

The Secondary Endosymbiont of the Cryptomonad *Guillardia theta* Contains Alpha-, Beta-, and Gamma-Tubulin Genes

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Cryptomonads have acquired photosynthesis through secondary endosymbiosis: they have engulfed and retained a photosynthetic eukaryote. The remnants of this autotrophic symbiont are severely reduced, but a small volume of cytoplasm surrounding the plastid persists, along with a residual nucleus (the nucleomorph) that encodes only a few hundred genes. We characterized tubulin genes from the cryptomonad *Guillardia theta*. Despite the apparent absence of microtubules in the endosymbiont, we recovered genes encoding alpha-, beta-, and gamma-tubulins from the nucleomorph genome of *G. theta*. The presence of tubulin genes in the nucleomorph indicates that some component of the cytoskeleton is still present in the cryptomonad symbiont despite the fact that very little cytoplasm remains, no mitosis is known in the nucleomorph, and microtubules have never been observed anywhere in the symbiont. Phylogenetic analyses with nucleomorph alpha- and beta-tubulins support the origin of the cryptomonad nucleomorph from a red alga. We also characterized alpha and beta-tubulins from the host nucleus of *G. theta* and compared these with tubulins we isolated from two flagellates, *Goniomonas truncata* and *Cyanophora paradoxa*, previously proposed to be related to the cryptomonad host. Phylogenetic analyses support a relationship between the cryptomonad host and *Goniomonas* but do not support any relationship between cryptomonads and *Cyanophora*.

Introduction

All plastids arose by endosymbiosis, but there have been two fundamentally different types of plastid origin. Primary plastids (which are found in land plants, green algae, red algae, and glaucocystophytes) originate from the uptake of a cyanobacterium by a heterotrophic eukaryote. Secondary plastids were acquired by the subsequent uptake of one of these primary phototrophs by a second eukaryote, presumably a phagotroph. Secondary plastids are found in Euglenozoa, dinoflagellates, Apicomplexa, heterokonts, haptophytes, chlorarachniophytes, and cryptomonads. In most instances, secondary plastids are only recognizable by the presence of more than two surrounding membranes, all other traces of the engulfed phototroph having disappeared. Cryptomonads and chlorarachniophytes are exceptional, though, as the reduction of the endosymbiotic algae has been less complete. In these lineages, the algal endosymbiont retains a small portion of cytoplasm and a remnant nucleus known as the nucleomorph (see Gibbs [1992] and McFadden and Gilson [1995] for reviews). Although chlorarachniophyte and cryptophyte symbionts are still recognizable as eukaryotic symbionts, they are severely reduced compared to their free-living algal ancestors. Nucleomorph genomes, for instance, contain only about 10% as much DNA as the smallest genome

of free-living eukaryotes and encode only a few hundred genes (McFadden et al. 1997). Reduction of the symbionts has also eliminated any visible trace of an endomembrane system (except for the nucleomorph), mitochondria, peroxisomes, a cell wall, or a cytoskeleton. The apparent absence of a cytoskeleton in these endosymbionts is most unusual, since components of the cytoskeleton are integral to so many essential activities of eukaryotic cells. Nevertheless, comprehensive electron microscopic surveys of both chlorarachniophyte and cryptomonad endosymbionts reveal no trace of classical cytoskeletal elements such as microtubules, actin, or intermediate filaments (Gillott and Gibbs 1980; McKerracher and Gibbs 1982; Morrall and Greenwood 1982; Ludwig and Gibbs 1989). This is particularly striking in the case of nucleomorph division, which occurs without a discernible spindle. This has led to the belief that nucleomorph division takes place amitotically and that the endosymbionts no longer use microtubules (McKerracher and Gibbs 1982; Morrall and Greenwood 1982; Ludwig and Gibbs 1989). To address the question of whether cryptomonad endosymbionts function without a cytoskeleton, we characterized genes encoding tubulins, the major microtubule proteins, from the cryptomonad *Guillardia theta*. We identified genes for each of the three tubulin paralogs, alpha-, beta-, and gamma-tubulin, from the nucleomorph genome of *Guillardia*. The presence of these genes in the nucleomorph strongly suggests that the endosymbiont utilizes some form of microtubule or microfilament structure at some stage of its life cycle. Phylogenetic analyses show that the *Guillardia* nucleomorph tubulins are most closely related to those of red algae, confirming previous hypotheses, based on pigments and gene sequences, that the cryptomonad endosymbiont was a red alga. We also characterized tubulin genes from the nuclear (host) genome

