. Lengths are given at left in kilobases.

Table 1. Plastid DNA RFLPs in Parasite and Host Comparisons

Probe	Taxon	BamHI Fragments (kb)	EcoRI Fragments (kb)	PstI Fragments (kb)
p16	*Plocamiocolax* (parasite)	28 14.5 1.4	13.5 4.8 1.4	17.0 15.5
(Plastid ribosomal 16S gene)	*Plocamium* (host)	14.5 1.4	4.8 1.4	15.5
	Gardneriella (parasite)	12.2 3.3	7.7 1.6	8.9
	Sarcodiotheca (host)	12.2 3.3	7.7 1.6	8.9
	Gracilariophila (parasite)	11.1	5.5 3.1	4.7
	Gracilariopsis (host)	11.1	5.5 3.1	4.7
p67	*Plocamiocolax* (parasite)	15.0	7.5	8.0 1.2
(*rbcL* gene)	*Plocamium* (host)	15.0	7.5	8.0 1.2
	Gardneriella (parasite)	8.1 2.3	7.6	9.0 1.5
	Sarcodiotheca (host)	8.1 2.3	7.6	9.0 1.5
	Gracilariophila (parasite)	12.0	5.3	7.0 2.0
	Gracilariopsis (host)	12.0	5.3	7.0 2.0

in both *Plocamiocolax* and *Plocamium* and in the parasite *Gardneriella tuberifera* and its host *Sarcodiotheca gaudichaudii* and *Gracilariophila oryzoides* and its host *Gracilariopsis lemaneiformis* (Table 1). This probe did not hybridize to the unique DNA fragments in the parasite *Plocamiocolax*.

Red algal, plastid-specific DNA probes also were used to compare host and parasite plastid DNA RFLPs to provide greater sensitivity than the heterologous plastid 16S rDNA and *rbcL* probes. These probes were obtained by screening a plasmid library containing restricted plastid DNA of the host red alga *Gracilariopsis lemaneiformis*. Probes were selected that hybridized specifically with plastid DNA from a broad spectrum of red algal species. Clones containing plastid 16S rDNA were excluded from these analyses to eliminate confusion resulting from possible cross-hybridization with contaminant mitochondrial ribosomal sequences contained in the DNA fractions (Coleman et al., 1991). These plastid-specific probes were used individually or in combination to compare the plastid DNA of each of the three parasites and their hosts. In all cases, these probes hybridized with identical fragments of parasite and host plastid DNA (data shown only for *Plocamiocolax* and *Plocamium* in Figures 4A and 4B).

To compare further plastid DNA of parasites and hosts, the sequence of a highly variable plastid DNA spacer region that occurs between *rbcL* and the small subunit (SSU) *rbcS* genes along with flanking regions of both genes were amplified using polymerase chain reaction (PCR). This region was amplified and sequenced from 20 individual parasites of *Gracilariophila oryzoides* and *Gardneriella tuberifera* and compared with that of their respective hosts, *Gracilariopsis lemaneiformis and S. gaudichaudii*. To eliminate the problem of host tissue contamination, this region was amplified from released parasite spores. In both cases, the ~330-bp sequence of the parasite was exactly the same as the host's plastid spacer region (the host sequence data has been previously published [Goff et al., 1994] and has GenBank accession numbers U21347 and U21345).

Mitochondrial DNA Comparisons

The observation that DNA probes containing plastid 16S rDNA sequence hybridize with some of the unique bands in the plastid-enriched DNA fraction of the parasite *Plocamiocolax* suggested that these fragments may contain mitochondrial SSU rDNA and accordingly that these fragments are from the mitochondrial genome. Approximately two-thirds of the putative mitochondrial genome was cloned from *Plocamiocolax* as four fragments in the pBluescript SK+ vector: clone 80b (4400-bp EcoRI-EcoRI fragment), clone 11 (1500-bp EcoRI-EcoRI fragment), clone 32d (3000-bp EcoRI-BamHI fragment), and clone 70 (7000-bp EcoRI-BamHI fragment). An additional mitochondrial fragment (clone 55; 7500-bp HindIII-HindIII fragment) was obtained from a library made from plastid and mitochondrial DNA fragments of *Gracilariopsis lemaneiformis*.

Probes containing the mitochondrial SSU rDNA from the oomycete *Phytophthora infestans* (gift from F. Lang, University of Montreal, Quebec, Canada) hybridized strongly to clones 80b and 32d from *Plocamiocolax* and to clone 55 from *Gracilariopsis*, indicating the presence of mitochondrial rDNA on these fragments. In addition, the mitochondrial gene cytochrome oxidase 1 (*cox1*) from *Brassica* (gift from M. Mulligan, University of California, Irvine) hybridized to clones 80b and 55 and to the unique mitochondrial fragment in BamHI-digested DNA from *Plocamiocolax* (Figures 5A and 5B). Both clone 80b and clone 55 have been sequenced, confirming the presence of *cox1* and mitochondrial SSU rDNA on these fragments.

Mitochondrial genomes of parasites and hosts were compared directly in pulsed-field gel electrophoresis (Figures 6A and 6B). To provide enough host mitochondrial DNA for these

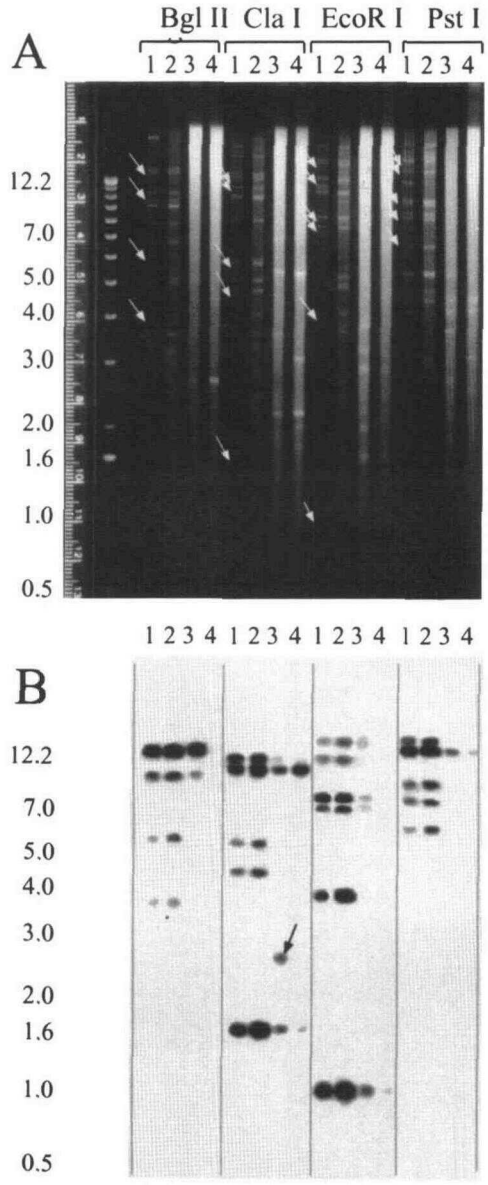

Figure 4. Restricted DNA Fractions from *Plocamiocolax* and *Plocamium* Probed with a Mixture of Red Algal Plastid DNA Probes.

