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Mitochondrial Genome of a Tertiary Endosymbiont Retains Genes for Electron Transport Proteins

BEHZAD IMANIAN,[a] KEVIN J. CARPENTER[b] and PATRICK J. KEELING[b]

[a]*Genetics Graduate Program, Department of Botany, University of British Columbia, 3529–6270 University Boulevard, Vancouver, BC, Canada V6T 1Z4, and*

[b]*Department of Botany, Canadian Institute for Advanced Research, University of British Columbia, 3529-6270 University Boulevard, Vancouver, BC, Canada V6T 1Z4*

ABSTRACT. Mitochondria and plastids originated through endosymbiosis, and subsequently became reduced and integrated with the host in similar ways. Plastids spread between lineages through further secondary or even tertiary endosymbioses, but mitochondria appear to have originated once and have not spread between lineages. Mitochondria are also generally lost in secondary and tertiary endosymbionts, with the single exception of the diatom tertiary endosymbiont of dinoflagellates like *Kryptoperidinium foliaceum,* where both host and endosymbiont are reported to contain mitochondria. Here we describe the first mitochondrial genes from this system: cytochrome c oxidase 1 (*cox1*), cytochrome oxidase 3 (*cox3*), and cytochrome b (*cob*). Phylogenetic analyses demonstrated that all characterized genes were derived from the pennate diatom endosymbiont, and not the host. We also demonstrated that all three genes are expressed, that *cox1* contains spliced group II introns, and that *cob* and *cox3* form an operon, all like their diatom relatives. The endosymbiont mitochondria not only retain a genome, but also express their genes, and are therefore likely involved in electron transport. Ultrastructural examination confirmed the endosymbiont mitochondria retain normal tubular cristae. Overall, these data suggest the endosymbiont mitochondria have not reduced at the genomic or functional level.

Key Words. Cytochrome b, cytochrome c oxidase 1, cytochrome oxidase 3, endosymbiosis, *Kryptoperidinium foliaceum*, reductive evolution, type II introns, ultrastructure.

IT is now well established that mitochondria and plastids (chloroplasts) arose through the endosymbiotic uptake of an α-proteobacterium and cyanobacterium, respectively (Archibald and Keeling 2002; Gray, Burger, and Lang 1999; Palmer 2003). In the case of the mitochondrion, this is thought to have happened very early in eukaryotic evolution, and no extant eukaryote is believed to have originated before the mitochondrial endosymbiosis (Gray et al. 1999). Plastids arose recently relative to mitochondria, in the ancestor of glaucophytes, red algae, green algae, and plants (Archibald and Keeling 2002; Bhattacharya, Yoon, and Hackett 2004; McFadden 2001). This endosymbiosis was only the beginning of plastid evolution, however, because plastids subsequently spread between eukaryotic groups by secondary endosymbiotic events, in which eukaryotic algae are themselves taken up by other eukaryotes and undergo reduction so that all that typically remains is the plastid (Archibald and Keeling 2002; Bhattacharya et al. 2004; McFadden 2001). The endosymbiotic histories of plastids and mitochondria share a good deal in common, such as the way the endosymbiont genome became reduced and the nature of the host-to-organelle targeting system that evolved (McFadden 2001). However, the secondary spread of plastids marks one major difference between the evolution of the two organelles; secondary endosymbiosis played a significant role in the evolution of plastid diversity, but there is no known case of the secondary endosymbiotic uptake of a mitochondrion.

Dinoflagellate algae have taken plastid evolution one step further. Dinoflagellates typically have a secondary plastid derived from a red alga (Fast et al. 2001; Zhang, Green, and Cavalier-Smith 2000), but certain lineages have lost or degraded this plastid, and acquired a new one from either another primary alga (serial secondary endosymbiosis) or another secondary alga (tertiary endosymbiosis). These complex cells are therefore like matryoshka dolls; in the most extreme cases, endosymbiotic plastids are found within a eukaryote (the primary alga), which is within another eukaryote (the secondary alga), which is itself within the dinoflagellate. *Gymnochlora* has a serial secondary plastid derived from a green alga (Ishida et al. 1997), whereas tertiary plastids have originated at least three times independently: *Karenia* and *Karlodinium* have plastids derived from a haptophyte (Tengs et al. 2000); *Dinophysis* has a plastid derived from a cryptophyte (Hewes et al. 1998; Schnepf and Elbraechter 1988; Hackett et al. 2003); and *Kryptoperidinium* and several related genera have a plastid derived from a diatom (Chesnick et al. 1997; Inagaki et al. 2000; Tamura, Shimada, and Horiguchi 2005). As is the case with secondary endosymbiosis, the tertiary endosymbiont is typically highly reduced; in most cases the plastid itself, and perhaps one or more extra membranes, are all that remain to indicate what has taken place.