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of *Guillardia*, and we compare these genes to tubulin genes from flagellates thought to be related to cryptomonads, *Goniomonas truncata*, and *Cyanophora paradoxa* (Kugrens and Lee 1991; McFadden, Gilson, and Hill 1994; Bhattacharya et al. 1995; Fraunholz et al. 1997).

Materials and Methods

Strains, Culture Conditions, and DNA Preparations

Guillardia theta (Hill et Wetherbee) strain CCMP 327 (Bigelow Culture Collection of Marine Phytoplankton) was grown in H/2 seawater medium, and DNA was prepared as previously described (Deane et al. 1998). *Goniomonas truncata* (Fresenius) cells were provided by D. R. A. Hill. Cultures were grown in WARIS-H medium, and DNA was prepared as previously described (McFadden, Gilson, and Hill 1994). *Cyanophora paradoxa* (Pringsheim) strain UTEX LB555 (University of Texas Collection of Algae) was grown in mineral medium, and nuclear DNA was isolated as previously described (Bohnert et al. 1982). *Cyanophora* mRNA was isolated using Oligotex mRNA isolation resin (QIAGEN) according to the manufacturer's protocols. Supplementary DNA was also generously donated by D. Bhattacharya. The *Cyanophora* cDNA-library was constructed using the Uni-ZAP XR cDNA Synthesis kit (Stratagene) and packaged into Lambda-phage with Gigapack II Gold Packaging Extract (Stratagene). Phage and phagemid manipulations were carried out according to the manufacturer's protocols.

Identification and Sequencing of Novel Tubulin Genes

With the exceptions noted below, all alpha-tubulin genes were amplified with the primers TCCGAATTCA-RGTNGGNAAYGCNGGGYTGGGA and CGCGCCAT-NCCYTCNCCNACRTACCA and all beta-tubulin genes were amplified with the primers GCCTGCAGG-NCARTGYGGNAAYCA and TCCTCGAGTRAAYT-CCATYTCRTCCAT. *Goniomonas* alpha-tubulin was amplified using GGGCCCCAGGTCGGCAAYGCNTG-YTGG and GGGCCCCGAGAACTCSCCYTCYTCAT. Beta-tubulin sequences from the *Guillardia* nucleomorph were amplified in two overlapping pieces, first with the primers GATACCGTNGTNGARCCNTAYAA and TCCTCGAGTRAAYTCCATYTCRTCCAT for the 3' end, and then with GCCTGCAGGNCARTGYGGN-AAYCA with a gene-specific primer for the remainder. *Cyanophora* alpha-tubulin and *Goniomonas* beta-tubulin 2 fragments were amplified using the primer combinations GATACCGTNGTNGARCCNTAYAA and CGCGCCATNCCYTCNCCNACRTACCA, and GATACCGTNGTNGARCCNTAYAA and TCCTCGAGTRAAYT-CCATYTCRTCCAT, respectively. All amplification reactions consisted of 35 cycles with an annealing temperature of 50°C and an extension time of 1.5 min. PCR products were purified from agarose gels as previously described (Keeling and Doolittle 1996) and cloned into pGEM-T vector (Promega). Sequencing was carried out on an ABI 373a automated sequencer using dye-terminator chemistry (Perkin Elmer). The *G. theta* nucleomorph

gamma-tubulin was encoded within a 4.3-kb *Xba*I fragment isolated from a nucleomorph chromosome I library constructed in pUC18. The *C. paradoxa* beta-tubulin was isolated from a cDNA screen for peptidoglycan synthesis genes by complementation in *Escherichia coli*. One clone from this screen was sequenced and found not to encode a peptidoglycan synthesis gene, but to encode beta-tubulin instead. The complete sequence of this clone was determined as above.