All probes hybridized with the same fragments in parasite- and host-digested DNA.

(A) Lanes 1 contain *Plocamiocolax* (parasite) plastid and mitochondrial DNA; lanes 2 contain *Plocamium* (host), plastid, and mitochondrial DNA; lanes 3 contain nuclear DNA of *Plocamiocolax* and contaminant plastid–mitochondrial DNA; lanes 4 contain the nuclear DNA of *Plocamium*. The white arrows correspond to the fragments recognized by the probes in **(B)**. The fragment length markers at left are a 1-kb standard ladder.

(B) Shown is a blot of **(A)** probed with a mixture of six red algal–specific plastid DNA probes. The nuclear fraction of *Plocamiocolax* (lane 3 in each digest) contains a considerable amount of contaminant plastid DNA, whereas little or none can be detected in the nuclear fraction from *Plocamium*. The black arrow indicates a unique fragment recognized by this plastid probe mixture, but this occurs only in the contaminanted nuclear fraction of *Plocamiocolax*.

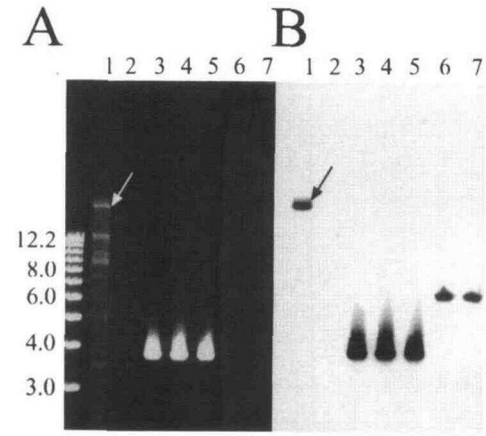

Figure 5. Hybridization of the *Brassica cox1* Probe with Putative Mitochondrial DNA of *Plocamiocolax*.

(A) Upper fraction of *Plocamiocolax* DNA cut with BamHI (lane 2) and cloned mitochondrial fragments (*Plocamiocolax* mitochondrial DNA clone 80b in lanes 3 to 5 and *Gracilariopsis* mitochondrial clone 55 in lanes 6 and 7). The length standard at left is a 1-kb ladder, and lane 2 contains no DNA.

(B) Blot of **(A)** probed with a *cox1* DNA probe.

The white arrow in **(A)** and the black arrow in **(B)** indicate the large ~25,000-bp fragment in the BamHI digest that most likely represents the entire linearized mitochondrial genome of *Plocamiocolax*.

analyses, mitochondrial DNA was purified from host cystocarps (the site of zygote amplification), which are rich in mitochondria. Enough mitochondrial DNA was obtained from parasite vegetative cells, which contain 10 to 100 times more mitochondria than host cells. In both the parasite and host, two bands are evident in pulsed-field electrophoresis gels (Figure 6A). The two bands from *Plocamiocolax* migrated at ~28,000 and 32,000 bp, whereas the two host bands migrated at ~27,000 and 31,000 bp. The *coxI* probe hybridized with both of these bands, indicating that they probably represent different conformation of the same mitochondrial molecule.

To examine the conformation of the mitochondrial genome, the mitochondrial DNA–enriched fraction from Hoechst gradients of *Plocamiocolax* was coated with cytochrome, spread on grids, and imaged with a transmission electron microscope. These analyses revealed the presence of circular DNA molecules (Figure 6C) that correspond to a genome size of 25,278 ± 863 bp (n = 51). These data suggest that the shorter ~28,000-bp band seen in pulsed field electrophoresis gels is the broken, linear mitochondrial genome; the longer moiety is most likely the nicked, relaxed circular form. The length of the mitochondrial genome is very similar to the uppermost fragment (~25 kb) seen in BamHI digests of *Plocamiocolax* (Figure 3A, lanes

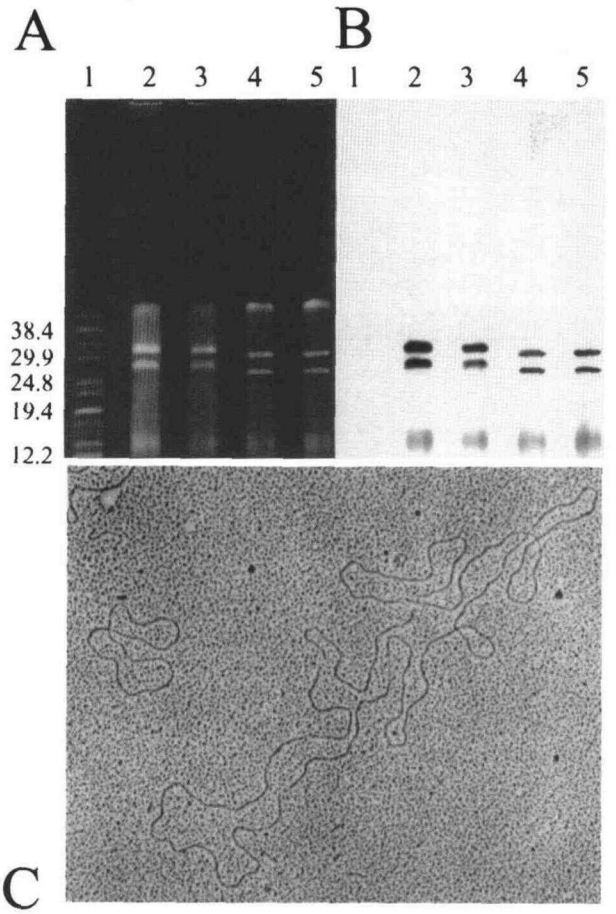

Figure 6. Pulsed-Field Gel Electrophoresis of *Plocamiocolax* and *Plocamium* Mitochondrial DNA.