The one exception to this is the diatom endosymbiont of *Kryptoperidinium* and its close relatives, which marks an intermediate stage in endosymbiont reduction. Integrating an endosymbiont into a host cell is a complex process, and different levels of integration are seen in different photosynthetic-based partnerships, ranging from transient associations (e.g. Fields and Rhodes 1991; Lewitus, Glasgow, and Burkholder 1999; Rumpho et al. 2001), to complex adaptations between cells that remain able to live on their own (e.g. *Hatena* (Okamoto and Inouye 2005)), to fully integrated organelles where neither host nor endosymbiont can survive without the other. The diatom endosymbiont of *Kryptoperidinium* and its close relatives falls near the end of this spectrum: it is essential for and dependent on its host; it is found throughout all the stages of cell cycle; and its division is closely linked to the division of the host (Chesnick and Cox 1989; Tippit and Pickett-Heaps 1976). It is stable through evolutionary time and predates the divergence of a number of closely related genera. In addition to *Kryptoperidinium foliaceum* (Dodge 1971; Jeffrey and Vesk 1976) and *Durinskia baltica* (Carty and Cox 1986), this includes *Gymnodinium quadrilobatum* (Horiguchi and Pienaar 1994), *Podolampas bipes*, which accommodates several endosymbionts rather than just one (Schweikert and Elbraechter 1999), *Amphisolenia thrinax* and *Amphisolenia bidentata* (Lucas 1991), *Peridinium quinquecorne* (Horiguchi and Pienaar 1991), and *Galeidinium rugatum* (Tamura et al. 2005). In all cases, the endosymbiont has lost several features (e.g. the cell wall and motility) and has generally been structurally transformed so it no longer resembles free-living diatoms. What sets it apart from other endosymbionts, however, is not what it has lost, but what it has

Corresponding Author: P. J. Keeling, Department of Botany, Canadian Institute for Advanced Research, University of British Columbia, 3529-6270 University Boulevard, Vancouver, BC, Canada, V6T 1Z4—Telephone number: +1 604 822 4906; FAX number: +1 604 822 6089; e-mail: pkeeling@interchange.ubc.ca

retained. In addition to the plastid, the endosymbiont retains a nucleus with a genome and, most interestingly, mitochondria.

In many ways, *K. foliaceum* is one of the most complex cells known. It is currently composed of five or six genome-containing compartments (Fig. 1), the uncertainty being the original plastid, which is believed to have been retained and converted to an eyespot (Dodge 1983). Altogether, if one traces back through the complex history of endosymbiosis that led to this cell, there are footprints of no less than 10 distinct genomes that contributed to this cell.

The retention of mitochondria, however, is the most exceptional characteristic of the *K. foliaceum* endosymbiont, because if the endosymbiont is sufficiently integrated to be considered an organelle, then this is the only known example of a eukaryote with two evolutionarily distinct mitochondria. In other secondary and tertiary endosymbiotic events, the mitochondrion is lost, so this is assumed to be one of the first steps in endosymbiotic reduction. Outside of these eukaryote-derived organelles, however, this is unique, as mitochondria are not known to have been lost outright in any other eukaryote. Even in anaerobic, highly reduced parasites that were formerly believed to have lost their mitochondria or to have evolved before the endosymbiosis from which mitochondria originated, relic organelles have now been found (Bui and Johnson 1996; Roger 1999; Williams et al. 2002; Williams and Keeling 2003). In these cases, electron transport and oxidative phosphorylation have been lost, as has the genome, but the organelle has been retained to carry out other activities, such as iron–sulphur cluster assembly (Tachezy, Sanchez, and Müller 2001; van der Giezen, Tovar, and Clark 2005; Williams and Keeling 2003). Interestingly, the dinoflagellate host mitochondria are also unusual. They have the most reduced mitochondrial genomes known in terms of their gene contents, encoding only three genes: cytochrome c oxidase subunit 1 (*cox1*), cytochrome c oxidase subunit 3 (*cox3*), and cytochrome b (*cob*). These genes are dispersed on several DNA fragments and are subject to extensive RNA editing (Lin et al. 2002; Zhang and Lin 2005).

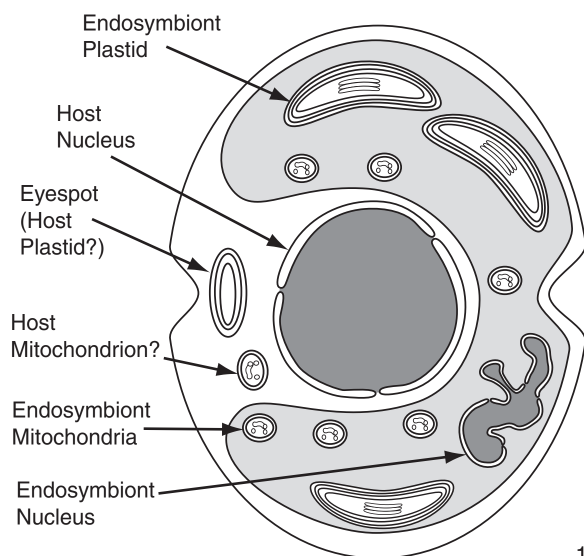

Fig. **1.** Schematic view of the genome-containing organelles in the dinoflagellate *Kryptoperidinium foliaceum*. In addition to the host dinoflagellate nucleus (the dinokaryon) and the large and branched nucleus of the diatom endosymbiont, there are potentially four organelles derived through endosymbiosis. Ironically, the two most obvious and abundant, the multiple plastids and mitochondria, are in the endosymbiont. The host eyespot has been hypothesized to be a relic of the three-membrane peridinin-containing plastid characteristic of dinoflagellates. Genomes have been found in the two nuclei, the plastid, and here we show that the endosymbiont mitochondrion retains a genome, but the putative host plastid and mitochondrial genomes have not been identified.