Phylogenetic Analysis

New tubulin amino acid sequences were added to alignments of known homologs, and phylogenetic trees were inferred using distance and maximum likelihood by quartet puzzling. Distances were calculated using the Dayhoff PAM250 substitution matrix by PROTDIST using PHYLIP 3.57c (Felsenstein 1993). In addition, maximum-likelihood distances were calculated using PUZZLE 4.0 with the JTT correction matrix and the frequency of amino acid usage estimated from the data. Site-to-site rate variation was modeled on a gamma distribution with invariant sites plus six rate categories and the shape parameter estimated from the data (Strimmer and von Haeseler 1996). Trees were inferred from these distances using neighbor joining and Fitch-Margoliash analyses (Felsenstein 1993). In Fitch-Margoliash analyses, the addition sequence was jumbled 10 times, and the global rearrangements option was also used. Protein distance trees were also inferred from 100 bootstrap replicates as described above, except that the sequence addition order was randomized five times in Fitch-Margoliash trees. Maximum-likelihood trees were inferred using quartet puzzling with PUZZLE 4.0. All data sets were analyzed by 1,000 puzzling steps, with the conditions described for maximum-likelihood distance calculations.

Results and Discussion

Alpha-, Beta-, and Gamma-Tubulin Genes from *Guillardia*

Amplification of alpha- and beta-tubulin-coding regions from *Guillardia* yielded two distantly related classes of genes with characteristics strongly suggesting that one class derived from the nucleus (host) and the other derived from the nucleomorph (endosymbiont). The first class of genes was not AT-rich (about 50% AT) and not particularly divergent, and the beta-tubulin contained spliceosomal introns, altogether suggesting that this class of genes is encoded in the nucleus. The second class resembled nucleomorph genes in being markedly AT-rich (about 65% AT), very divergent compared with other tubulins, and free of introns. Probing Southern blots of pulsed field-separated *Guillardia* chromosomes, combined with genomic sequencing of the nucleomorph (data not shown), demonstrated that nucleomorph chromosome II carries an alpha tubulin gene and nucleomorph chromosome III carries a beta tubulin gene. A third tubulin gene on chromosome I of the nucleomorph of *Guillardia* was also uncovered by the nucleomorph genome sequencing project. Although this gene is ob-