(A) Lanes 2 and 3 contain *Plocamiocolax* (parasite) mitochondrial DNA with some contaminant plastid DNA. Lanes 4 and 5 contain mitochondrial DNA (and some plastid DNA) isolated from the host *Plocamium*. Lane 1 contains high molecular weight markers (units are given at left in kilobases).

(B) A blot of **(A)** probed with *Brassica cox1* shows that both fragments are mitochondrial. The diffuse, shorter DNA fragments between 12 to 19 kb are probably broken pieces of mitochondrial DNA because the *cox1* probe also hybridized with these fragments.

(C) Shown is a circular mitochondrial DNA molecule from the parasite *Plocamiocolax* (uppermost fraction of DNA from the cesium chloride gradient) that was spread using Kleinschmidt (1968) cytochrome methods and imaged with a transmission electron microscope. The size of this circle was determined by comparing it with a 4.755-kb plasmid shown to the left of the mitochondrial genome.

3 and 4) and suggests that this fragment may be the entire mitochondrial genome.

Mitochondrial DNA RFLP of parasites and their hosts were compared by hybridizing cloned mitochondrial DNA fragments with blots of restricted host and parasite DNA containing both mitochondrial and plastid DNA. For these analyses, both cloned

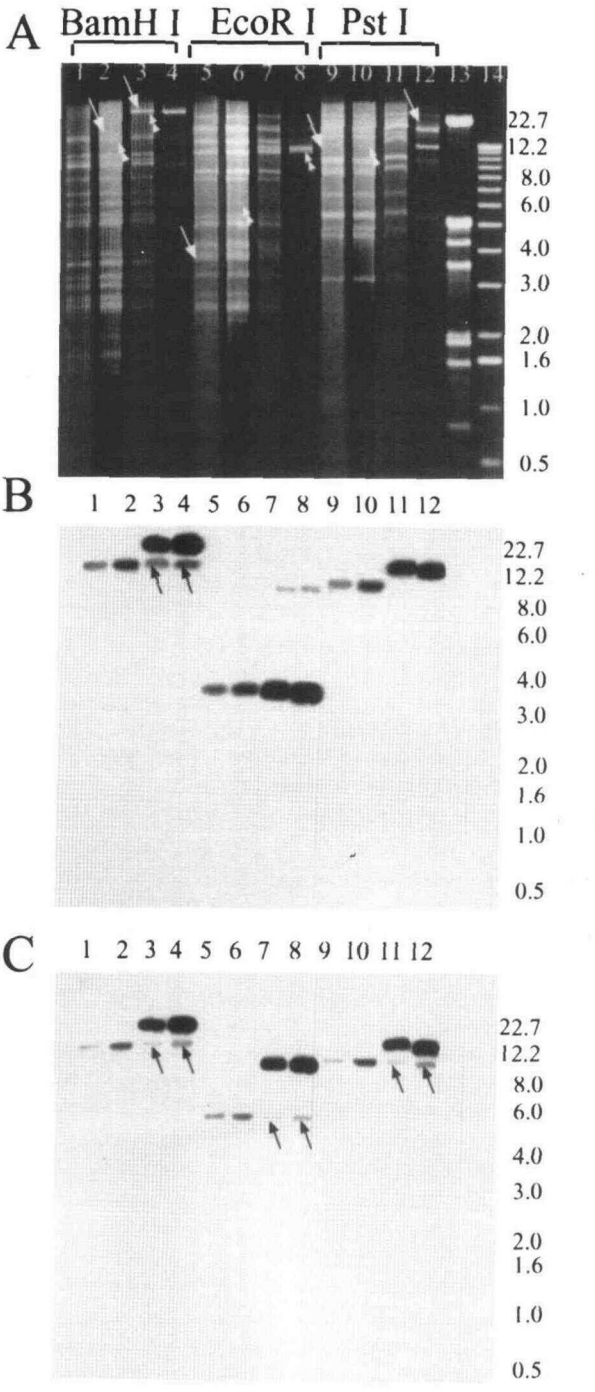

Figure 7. Mitochondrial RFLP Patterns of *Plocamiocolax* and *Plocamium*.

(A) *Plocamiocolax* and *Plocamium* restricted plastid and mitochondrial DNA. All fractions except lanes 4, 8, and 12 are from a mixed plastid (primarily) and mitochondrial (secondarily) DNA fraction isolated from the top of a Hoechst 33258–cesium chloride gradient. Lanes 4, 8, and 12 are from a fraction that occurs just above the mixed plastid/mitochondrial fraction in *Plocamiocolax* and contains primarily mitochondrial

Table 2. Mitochondrial DNA RFLPs in Parasites and Hosts[a]

Taxon	BglII Fragments (kb)	ClaI Fragments (kb)	EcoRI Fragments (kb)	PstI Fragments (kb)
Plocamiocolax (parasite)	28 22	25 12 5 2.6	11 8 6 3.2	26 24
Plocamium (host)	22	25 12 5 2.6	8 6 3.2	26 24
Gardneriella (parasite)	12 3.8	14 4.3	9 5.5	8
Sarcodiotheca (host)	12 3.8	14 4.3	9 5.5	8
Gracilariophila (parasite)	10.5	4.5 2.8	5.8 3.5 2.2 1.7	4.5 2.8 2 1 0.2
Gracilariopsis (host)	12	2.8	3.5 1.7	2.8 1 0.2

[a] The probes used are a mixture of cloned red algal mitochondrial fragments.

mitochondrial DNA from the parasite *Plocamiocolax* (clone 80b containing both *cox1* and mitochondrial SSU rDNA, clone 32d containing some mitochondrial SSU rDNA, and clones 70 and 11) and clone 55 from the red alga *Gracilariopsis lemaneiformis* (a 7.5-kb fragment containing both *cox1* and mitochondrial SSU rDNA) were used as probes.