In order to investigate the nature of the *K. foliaceum* endosymbiont mitochondria, their relationship to host mitochondria, and the reasons for their retention, we characterized *K. foliaceum* homologues of the three genes presently known to have been retained by dinoflagellate mitochondrial genomes. We found one copy of all three genes in *K. foliaceum*, and surprisingly, phylogenetic analyses, along with the identification and characterization of two group IIA introns within the *cox1* gene, reveal that all three genes originated from the tertiary endosymbiont mitochondria, and not those of the host. We show that all three genes are expressed, two of them (*cob* and *cox3*) forming part of an operon. The presence and expression of these three genes suggest the endosymbiont mitochondria retain electron transport, and thus are functional in energy generation. This is unexpected given the apparent age and level of integration of this endosymbiont, and raises the intriguing possibilities that the two distinct mitochondria share functions, or that the host mitochondrion might have attenuated function. These hypotheses are corroborated by ultrastructural data, which show endosymbiont mitochondria are common and have well-developed cristae whereas host mitochondria were not observed and are therefore potentially rare.

MATERIALS AND METHODS

Culture conditions, DNA and RNA extraction, amplification, and sequencing. Cultures of *Kryptoperidinium foliaceum* CCMP 1326 were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (West Boothbay Harbor, ME) and maintained in F/2-Si medium at 22 °C/19 °C (13:11 light:dark cycle). Cultures were grown both with and without antibiotics to reduce the number of bacteria: 500 μg/ml penicillin G, 200 μg/ml ampicillin, 50 μg/ml streptomycin sulphate, and 50 μg/ml neomycin, modified from (Kite, Rothschild, and Dodge 1988). Cultures for electron micsroscopy did not include antibiotics, while cultures used in some molecular experiments did while others did not. Exponentially growing cells were harvested by centrifugation at 3,220 *g* for 5 min at 8 °C, and the pellet was frozen and ground under liquid nitrogen. The total genomic DNA was extracted from about 100 mg of the ground cells using DNeasy Plant DNA isolation kit (Qiagen, Mississauga, ON). Total RNA was isolated using TRIzol Reagent (Invitrogen, Burlington, ON) from the pelleted cells following manufacturer's instructions, and it was treated with Deoxyribonuclease I (Invitrogen, Burlington, Ontario). Polymerase chain reaction (PCR) was carried out using PuReTaq (Amersham Biosciences, Baie d'Urfé, QC) and long range PCR using Elongase Enzyme Mix (Invitrogen). Reverse-transcriptase polymerase chain reaction (RT-PCR) was carried out using SuperScript III One-Step System with platinum Taq DNA polymerase (Invitrogen).

Amplification of *cob* and *cox3* from genomic DNA used the following degenerate primers: for *cob*, 5′-CAGATGTCGTTTTGGGGNGCNACNGTNATHAC-3′ and 5′-GGGGAGGAAGTACCAYTCNGGNACDATRTG-3′ and for *cox3*, 5′-TTCCACCTTGTTGACCCNWSNCCNTGGCC-3′, and 5′-CCAAGCTGCCGCCTCRAANCCRAARTGRTG-3′. Transcripts of both genes were characterized by RT-PCR using exact-match primers: for cob 5′-ACAGCAATTCCATTCGGAGGTCAAACAATC-3′ and 5′-CTGGAATACAATTATCAGGATGGTTCAAAA-3′ and for *cox3* 5′-TTACAGGTGGTGTTCTTTATATGCACAAAA-3′ and 5′-AGCCGAAGTGGTGGGTATTTGTTGAGTGGT-3′. Long-range PCR and RT-PCR using DNA-free RNA were also used to amplify the region between the *cob* and *cox3* gene using the primers 5′-ACCACTCAACAAATACCCACCACTTCGGCT-3′

and 5′-GATTGTTTGACCTCCGAATGGAATTGCTGT-3′. In the case of RT-PCR (and that of *cox1* below), all amplifications were carried out with controls lacking RT enzyme, from which no products were acquired.

Transcripts of *cox1* were amplified by RT-PCR using primers 5′-GGCGCCCCCGACATGGCNTTYCCNMG-3′ and 5′-TGATGGAAAAACCAGAANARRTGYTGRTA-3′, and the genomic copy then amplified using long-range PCR and the exact-match primers 5′-GGTTGTTACCACCTTCTCTTTTACTACTGATTG-3′ and 5′-CTGGTACAATACAGGATCACCTCCACCCGC-3′. The 3′-end of the *cox1* fragment, including the second intron, was amplified by long-range PCR from genomic DNA using the primers 5′-GCGGGTGGAGGTGATCCTGTATTGTACCAG-3′ and 5′-TATAAAGAACACCACCTGTAACGAACATAA-3′.

We also used a variety of dinoflagellate-specific primers to search for the host mitochondrial genes: 10 degenerate primers for *cox1*, six for *cob*, and four for *cox3* were based on the most conserved regions of these genes found in dinoflagellate mitochondria. The *cox1* primers were tested successfully to amplify this gene from several other species of dinoflagellates (data not shown). However, no product was obtained with any of these primers from the total DNA or RNA extracted from *K. foliaceum* used in PCR and RT-PCR, respectively.