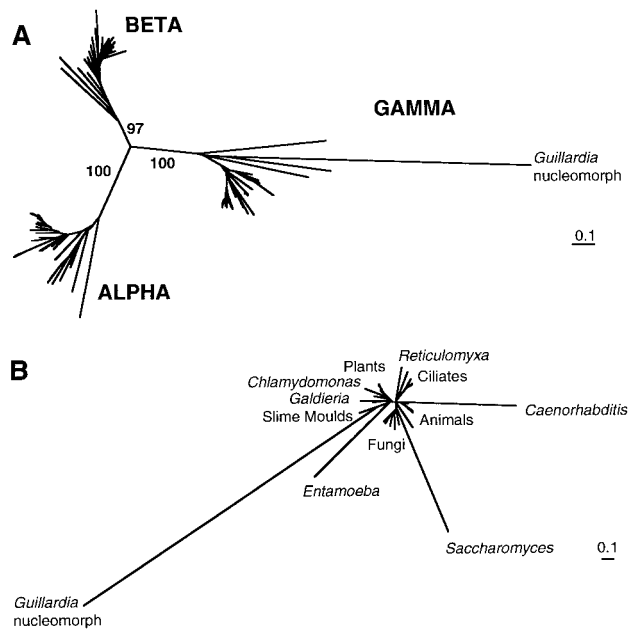


FIG. 1.—Extreme divergence of the *Guillardia* nucleomorph gamma-tubulin. *A*, Neighbor-joining tree of 92 alpha-, beta-, and gamma-tubulins, showing the very high level of divergence characterizing the gamma-tubulin from the nucleomorph (labeled). The numbers at the base of each subfamily correspond to bootstrap support for that subfamily, and the support for the *Guillardia* nucleomorph sequence in question branching with the gamma-tubulins is 100%. *B*, Fitch-Margoliash tree of gamma-tubulins based on maximum-likelihood distances with correction for site-to-site rate variation. The nucleomorph sequence shows no affinity to the red algal sequence (*Galdieria*), even though the other extremely divergent sequences from *Saccharomyces* and *Caenorhabditis* do fall with fungi and animals, respectively, as expected.

viously a member of the tubulin family, it is so divergent that it is difficult to assign it to a particular subfamily. Phylogenetic analyses with representatives of all three tubulins suggest that this third gene is a gamma-tubulin (fig. 1A), but one of extraordinary structure. Indeed, although this *Guillardia* gene branches strongly with the gamma-tubulin subtree, the evolutionary distance between the *Guillardia* gamma-tubulin and other gamma-tubulins is greater even than the distance between each of the tubulin families. In trees of gamma-tubulin alone (fig. 1B), the *Guillardia* nucleomorph gene did not branch with the gamma-tubulin from the red alga *Galdieria*, but nearly always branched with other highly divergent sequences from *Saccharomyces* and *Caenorhabditis*. This is almost certainly an artifact of the extraordinary divergence of these three sequences, since the very weak phylogenetic signal uniting the divergent *Saccharomyces* and *Caenorhabditis* with fungi and animals, respectively, can be recovered by removing other divergent taxa (Keeling and Doolittle 1996) or by correcting maximum-likelihood distances for site-to-site rate variation (fig. 1B). However, even under these conditions, the *Guillardia* gene does not branch with *Galdieria*. Gamma-tubulins are often the most divergent of the three tubulin families, but the divergence level of the nucleomorph gamma-tubulin surpasses even that of the highly unusual *Saccharomyces* and *Caenorhabditis*

genes. This divergence appears to have extinguished any phylogenetic signal beyond simply showing that this gene is a gamma-tubulin.

Tubulins from *Goniomonas* and *Cyanophora* and the Phylogeny of Alpha- and Beta-Tubulins

In contrast to gamma-tubulin, both alpha- and beta-tubulins have been sampled widely, and the phylogeny of these genes has been useful for determining many eukaryotic relationships (Baldauf and Palmer 1993; Edlind et al. 1996; Keeling and Doolittle 1996; Keeling, Deane, and McFadden 1998). Having identified both the host and the endosymbiont alpha- and beta-tubulins from *Guillardia*, we decided to investigate how these tubulins, each derived from a different eukaryotic lineage via secondary endosymbiosis, relate to tubulin genes of other eukaryotes. The symbiont of cryptomonads is thought to be derived from a red alga (Gantt 1979; Gilloft and Gibbs 1980; Douglas et al. 1991; Maier et al. 1991; McFadden, Gilson, and Hill 1994; Cavalier-Smith et al. 1996; Van de Peer et al. 1996), and a variety of evolutionary positions have been proposed for the host (e.g., Roberts, Stewart, and Mattox 1981; Gilliott 1990; Douglas et al. 1991; Cavalier-Smith, Allsopp, and Chao 1994; Häuber et al. 1994). Two lineages repeatedly suggested to be close relatives of cryptomonad hosts are *G. truncata* and glaucocystophytes.