Differences in restriction fragment patterns of parasite compared with host mitochondrial DNA are clearly evident in comparison of *Plocamiocolax* (parasite) and *Plocamium* (host) DNA cut with BamHI, EcoRI, and PstI (Figure 7A). The enhanced mitochondrial content of parasite tissue is also obvious from the band intensities. Hybridization with *Plocamiocolax* mitochondrial clone 80b revealed polymorphisms in mitochondrial DNA restricted with BamHI and PstI (Figure 7B), whereas clone 32d revealed polymorphisms in mitochondrial DNA cut with BamHI, EcoRI and PstI (Figure 7C). Both clones 80b and 32d contain regions of the mitochondrial SSU rDNA, and these hybridized strongly with the conspicuous mitochondrial DNA fragment in BamHI-digested parasite DNA that was recognized (weak hybridization) by the plastid 16S (p16) rDNA probe (compare Figures 7B and 7C, lanes 3 and 4 to Figure 3B, lanes 3 and 4). Because of host contamination in parasite DNA, these probes also detected some host mitochondrial DNA in the parasite DNA (Figure 7B, lanes 3 and 4 and Figure 7C lanes 3 and 4, 7 and 8, 11 and 12, arrows).

Using mixtures of red algal mitochondrial fragments as probes, RFLP were apparent in comparisons of mitochondrial DNAs (Table 2) of *Plocamiocolax* and its host *Plocamium* (polymorphisms in BglII, EcoRI, and PstI) and *Gracilariophila oryzoides* and its host *Gracilariopsis lemaneiformis* (polymorphisms in BglII, ClaI, EcoRI and PstI). However, no polymorphisms were apparent in comparisons of restricted mitochondrial DNA from the parasite *Gardneriella tuberifera* and its host *S. gaudichaudii* (Table 2).

Nuclear DNA Comparisons

The nuclear genomes of several red algal parasites and their hosts were compared by hybridizing restricted host and parasite nuclear DNA with a DNA probe from pea containing the entire nuclear ribosomal repeat region (both small and large subunits of rDNA, 5.8S rDNA, and internal transcribed spacers ITS1 and ITS2). This probe was chosen for these comparisons because it contains both highly conserved rDNA sequences and more variable ITS sequences and accordingly might indicate which subregions of the ribosomal repeat (spacers versus rDNA) might be most useful for subsequent evolutionary sequence comparisons of parasites and their hosts.

Comparison of the nuclear DNA of the parasite *Gracilariophila oryzoides* and its host *Gracilariopsis lemaneiformis*, cut with six restriction endonucleases, revealed RFLP in the ribosomal repeat region polymorphisms using BamHI, BglII, ClaI, EcoRI, HindIII, and PstI (Figures 8A and 8B and Table 3). Additional probing with a DNA fragment containing the PCR-amplified ITS regions and the 5.8S rDNA from the host *G. lemaneiformis* (Goff et al. 1994) revealed that some of the RFLP seen between this host and its parasite occurs in regions containing the ITS and the 5.8S rDNA (Figures 9A and 9B). Nuclear DNA RFLP were seen also in comparisons of *Plocamiocolax* and *Plocamium* and *Gardneriella tuberifera* and its host *S. gaudichaudii*; these are summarized in Table 3.

Figure 7. (continued).

DNA (plastid DNA is secondary). Lanes 1, 5, and 9 contain digested host (*Plocamium*) DNA collected in San Juan County, WA, and lanes 2, 6, and 10 contain digested host DNA from Santa Cruz County, CA. Lanes 3, 7, and 11 contain digested DNAs from the parasite (*Plocamiocolax*) collected in Santa Cruz County, CA. The white single arrows indicate the DNA fragments recognized by clone 80b in **(B)**, and the white double arrows indicate those fragments recognized by clone 32d in **(C)**. Lane 13 contains an EcoRI-HindIII marker, and lane 14 contains fragment markers in a 1-kb ladder. Fragment lengths are given at right in kilobases.

(B) Blot of **(A)** probed with mitochondrial probe 80b from *Plocamiocolax*.

(C) Blot of **(A)** probed with mitochondrial probe 32d from *Plocamiocolax*.

The fragments in **(B)** and **(C)** were cloned from the conspicuous long (~25,000-bp) BamHI fragment (lane 4). The black arrows indicate host DNA-contaminating DNA isolated from the parasite.

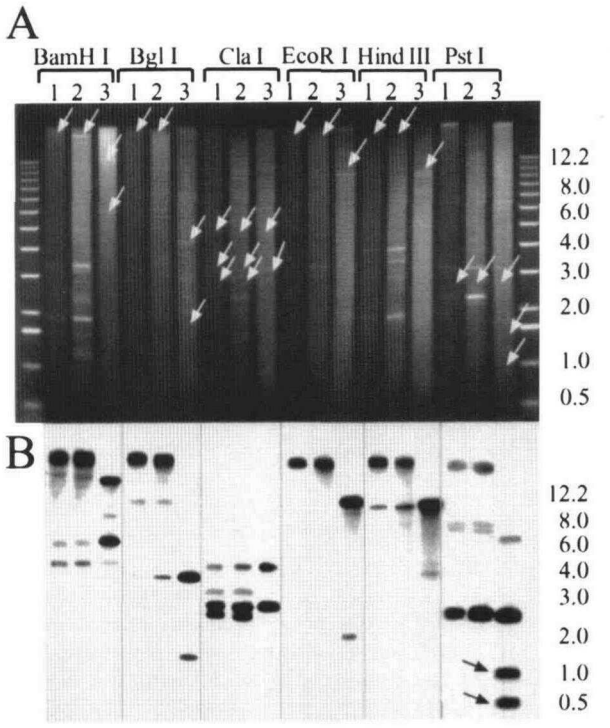

Figure 8. Nuclear RFLP Patterns of the Parasite *Gracilariophila* and Its Host *Gracilariopsis*.

(A) Nuclear DNA of the parasite *Gracilariophila* (lanes 1 and 2 from different populations) and its host *Gracilariopsis* (lane 3 from each digest) cut with six endonucleases. The white arrows indicate the fragments shown in **(B)** with which *PHA2* (nuclear ribosomal repeat region) hybridized. The outside lanes are the 1-kb markers, and the lengths indicated at right are given in kilobases.

(B) Blot in **(A)** probed with *PHA2*. The black arrows indicate a PstI polymorphism in this host nuclear rDNA.

DISCUSSION

Comparisons of plastid, mitochondrial, and nuclear DNAs from three adelphoparasites and their hosts revealed (1) no discernible difference in the RFLP patterns of restricted plastid DNA of parasites and their hosts, (2) differences in RFLP patterns of mitochondrial DNA in two of the parasites and their hosts, and (3) differences in the restriction fragment patterns of nuclear ribosomal DNA between all three adelphoparasites and their hosts (Table 4).