All PCR and RT-PCR products were gel purified and cloned using PCR 2.1 TOPO Cloning kit (Invitrogen). In each case, several clones were sequenced on both strands using BigDye terminator chemistry. New sequences have been deposited into GenBank, accession numbers DQ831826 and DQ831827.

Phylogenetic analyses. The conceptual translations of *cob*, *cox3*, and *cox1* from *K. foliaceum* were aligned with homologues from public database using ClustalX 1.83.1 (Thompson, Higgins, and Gibson 1994) under the default gap opening and gap extension penalties and the alignment edited manually. *Phaeodactylum tricornutum* homologues were kindly provided by Marie–Pierre Oudot–Le Secq from the *P. tricornutum* genome sequencing project (DOEs Joint Genome Institute: http://www.jgi.doe.gov/index.html). Phylogenetic analyses were carried out including a diversity of eukaryotes to determine the overall position of new sequences, and subsequently restricted to homologues from chromalveolate taxa (dinoflagellates, apicomplexans, ciliates, heterokonts, haptophytes, and cryptomonads), as both the host and endosymbiont are members of this supergroup (Keeling et al. 2005). These alignments consisted of 24, 20, and 49 sequences with 264, 252, and 291 unambiguously aligned sites for *cob*, *cox3*, and *cox1*, respectively. Phylogenetic trees were inferred using maximum likelihood. The proportion of invariable sites (i) and shape parameter alpha (α) with eight variable rate categories were estimated from the data with PhyML 2.4.4 (Guindon and Gascuel 2003) under the Whelan and Goldman model of substitution with the frequency of amino acid usage calculated from the data. The i and α parameters estimated from the data were 0.037, 0.000, and 0.053, and 1.544, 1.499, and 1.173 for *cob*, *cox3*, and *cox1*, respectively. For all three data sets 1,000 bootstrap replicates were analysed using PhyML. For distance trees, distances were calculated using TREE-PUZZLE 5.2 (Schmidt et al. 2002) with eight variable rate categories and invariable sites. The i and α parameters were estimated by TREE-PUZZLE to be 0.020, 0.000, and 0.000, and 1.190, 1.140, and 0.089 for *cob*, *cox3*, and *cox1*, respectively. Trees were constructed using weighted neighbor-joining using WEIGHBOR 1.0.1a (Bruno, Socci, and Halpern 2000). Distance bootstrapping of 1,000 replicates was carried out using PUZZLEBOOT (shell script by A. Roger and M. Holder, http://www.tree-puzzle.de).

Transmission electron microscopy. Cells were collected by centrifugation at 3,220 g for 5 min at 8 °C and fixed with a solution of 2% (v/v) glutaraldehyde in seawater, rinsed in seawater, and postfixed in 2% (w/v) osmium tetroxide (OsO_4). Cells were dehydrated in an ethanol series (30%, 50%, 70%, 90%, 100%, 100%) and infiltrated with increasing concentrations of Spurr resin in acetone (1:3, 1:1, 3:1, 100%, 100%, 100%). All fixation, dehydration, and infiltration steps were carried out using microwave processing. Cells were embedded in 100% Spurr resin overnight at 60 °C. Thin (60 nm) sections were cut with a Leica Ultracut E Ultramicrotome, placed on formvar-coated grids, and stained with 1% (w/v) uranyl acetate and lead citrate. Approximately 100 different *K. foliaceum* cells from four different grids (i.e. approximately 25 from each grid) were observed and photographed using a Hitachi S7600 transmission electron microscope (Tokyo, JP) at 80 kV.

RESULTS AND DISCUSSION

The endosymbiont mitochondrion encodes three genes for electron transport proteins. To examine the potential reduction or functional relationship between the two mitochondria in *K. foliaceum*, we characterized the first mitochondrial genes from this organism. We chose to focus on the only three genes that have been retained in the mitochondrial genome of other dinoflagellates: *cox1*, *cox3*, and *cob*. PCR amplification from total DNA (or RNA) resulted in fragments of the expected size, and sequencing multiple clones yielded a single copy of each gene (from DNA, three clones for *cob*, three clones for *cox3*, two clones for the intergenic space between *cob* and *cox3*, and four clones for *cox1*; from RNA, one clone for each of the *cob, cox3*, and the spacer between the two, and four clones for *cox1*). Phylogenetic analyses were carried out on all three genes including representatives of both the host (dinoflagellate) and endosymbiont (diatom) sequences as well as all other major groups of chromalveolates. Overall, these trees resemble analyses based on other genes, with generally strong support for the monophyly of alveolates and sister relationship between dinoflagellates and apicomplexans (Fig. 2–4) (Fast et al. 2001; Harper, Waanders, and Keeling 2005; Van de Peer and De Wachter 1997), and a sister relationship between haptophytes and cryptophytes in *cox3* and *cob* trees (Fig. 3 and 4) (Harper et al. 2005). Most importantly, in all three phylogenies the distinction between the expected positions of host and endosymbiont-derived genes was clear, and in all three phylogenies the *K. foliaceum* gene branched within the diatom clade with strong support, and not with the dinoflagellates. Moreover, in all three phylogenies, the *K. foliaceum* gene grouped specifically with pennate diatoms (i.e. *Phaodactylum*, *Nitzschia* or *Cylindrotheca*) to the exclusion of the centric diatoms (i.e. *Thalassiosira*, *Ditylum* or *Fragilaria*), which is consistent with evidence that the endosymbiont is derived from a pennate diatom (Chesnick et al. 1997; McEwan and Keeling 2004). Altogether, these trees strongly support the conclusion that all three genes characterized belong to the endosymbiont mitochondrion, and not that of the host.