Goniomonas is a heterotrophic, plastid-lacking flagellate with strong morphological resemblance to cryptomonads (Kugrens and Lee 1991), and trees inferred from rRNA sequences demonstrate that *Goniomonas* is a close relative of the cryptomonad host lineage (McFadden, Gilson, and Hill 1994). Various analyses of nuclear rRNA sequences also ally the glaucocystophyte *Cyanophora* with the cryptomonad host, perhaps suggesting a phylogenetic link (Bhattacharya et al. 1995; Cavalier-Smith et al. 1996; Van de Peer et al. 1996; Bhattacharya and Schmidt 1997; Cavalier-Smith and Chao 1997); however, the alliance in rRNA trees is weak, and there are no biochemical or morphological features to ally cryptomonads and glaucocystophytes. Likewise, analyses of Hsp70 sequences did not confirm any close relationship between cryptomonads and glaucocystophytes (Rensing et al. 1997). To test whether tubulins from either or both of these organisms are closely related to *Guillardia* host tubulins, we isolated both alpha- and beta-tubulin sequences from *G. truncata* and *C. paradoxa*. Single copies of each of alpha- and beta-tubulin were found in *Cyanophora*, and a single *Goniomonas* alpha-tubulin was found. Two copies of beta-tubulin were amplified from *Goniomonas*, one highly divergent and a second more conserved. Despite their divergence, both of the *Goniomonas* beta-tubulin sequences group together in trees, suggesting that they evolved recently in the *Goniomonas* lineage and that one (*Goniomonas* beta 1) has an accelerated rate of substitution.

Phylogenetic trees of alpha- and beta-tubulins, including a broad representation of eukaryotes and our new sequences, are shown in figs. 2 and 3, respectively.

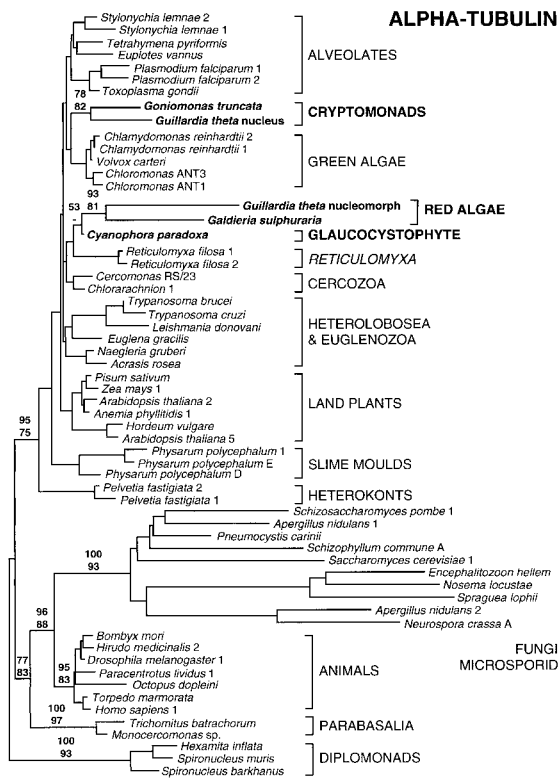


FIG. 2.—Alpha-tubulin tree of 60 taxa, with sequences from cryptomonads, red algae, and *Cyanophora* shown in bold. Numbers at nodes correspond to Fitch-Margoliash bootstrap percentages (top) and percentages of occurrence in 1,000 quartet puzzling steps (bottom), and dashes represent support less than 50%. These numbers are only shown at major nodes and nodes important to this study. A Fitch-Margoliash tree of protein distances is shown. In other analyses, the overall structure of the tree and the strongly supported features are maintained, including the general positions of the *Guillardia*, *Goniomonas*, and *Cyanophora* sequences.