The fact that the plastid DNA of parasites appears identical with that of their hosts may indicate that parasites and their hosts are as similar genetically as are members of the same species. Earlier studies of plastid DNA in red algae have shown that individuals of the same red algal species have identical or near-identical plastid DNA restriction fragment patterns (Goff and Coleman, 1988; Rice and Bird, 1990; Maggs et al., 1992). Alternatively, parasites and their hosts may have identical plastid restriction fragment patterns if the plastids seen in parasite cells are acquired from their host during cellular transformation.

This alternative explanation is supported by microscopic studies of host cellular transformation in the parasites *Gardneriella* and *Gracilariophila* (Goff and Zuccarello, 1994). Similar to all other parasitic red algae, these parasites harbor proplastids with conspicuous plastid DNA nucleoids. These proplastids lack thylakoids, phycobilisomes, and photosynthetic pigments that are characteristic of a differentiated red algal plastid. Proplastids occur in the infecting spores of the parasite, and upon host infection, they are injected into the recipient host cell's cytoplasm, along with the parasite nucleus. Once the parasite organelles enter the host's cytoplasm, the host's plastids rapidly dedifferentiate by a process of budding, followed by fission, to form small proplastids with one or more plastid nucleoids and few or no thylakoids.

The host proplastids that occur in heterokaryotic cells (containing both host and parasite nuclei) are transferred, along

Table 3. Nuclear DNA RFLP in Parasites and Hosts[a]

Taxon	BamHI Fragments	BglII Fragments	ClaI Fragments	EcoRI Fragments	HindIII Fragments	PstI Fragments
Plocamiocolax (parasite)	ND[b]	ND	ND	15 11	ND	15.5 7
Plocamium (host)	ND	ND	ND	15	ND	7
Garneriella (parasite)	12.5	12.5	12.6	5 1.4	12.7	7.5 3.0
Sarcodiotheca (host)	13	13	13.8 13.5	5	2.5 1.3	8.0 8.5 3.0
Gracilariophila (parasite)	>23	>23	5 3.3 3	>23	>23	2.8
Gracilariopsis (host)	20 6	4.5	5 3.3	10	9	2.8 1.6 1

[a] The probes that were used contained the entire nuclear rDNA repeat region from pea. The approximate lengths of fragments are given in kilobases.

[b] ND, no data.

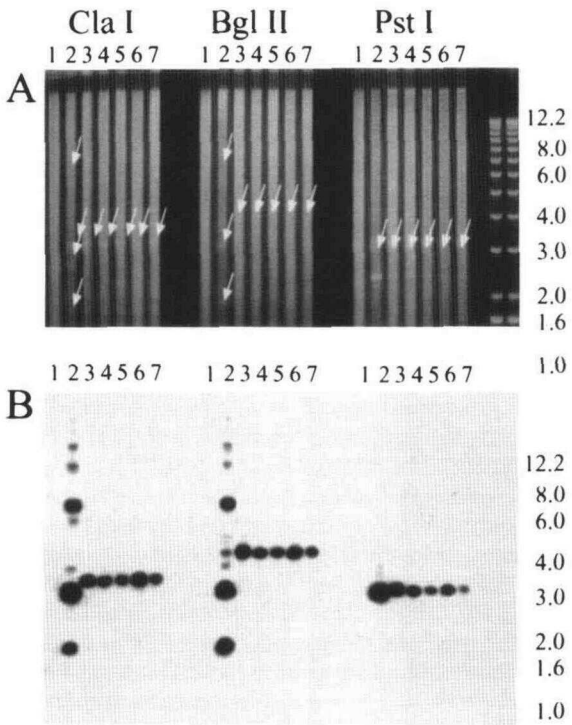

Figure 9. Polymorphisms in the Nuclear rDNA Regions Occur within the Internal (ITS) Regions.

(A) Nuclear DNA of the parasite *Gracilariophila* (lanes 2), its host *Gracilariopsis* (lanes 3 to 7 from different populations), and a non-host species, *Gracilaria robusta* (lanes 1), cut with three endonucleases. The white arrows indicate the fragments shown in **(B)** with which the ITS probe hybridized. The two lanes at the right contain the 1-kb makers, and the lenghts at right are given in kilobases.

(B) Shown is a blot of **(A)** probed with the PCR-amplified ITS regions of the nuclear ribosomal repeat (ITS1, 5.8S rDNA, and ITS2) from the host *Gracilariopsis lemaneiformis*. At high stringency, the probe does not recognize the non-host DNA but shows host and parasite RFLP when ClaI and BglII were used.

with parasite nuclei, to adjacent, uninfected host cells either upon the direct fusion of the heterokaryotic cell with an adjacent host cell or via the fusion of a conjunctor cell, which is formed by the heterokaryon (Figures 10A to 10C). Once the proplastids and parasite nuclei enter into host cells, the resident host plastids dedifferentiate and divide to form more proplastids (Figure 10B). From the mass of heterokaryotic tissue, cells are formed that contain parasite nuclei, proplastids derived from the host plastids, and mitochondria. These heterokaryotic cells give rise to gametes, 2n carpospores, or 1n tetraspores that disperse the parasite nuclear and, as shown in this study, mitochondrial genomes. Proplastids have been observed in all cell stages examined, but as far as we can determine, neither host nuclear DNA nor host mitochondrial DNA occurs in detectable quantities in the parasite spores or gametes. Whether these host organelles are destroyed upon cellular transformation or whether they simply become outnumbered by the differential replication of parasite nuclei and mitochondria remains unknown.

These cellular and developmental observations and the RFLP data presented are in agreement: there is no evidence that *Plocamiocolax*, *Gracilariophila*, and *Gardneriella* possess their own genetically unique plastids. Rather, the proplastids seen in the infective spore stages stem from proplastids formed during host cellular transformation processes in their previous host. The process whereby the parasite acquires the plastids from its host may be similar to the means by which maternally derived organelles are acquired in normal red algal postfertilization development. In red algal postfertilization, the 2n zygote nucleus is transferred from the carpogonium (which contains a few proplastids), frequently via connecting filaments, to a specialized cell termed the auxiliary cell. This cell is rich in plastids and mitochondria and may serve as the source of organelles for the developing carposporophyte (2n) generation (Wilce and Sears, 1991).