Endosymbiont mitochondrial genes are expressed and organized as operons. Long-range PCR was carried out between all possible combinations of the six gene ends to determine if any or all of the three genes resided on the same chromosome. No linkage of *cox1* was found, but a fragment linking *cob* and *cox3* genes was amplified and sequenced, showing these two genes are adjacent, encoded on the same strand, and separated by a short spacer of 70 bp. To determine whether these two genes form part of an operon, RT-PCR was carried out using DNA-free RNA. Primer pairs for the *cob* and *cox3* genes individually and a pair that spanned both genes all yielded fragments of the expected size that were cloned and sequenced, confirming their identity and the presence of an operon including *cob* and *cox3* (data not shown). Operons are consistent with (prokaryotic) mitochondrial genomes, but not with nucleus-encoded genes derived from the mitochondrial genome, so this operon confirms these genes to be

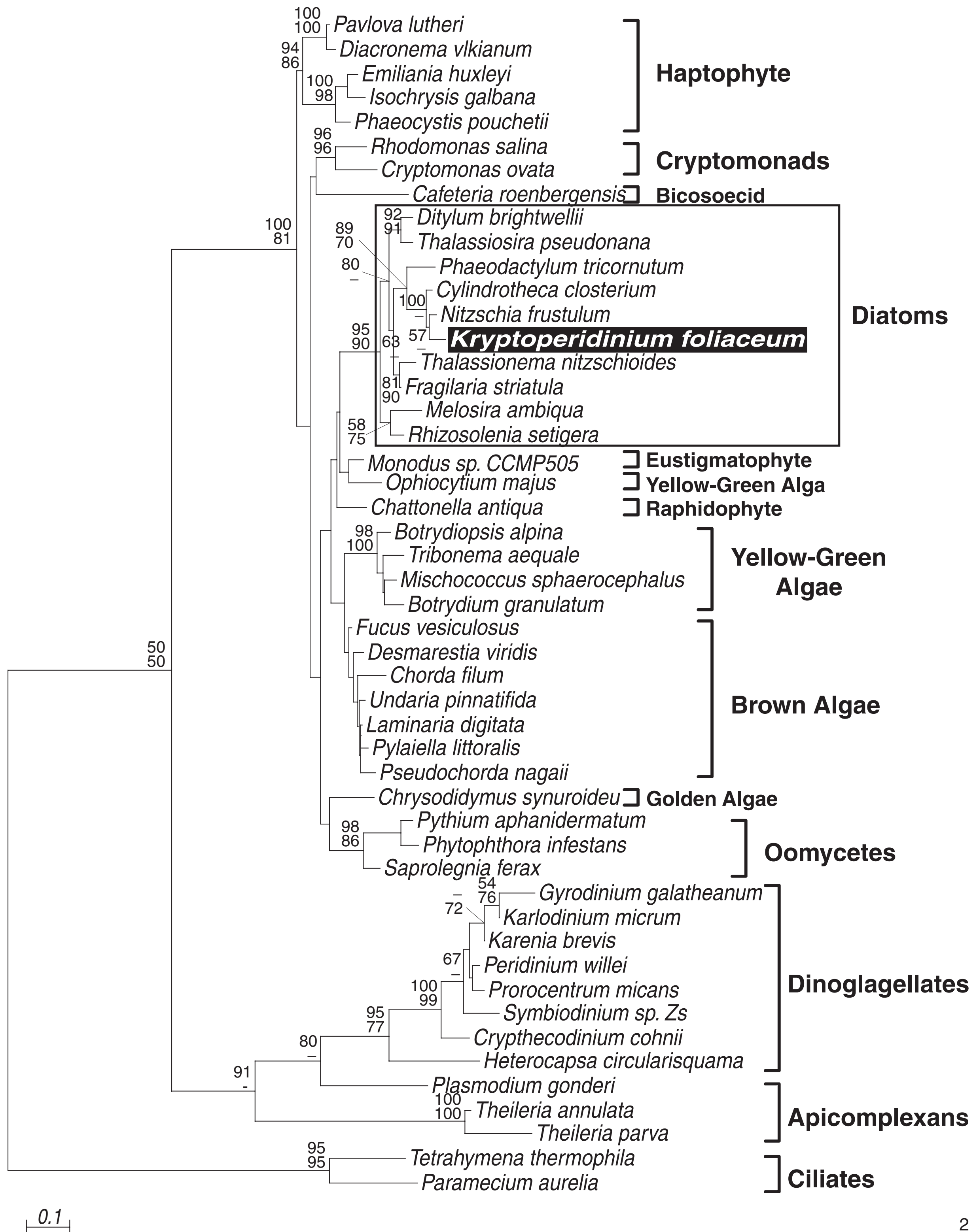

Fig. **2.** Protein maximum likelihood phylogeny of cytochrome c oxidase 1 (*cox1*). Numbers at nodes indicate bootstrap support for major nodes over 50% from ML (top) and distance (bottom). A dash (-) indicates support less than 50%. Major groups are labelled to the right, with diatoms indicated by a box. The scale bar is proportional to the number of character change.