The overall topology of alpha- and beta-tubulin trees is quite similar: diplomonads and parabasalia branch independently from other organisms (although the relative positions of the two groups vary between the two molecules) and the microsporidia emerge from the fungi, and together these are a sister group to animals, and the remainder of the sequences shown (with the exception of the red algae as described below) form a large clade that includes alveolates, plants, green algae, Euglenozoa and Heterolobosea, heterokonts, and Cercozoa. The *Guillardia* nuclear tubulins also branch within this large and heterogeneous clade, and—not surprisingly—are most closely related to those of *Goniomonas* in all analyses (figs. 2 and 3). Conversely, in no analysis of either molecule does *Cyanophora* show any affinity for the cryptomonad clade, instead branching weakly with the alveolates in all analyses of beta-tubulin (fig. 3) and, perhaps more believably, with the red algae in alpha-tubulin trees (fig. 2). Although there is only weak statistical support for the position of *Cyanophora* in either tree, there is no evidence for a relationship with the cryptomonad host (figs. 2 and 3).

The nucleomorph alpha- and beta-tubulins present quite different and interesting stories. Both genes are

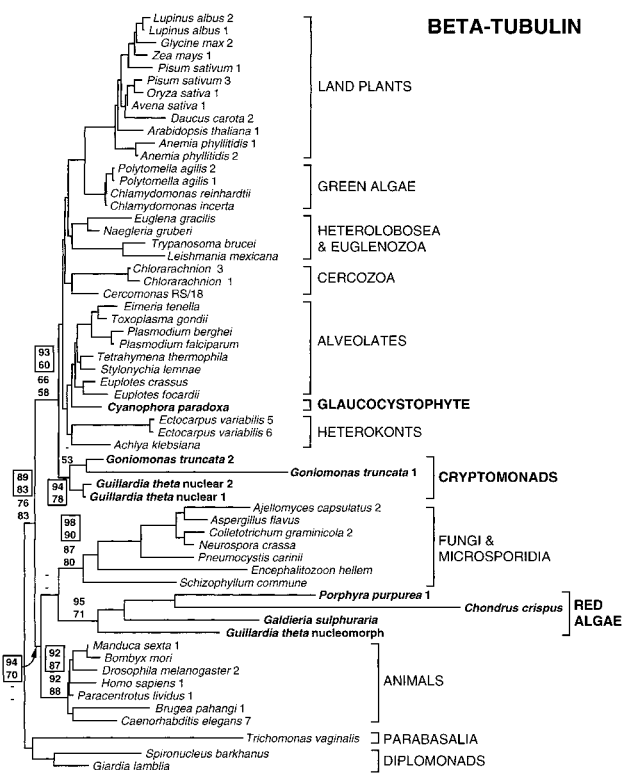


FIG. 3.—Beta-tubulin tree of 60 taxa, with cryptomonads, red algae, and *Cyanophora* shown in bold. *Porphyra* beta-tubulin genes 2–4 are excluded, but when they are included they branch with heterokonts in all analyses. Details of tree construction and the meanings of the numbers at nodes are as in figure 2. Numbers in boxes correspond to the significance values found when the three divergent red algal sequences, the *Guillardia* nucleomorph tubulin, and *Goniomonas* beta-tubulin 1 are excluded from the analyses.

divergent relative to most other tubulins, but the alpha-tubulin is markedly less so. Reflecting this, the phylogenetic position of the nucleomorph alpha-tubulin is quite straightforward: it branches strongly with the red alga *Galdieria*, and together these branch within the large and heterogeneous clade, although it is not strongly related to green algae and land plants as might be expected (fig. 2). The nucleomorph beta-tubulin phylogeny is more complicated. It also branches with red algal genes from *Porphyra*, *Chondrus*, and *Galdieria* (fig. 3), confirming once again the red algal ancestry of the nucleomorph tubulins. However, the position of this clade as a sister group to the fungal tubulins is unexpected and is likely an artifact of long-branch attraction. Indeed, the presence of these four divergent red algal/cryptomonad endosymbiont sequences adversely affects support for surrounding nodes, even nodes previously shown to be quite sound in beta-tubulin trees (Baldauf and Palmer 1993; Edlind et al. 1996; Keeling and Doolittle 1996; Keeling, Deane, and McFadden 1998). Accordingly, we removed these four red algal/cryptomonad endosymbiont sequences (as well as *Goniomonas* beta-tubulin 1, which is also very divergent) and repeated the analyses. The result of this pruning was a tree with much the same topology but considerably higher support values from bootstrapping and quartet puzzling (see boxed