The transfer of a parasite nucleus into a host cell triggers the dedifferentiation processes that result in the formation of proplastids from host plastids. Fluorescence microscopic examination of host cell transformation by parasite nuclei has shown that loss of plastid structure is accompanied by loss of plastid pigmentation (loss of pigment autofluorescence) and presumably photosynthetic ability (Goff and Zuccarello, 1994). As a consequence, transformed host tissue becomes colorless. In *Gardneriella*, the regions of pigmented (red) tissue in the otherwise colorless thallus are composed of host cells that have not yet received parasite nuclei. Eventually, all pigmented host cells receive parasite nuclei and the entire erumpent mass of tissue becomes colorless.

But why would a parasite maintain host plastids, albeit as proplastids, throughout its life cycle stages? And why, if the parasite is dependent upon its host as a source of photosynthate, would the process of host cellular transformation by red algal parasites result in the dedifferentiation of host plastids into photosynthetically incompetent plastids?

Table 4. RFLP Differences in Parasites and Hosts

Parasite vs Host	Plastid Genome		Mitochondrial Genome	Nuclear Genome
	RFLP	Sequence[a]		
Plocamiocolax vs *Plocamium*	0/5[b]	No data	3/4	2/2
Gardneriella vs *Sarcodiotheca*	0/5	Identical	0/5	6/6
Gracilariophila vs *Gracilariopsis*	0/3	Identical	4/5	6/6

[a] Sequence of the plastid DNA Rubisco spacer and flanking regions of the *rbcL* and *rbcS* genes.

[b] Number of RFLP found per number of restriction enzymes tested.

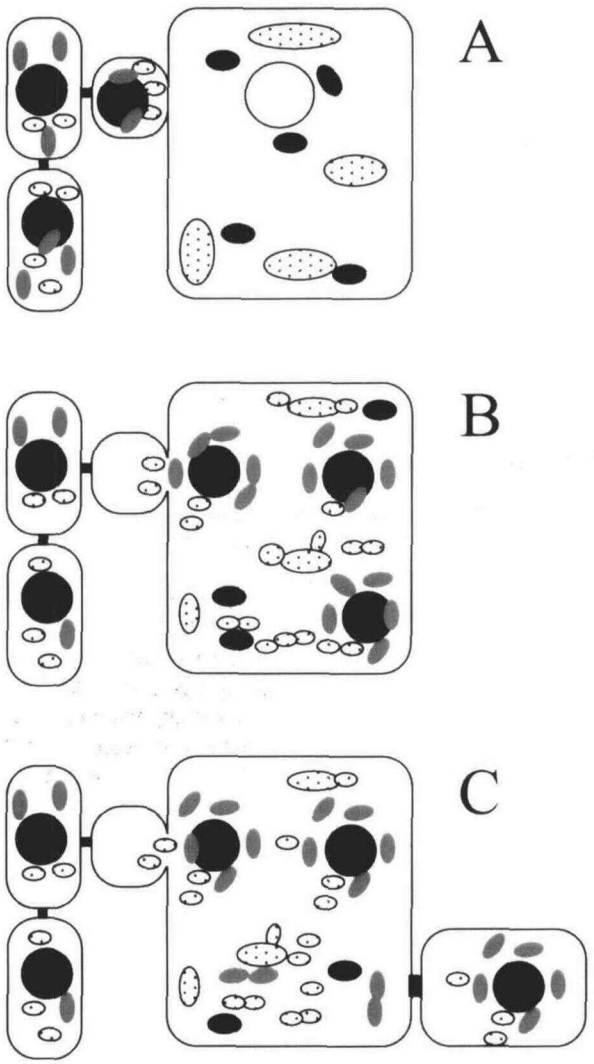

Figure 10. Transfer of Parasite Nuclei, Mitochondria, and Proplastids into a Host Cell.

(A) The parasite cells (left) have formed a conjunctor cell containing a parasite nucleus (black), mitochondria (gray), and proplastids (white with dots). This conjunctor cell fuses with the host cell (host nuclei are white, host plastids are dotted, and host mitochondria are black ovals) delivering the parasite organelles into the host's cytoplasm.
(B) The parasite nucleus and mitochondria replicate in the host cell, and the host plastids divide to form numerous proplastids. The host nuclei and mitochondria replicate in the host cell. The host nucleus may disappear or persist.
(C) Ultimately, a cell is cut off from the heterokaryotic host plus parasite cell. This cell contains a parasite nucleus, parasite mitochondria, and proplastids derived from the host plastids.

The development of parasite tissue from transformed host cells may require maintenance of the plastid genome for functions other than photosynthesis, such as pyrimidine biosynthesis (Doremus and Jagendorf, 1995) and amino acid metabolism and fatty acid biosynthesis (Hrazdina and Jensen, 1992; Reith and Munholland, 1993; Gillham, 1994). Alternatively, these proplastids may serve the parasite no function whatsoever. They may merely be the by-product of host cellular fusion events, and the signal for their production may be linked to the developmental program allowing a nucleus to invade another cell and propagate, as occurs during postfertilization development in red algae (Goff and Zuccarello, 1994).

Dedifferentiation of host plastids into proplastids may be a result of parasite nuclear and host plastid incompatibility. Once a parasite nucleus enters the cytoplasm of its host cell, it replicates rapidly and outnumbers the resident host nuclei, which in some cases appear to degenerate. Parasite nuclei may lack genes required for synthesis of proteins required by the plastids, or alternatively, targeting these proteins to the host plastid may be affected by the presence of parasite nuclei.

Two observations argue against plastid–nuclear incompatibility as a cause of plastid dedifferentiation in parasitic red algae. First, not all parasites or all stages of specific parasitic red algae lack photosynthetically competent plastids. Although all the vegetative tissues of *Plocamiocolax* are colorless, mature carpospores may be pigmented and presumably photosynthetically competent. Other adelphoparasites, such as *Janczewskia* spp and *Gonimophyllum* spp, may be highly pigmented at maturity and, in the case of *Janczewskia*, photosynthetically competent (Court, 1980). However, photosynthetic pigments are expressed in these organisms only after they have become reproductively mature. During earlier stages of development, the tissues of the parasite are devoid of pigmentation.