present in the endosymbiont mitochondrial genome. Expression of *cox1* was also confirmed using RT-PCR, and also by the demonstration that it contains spliced introns. Dinoflagellate mitochondrial *cox1*, *cox3*, and *cob* genes are extensively edited at the RNA level (Lin et al. 2002), but RNA editing was not found in any of the genes reported here (data not shown). These data demonstrate

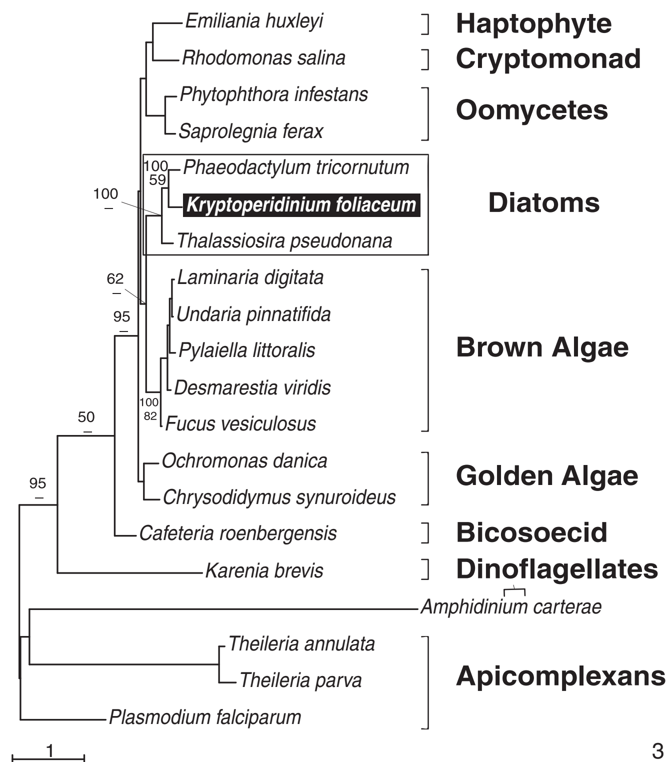

Fig. **3.** Protein maximum likelihood phylogeny of cytochrome oxidase 3 (*cox3*). Numbers at nodes indicate bootstrap support for major nodes over 50% from ML (top) and distance (bottom). A dash (-) indicates support less than 50%. Major groups are labelled to the right, with diatoms indicated by a box. The scale bar is proportional to the number of character change.

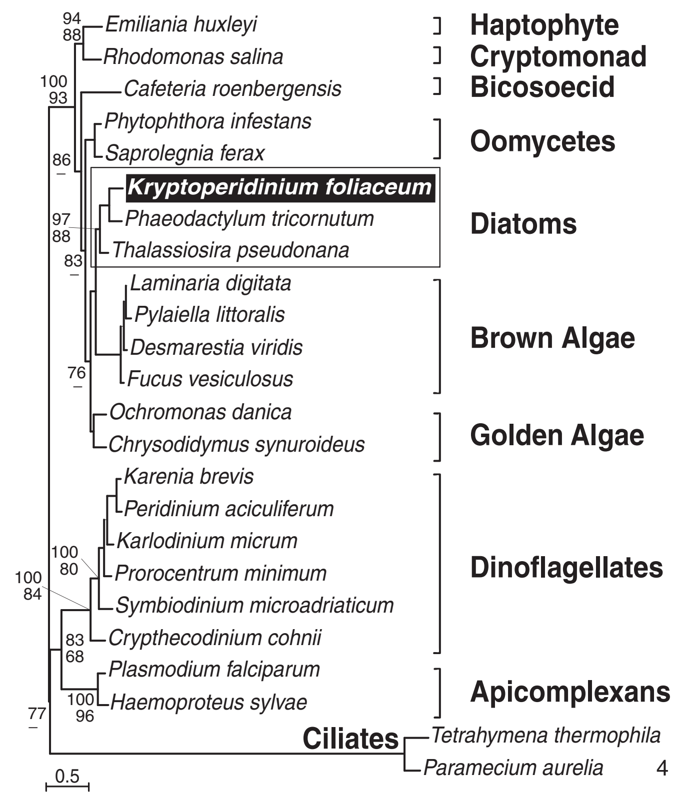

Fig. **4.** Protein maximum likelihood phylogeny of cytochrome b (*cob*). Numbers at nodes indicate bootstrap support for major nodes over 50% from ML (top) and distance (bottom). A dash (-) indicates support less than 50%. Major groups are labelled to the right, with diatoms indicated by a box. The scale bar is proportional to the number of character change.

the diatom endosymbiont mitochondrial genome is expressing genes for electron transport proteins.