values in fig. 3). This was especially true for nodes uniting animals and fungi and for those uniting the large and heterogeneous clade to which red algal alpha-tubulins belong. It is probable that the high divergence of the red algal sequences leads to their branching with the fungi because the fungal tubulins are also more divergent than most. In support of this hypothesis, the red algae do not branch with fungi when the more conservative sequences from chytrid fungi are used (unpublished data), and they branch with the green algae and land plants when only the red algae and the large and heterogeneous clade are used (data not shown). It appears that these divergent sequences, although clearly representing a red algal lineage, are not useful for reliably determining the position of red algae in the beta-tubulin tree. These uncertainties notwithstanding, the strong support for the inclusion of the *Guillardia* beta-tubulin as sister to this clade of red algal genes does show that the nucleomorph beta-tubulin retains evidence of its red algal ancestry.

In addition to the red algal and nucleomorph genes shown in figure 3, there are three additional *Porphyra* beta-tubulins in GenBank (accession numbers Z67992–Z67994). These genes are markedly less divergent than the other red algal genes, and in all phylogenetic analyses in which they were included, they branched specifically with the heterokonts (data not shown; see, e.g., Keeling, Deane, and McFadden 1998). This relationship to heterokont tubulins is suspicious, and no closely related paralog has been found in *Chondrus*, *Galdieria*, or the *Guillardia* nucleomorph. The origin of these genes from *Porphyra* is therefore questionable, and they have accordingly been excluded. If, however, these genes are from *Porphyra*, then it is interesting that they do not show the same accelerated substitution rate as other red algal beta-tubulins.

The Role of Tubulins in the Cryptomonad Endosymbiont

The process of nucleomorph division has been examined in some detail for various cryptomonads (McKerracher and Gibbs 1982; Morrall and Greenwood 1982). These studies have shown that neither chromatin nor spindles are present and support the conclusion that the genome is segregated amitotically. Indeed, in all of the many ultrastructural characterizations of cryptomonad endosymbionts, microtubules have never been found and are presumed lost. Contrary to these expectations, we have shown here that genes encoding all three paralogs of the tubulin family are found in the *Guillardia* nucleomorph genome. It is difficult to argue that three intact but nonfunctional genes would be maintained in a genome primarily distinguished by its severe reduction in content, so the presence of these genes in the nucleomorph must indicate that tubulins are essential to the endosymbiont. The discovery of these three genes begs the question: what are they doing? As we have mentioned, endosymbiont division has been studied at the ultrastructural level, and no microtubules have been observed. Moreover, when cryptomonads were treated with the microtubule polymerization-inhibitor colchi-

cine, host nuclear division was arrested but nucleomorph division proceeded unperturbed (McKerracher and Gibbs 1982), again suggesting that microtubules are not required for nucleomorph division. Furthermore, the two resulting nucleomorphs appear to be housed in two distinct symbionts, suggesting that the cytokinesis of the symbiont had also proceeded unperturbed (S. P. Gibbs, personal communication). These studies do seem to indicate that nucleomorph and symbiont division are independent of microtubules, but it is very difficult to predict whether the extremely divergent nucleomorph tubulins would even be susceptible to colchicine. It is also possible that these tubulin proteins are making microtubules at some other stage of the endosymbiont life cycle, or they could be making some structure other than a microtubule (such as a filament). In any case, our identification of tubulin genes in the cryptomonad endosymbiont genome provides renewed impetus for exploring the structure and biological roles of these tubulins in this vestigial cell.

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