Second, observations that colorless tissues of the parasitic red alga *Choreocolax* rapidly develop pigmentation, plastid structure, and photosynthetic ability upon excision from its underlying host tissue (Callow et al., 1979) also support the conclusion that parasite nuclei and host plastids are not genetically incompatible. Furthermore, this observation suggests that that dedifferentiation of host plastids into proplastids may be required to establish source–sink gradients for the transport of photosynthetically fixed carbon compounds. These compounds are translocated from photosynthetically active host tissue to colorless parasite tissue (Evans et al., 1973; Goff, 1979; Kremer, 1983). In the case of pigmented *Janczewskia* and *Gonimophyllum*, maintenance of the source–sink gradients may be very important during the initial rapid growth phase of the parasitism; however, once reproductive maturity is attained and vegetative growth slows or ceases, these gradients may not be required, and accordingly, some of the proplastids may differentiate into plastids.

In all red algal parasites, the presence of adjacent host tissue and/or source–sink gradients may inhibit the differentiation of proplastids into photosynthetically functional plastids. These processes may be similar to those encountered in the Em-

bryophyta, where colorless gametophytic tissue, once isolated from surrounding host tissues, becomes pigmented and photosynthetically competent (Tobin and Silverthorne, 1985).

In contrast with the proplastid that is acquired from host cells, differences in the mitochondrial DNA restriction fragment patterns of *Plocamiocolax* and its host and *Gracilariophila* and its host indicate that these parasites must retain their own mitochondrial genomes and not simply use those of their hosts. The parasite mitochondria enter into the cytoplasm of the host cell along with the parasite nucleus during initial host infection and are spread from cell to cell upon the fusion of infected heterokaryotic cells with uninfected host cells (Goff and Zuccarello, 1994). Once parasite mitochondria enter into the cytoplasm of host cells, they and their genomes replicate rapidly, as evidenced by the large numbers of mitochondria seen in transformed host cells. Consequently, the yield of mitochondrial DNA is much higher from parasite tissue than from uninfected host tissue.

Studies with the parasite *Gardneriella* show the presence of mitochondria attached to endoplasmic reticulum, which surrounds the parasite nucleus in heterokaryotic cells (Goff and Zuccarello, 1994). The physical attachment may provide the means to deliver parasite mitochondria with parasite nuclei to host cells. In flowering plants, mitochondria have also been described as attached to membranes surrounding the male nucleus during pollen tube growth and fertilization (Dickinson, 1986).

The fact that both *Gracilariophila* and *Plocamiocolax* maintain their own genetically unique mitochondria during host cellular transformation indicates that these parasites cannot utilize host mitochondria. If, as first postulated by Setchell (1918; reviewed in Goff and Zuccarello, 1994), these adelphoparasites evolved from their hosts, then they may have diverged genetically to the point where the parasite nuclei are not compatible with the mitochondria of the host. Evidence for the nuclear control of mitochondrial compatibility has been reported in *Paramecium*, where the mitochondria of certain species are not compatible with nuclei of very closely related species (Gillham, 1994). Likewise, interspecific hybrid studies of flowering plants (Perl et al., 1991) provide similar evidence for nuclear control of mitochondrial compatibility.

In contrast, RFLP analysis indicates that this mitochondrial genome of *Gardneriella* is indistinguishable from that of its host. Either this parasite is able to use the mitochondria of its host or its genome is so similar to that of the host as to appear identical in the RFLP genetic analyses. In either case, the data indicate that *Gardneriella* and its host *Sarcodiotheca* may be much more closely related to each other than *Plocamiocolax* is to its host *Plocamium*, and *Gracilariophila* is to its host *Gracilariopsis lemaneiformis*. These conclusions are supported by comparisons of ribosomal gene sequences (Goff et al., 1995).

Comparisons of RFLP patterns of the rDNA of parasites and hosts reveal restriction polymorphisms between all three parasites and their hosts and provide evidence that these parasites are genetically different from their hosts and are not merely aberrant accessory reproductive structures of the host. In a subsequent study (Goff et al., 1995), the sequences of the rDNA of these and other adelphoparasites have been compared with their hosts to determine whether these parasites evolved directly from their hosts.

If parasites have evolved from their hosts, then the frequency of RFLP (Table 4) indicates that the nuclear ribosomal repeat region of the nuclear genome of parasites is diverging from that of its host at a more rapid rate than its mitochondrial genome. Although nothing is known of the relative rates of evolution of red algal nuclear, mitochondrial, and plastid genomes, it is known that the nuclear genome of flowering plants is evolving ~12 times more quickly (relative substitution rates) than the flowering plant mitochondrion. Likewise, the plastid genome of flowering plants is evolving approximately three times more rapidly than the mitochondrial genome and approximately four times more slowly than the nuclear genome (Palmer, 1992). If similar relative substitution rates do occur in red algal genomes, then the plastid genome might be expected to exhibit more variation and consequently more RFLP than the mitochondrial genome. Accordingly, the absence of any detectable RFLP in any of the parasite plastid genomes relative to their hosts provides additional support for the conclusion that the plastid genome seen in parasites is that of the host.

The ability of parasitic red algae to make direct cytoplasmic connections with host cells, inject nuclei into their hosts, and genetically transform their hosts is almost without parallel in biology. Only in mycoparasitic interactions between closely related parasitic fungi and their fungal hosts are similar interactions observed. Although some mycoparasites form direct cytoplasmic connections across their haustoria with the cytoplasm of the fungal hosts, these micropore channels are too small for organelle transmission between hosts and parasites (Bauer and Oberwinkler, 1990). It is only in the interactions of the zygomycetous mycoparasites *Parasitiella parasitica* and *Chaetocladium brefeldii* with their closely related hosts *Absidia glauca* and *A. caerulea* that parasite nuclei and presumably mitochondria are transferred into the cytoplasm of their specific host, where they function to transfer genetic information from parasite to host (Kellner et al., 1993). We still do not know whether a similar horizontal gene flow occurs between red algal parasites and their hosts.