Group II introns in the *Kryptoperidinium foliaceum* endosymbiont *cox1*. Reverse-transcriptase polymerase chain reaction products of the *cox1* gene encoded a continuous open reading frame, but the genomic DNA sequence was found to contain two insertions resembling group II self-splicing introns (Lambowitz and Belfort 1993; Michel and Ferat 1995; Michel, Umesono, and Ozeki 1989). The first intron was completely sequenced and found to contain an open reading frame (ORF) encoding a putative DNA endonuclease, whereas the second intron encoded a reverse transcriptase (data not shown). Interestingly, group II introns are not found in dinoflagellate mitochondria, but they are common in the *cox1* genes of heterokonts (Ehara, Watanabe, and Ohama 2000; Fontaine et al. 1997). In diatoms, the *cox1* of the centric diatom, *Thalassiosira pseudonana* contains one intron while that of the pennate diatom, *Phaeodactylum tricornutum* has two. The positions of these introns were compared with those of the *K. foliaceum*, but none was found to occupy the same position. Phylogenetic analysis of the intron-encoded ORFs was also carried out, and intron 1 from *K. foliaceum* is not closely related to introns from other heterokont algae, but instead was more akin to several fungal introns (data not shown). Intron 2, however, shared a high level of similarity with the *T. pseudonana* intron, and in phylogenetic analyses the *K. foliaceum* and *T. pseudonana* introns formed a clade with 100% support (data not shown). Although the *K. foliaceum* endosymbiont is a pennate diatom, neither of its introns is closely related to the two introns in the *cox1* from *P. tricornutum*.

Electron microscopic observations of mitochondria. Endosymbiont mitochondria appear as double-membrane bounded, broadly circular to elliptical, or irregular structures with well-developed tubular cristae in a finely granular, somewhat dense medium (Fig. 5). Mitochondria in the various planes of section generally range from approximately 0.5–1 μm at their widest point.

All of the mitochondria we observed appeared to be located within the endosymbiont cytoplasm and not that of the host. Even those mitochondria that are quite close to the host's dinokaryotic nucleus can be seen, under sufficiently high magnification, to be separated from the dinokaryon by a membrane, presumably that separating the host's cytoplasm from that of the endosymbiont (Fig. 6). Further, we noted several qualitative differences between host and endosymbiont cytoplasm that reinforce this conclusion. As Jeffrey and Vesk (1976) noted, the host cytoplasm is generally less dense than that of the endosymbiont. In instances in which both can be seen, mitochondria are always associated with the denser (i.e. the endosymbiont) cytoplasm. More importantly, the endosymbiont cytoplasm was observed to contain a large number of hexagonal structures, approximately 25 nm in diameter and forming crystalline arrays, which we infer to be viruses. These are not seen in host cytoplasm (as expected for a virus, which would be associated with one nucleocytoplasmic system), and so are good markers for the endosymbiont cytoplasm. These crystalline arrays are often found near mitochondria (Fig. 5 and 6). Altogether, we did not observe any mitochondrion that was unambiguously in the host cytoplasm. This suggests that host mitochondria may be rare in comparison with those of the

endosymbiont or highly modified. Our data do not exclude the possibility that host mitochondria are absent altogether, but this is not consistent with previous reports (Jeffrey and Vesk 1976; Tamura et al. 2005).

Concluding remarks: endosymbiont reduction, host reduction, or both? The endosymbiont of *K. foliaceum* has been transformed substantially from its original state as a free-living pennate diatom, losing its diatom cell wall, distinctive arrangement of the nucleus and plastids, and motility system, overall transforming in appearance and losing any specific resemblance to diatoms. However, relative to most secondary and tertiary endosymbionts it is still remarkably well preserved. In most cases of secondary and tertiary endosymbiosis, nearly all signs that the endosymbiont was derived from a eukaryote are gone, the best exceptions being the relic nuclei known as nucleomorphs found in cryptomonads and chlorarachniophytes (Gilson and McFadden 2002). These nuclei and their tiny genomes have been the focus of much attention because they offer a rare opportunity to see the effects of secondary endosymbiosis and the integration of endosymbiont and host. Complete nucleomorph genome sequences are now known for representatives of both groups, and these are models of reduction in nuclear genomes (Douglas et al. 2001; Gilson et al. 2006). The endosymbiont of *K. foliaceum* is unique among tertiary endosymbiotic algae in retaining a nucleus that is not reduced in size (Fig. 7) like the nucleomorphs. However, as only handful of its genes have been sequenced from the endosymbiont nucleus (Chesnick, Morden, and Schmieg 1996; Chesnick et al. 1997; Inagaki et al. 2000; McEwan and Keeling 2004), little can be said about its level of genomic degeneration.

The unique feature of the *K. foliaceum* endosymbiont, however, is the retention of mitochondria and, as we demonstrate here, the mitochondrial genome. This might be attributed to the endosymbiotic event being very recent, so that there simply has not been sufficient time for the loss of its mitochondria. However, several lines of reasoning suggest otherwise. In addition to the presence of the symbiont in several morphologically and genetically distinct (but related) genera, molecular data showing that the endosymbiosis predates at least some of these divergence events suggest the endosymbiont is not particularly recent (Inagaki et al. 2000). Similarly, the synchronization of host and endosymbiont cell division observed in *Durinskia* (Tippit and Pickett-Heaps 1976) suggests they are highly integrated, and the morphology of the endosymbiont has been radically altered, none of which is consistent with the mitochondrion simply not having time to change or disappear.