METHODS

The parasites and hosts used in this study were collected from the locations listed in Table 5. These were collected during times of the year when epiphytes were minimal. After collecting, the few epiphytes present were removed by gentle (30-sec pulses) sonication. Parasite thalli were cut away from their hosts and frozen immediately in liquid nitrogen. For each DNA extraction, ~1000 to 3000 parasite individuals or ~1 to 5 g fresh weight of tissue was used. To obtain enough host mitochondrial DNA for the pulse gel electrophoresis analyses, ~3000 immature cystocarps were removed from female gametophytes of the host *Plocamium cartilagineum*. Only cystocarps with unpigmented

Table 5. Collecting Sites and Dates

Parasite and Host	Collection Date	Collection Site
Plocamiocolax pulvinata (parasite)	May 1988	Piegon Point, San Mateo County, CA
Plocamium cartilagineum (host)	June 1989	El Jarro Point, Santa Cruz County, CA
	June 1992	Salmon Bank, San Juan County, WA
Gracilariophila oryzoides (parasite)	June 1989	Pigeon Point, San Mateo County, CA
Gracilariopsis lemaneiformis (host)	June 1989	El Jarro Point, Santa Cruz County, CA
	June 1990	Sunset Beach, Coos Bay County, OR
	July 1992	Botany Beach, Port Renfrew, British Columbia
Gardneriella tuberifera (parasite)	July 1990	Stillwater Cove, Monterey County, CA
Sarcodiotheca gaudichaudii (host)	July 1990	Stillwater Cove, Monterey County, CA

gonimoblasts were used because these had the most mitochondria present. For each DNA extraction of host tissue, ~100 g (wet weight) of uninfected host species was used. Generally, only the tips of thalli or the youngest material was used because this reduced the amount of carbohydrates coextracted with the nucleic acids.

Nuclei acids were extracted using SDS-sarkosyl (1:1 sodium *N*-lauroylsarcosine) or sodium lauryl sulfate (IBI-Kodak, New Haven, CT) and phenol isolation procedures (Goff and Coleman, 1988) or by modified hexadecyltrimethylammonium bromide (CTAB) extraction procedures (Goff et al. 1994). Hoeschst 33258 (Sigma) cesium chloride gradients (Goff and Coleman, 1988) were used to separate DNA from the pelleted RNA and to separate the more GC-rich nuclear fraction from the more AT-rich upper fraction which contained primarily plastid DNA and contaminant mitochondrial DNA. The mitochondrial DNA banded just slightly above the plastid fraction and, because of its proximity, could not be separated cleanly from the plastid DNA.

After dialysis and removal of ethidium bromide (Sambrook et al., 1989), DNA fractions were restricted using Gibco BRL restriction endonucleases according to the manufacturer's recommended protocols. To facilitate cutting in DNA contaminated with polysaccharides, 2 to 5 μg of DNA was diluted in 200 μL of distilled water, and the appropriate restriction endonuclease buffer and enzyme and the fragments were precipitated by the procedures of Goff et al. (1992).

After electrophoresis in agarose gels (0.7% SeaKem LE; FMC Corp., Rockland, ME), DNA fragments were denatured and blotted onto nitrocellulose by using standard procedures (Sambrook et al., 1989), and ^{32}P-labeled probes were prepared by random priming (Boehringer Mannheim). Hybridization conditions were the same as presented by Goff et al. (1992).

Plastid probes used in this study were selected from a HindIII-HindIII library made from a fraction of *Gracilariopsis lemaneiformis* DNA containing primarily plastid DNA but also containing some contaminant mitochondrial DNA. These probes were screened against plastid DNA from a broad taxonomic assortment of red algae to obtain plastid probes for the hybridization comparisons of host and parasite plastid DNA restriction fragment length polymorphisms (RFLPs). Methods for polymerase chain reaction (PCR) amplification and sequencing of the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) plastid spacer are detailed in Goff and Moon (1993) and Destombe and Douglas (1991).

The mitochondrial genome of *Plocamiocolax* was cloned by digesting the mitochondrial DNA–enriched fraction from a Hoechst 33258–cesium chloride gradient with BamHI and EcoRI. The EcoRI-EcoRI fragments and the BamHI-EcoRI fragments were cloned either into a pBluescript SK+ vector, which had been digested with EcoRI and dephosphorylated using calf intestinal phosphatase (Stratagene), or into pBluescript SK+ cut with both EcoRI and BamHI. Clones containing mitochondrial fragments were delineated from clones containing plastid DNA by their hybridization to the large (~25,000 bp) putative mitochondrial band seen in BamHI digestion of the upper fraction from *Plocamiocolax* Hoechst 33258 gradients. In addition, an ~7500-bp HindIII-HindIII fragment of *G. lemaneiformis* mitochondrial DNA was identified from a shotgun (HindIII-HindIII) pUC library made from an upper Hoechst gradient fraction that contained both plastid DNA and mitochondrial DNA. This clone was identified as mitochondrial because it too hybridized to the long (~25,000-bp) mitochondrial fragment in BamHI-digested *Plocamiocolax* DNA. That these fragments from *Plocamiocolax* and *Gracilariopsis* were indeed mitochondrial was confirmed by probing the cloned fragments with DNA probes containing mitochondrial small subunit (SSU) sequences (from *Phytophthora infestans*) and cytochrome oxidase 1 (*cox1*; from *Brassica*).

Undigested mitochondrial DNA isolated from *Plocamiocolax* and immature cystocarps of its host *Plocamium cartilagineum* were pulse electrophoresed using a Bio-Rad CHEF-DRII apparatus. Approximately 1 to 2 μg of mitochondrial DNA was loaded per lane in a 1% GTE SeaKem agarose (FMC Corp.) gel made up in 0.5 × Tris-borate-EDTA buffer (Sambrook et al., 1989). The pulse and run time and voltage required to separate maximally fragments 20 to 60 kb in length were determined empirically using a λ 50-kb ladder (Stratagene) and a high molecular weight DNA ladder (Gibco BRL). The optimal voltage was determined to be 240 V, with cooling at 16°C with a 3:1 forward-to-reverse pulse ratio. The mitochondrial DNA of *Plocamiocolax* was spread using the techniques of Kleinschmidt (1968) and imaged using a Jeol 100-S electron microscope as described by Goff and Coleman (1988).

ACKNOWLEDGMENTS

We thank Elisabeth Harris (Duke University), Michael Mulligan (University of California, Irvine), William Thompson (North Carolina State University, Raleigh), and Franz Lang (University of Montreal, Quebec) for gifts of DNA probes used in this study. Thanks are also extended to the late Will Bentham (University of California, Berkeley) for his assistance in DNA spreading. Funds for this study were provided by the National Science Foundation (No. DEB-9355121) to L.J.G.

Received May 15, 1995; accepted September 12, 1995.

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