The alternative is that the endosymbiont mitochondrion plays some important role in the new conglomerate cell, and will not simply be lost given more time. Mitochondria have not been retained in any other secondary or tertiary plastid endosymbiont, so there is no obvious point of comparison with *K. foliaceum*; it is accordingly difficult to predict what their function might be and why they would be retained. One possible comparison is the relic mitochondria of protists formerly thought to be amitochondriate, such as *Giardia*, *Trichomonas*, and microsporidia. In these cases, the organelles have lost or totally transformed their role in metabolism, in some cases apparently functioning only in iron sulfur cluster assembly (Williams and Keeling 2003). In contrast, we have shown that the mitochondrial genome of the *K. foliaceum* endosymbiont encodes *cox1*, *cox3*, and *cob*. The fact that the genes are expressed, that all three proteins function in the electron transport chain, and that well-developed mitochondrial cristae remain, all strongly suggest that the endosymbiont mitochondria are still functional in aerobic respiration, and perhaps still perform the full suite of their ancestral functions. The presence of two type II introns in *cox1*, and the presence of similar introns within the same gene of other diatoms, further suggests that the mitochondrial genome of the *K. foliaceum* endosymbiont is not particularly reduced, as introns might be expected to be among the first genetic elements to be lost during degeneration (Douglas et al. 2001). We would predict that the endosymbiont mitochondrial genome is

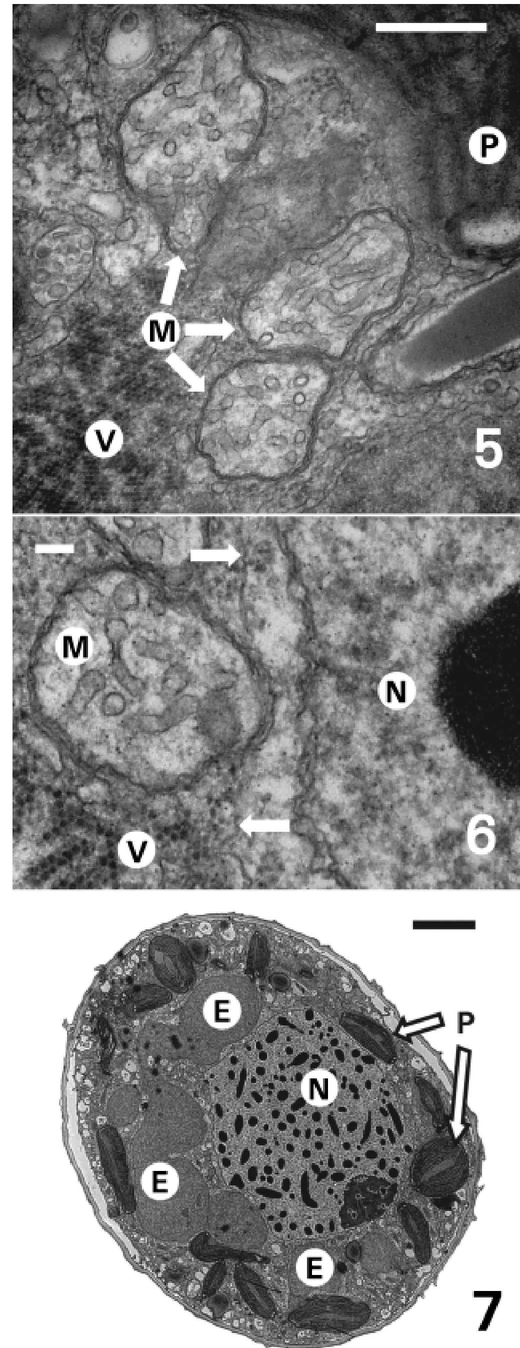

Fig. **5–7.** Transmission electron micrographs of *Kryptoperidinium foliaceum* **5.** Three mitochondria (m) with prominent tubular cristae in close association with a plastid (p) and crystalline assemblies of putative viral particles (v) often seen in the endosymbiont cytoplasm. Scale bar = 500 nm. **6.** Example of a mitochondrion (m) in cytoplasm associated with the viral particles (v) and in close proximity to the host nucleus (n), but separated from it by a membrane (arrows). Scale bar = 100 nm. **7.** Whole cell showing host nucleus (n), endosymboint nucleus (e), and endosymboint plastids (p) Scale bar = 5 μm.

most likely comparable in content and size with that of free-living pennate diatoms, and that its functional complexity may be comparable with canonical mitochondria.

All of this leads to one question: why does the endosymbiont retain apparently functional mitochondria? Or perhaps more importantly, why would an organism retain two mitochondria from two different sources? The host dinoflagellate presumably had fully functional mitochondria, and there is evidence that the host organelle has been retained (Jeffrey and Vesk 1976; Tamura et al. 2005). In our examination of ultrastructure, we were unable to identify a single mitochondrion that we could unambiguously attribute to the host. This suggests a spectrum of possibilities. On one hand, *K. foliaceum* may have retained both mitochondria with redundant metabolic functions. This would mean the host organelle contains a typical dinoflagellate mitochondrial genome with all three genes analysed here, but we failed to detect them (perhaps because they are divergent, edited unusually, or very rare compared with those of the endosymbiont). It is also possible that the host organelle has lost metabolic functions, making the endosymbiont mitochondrion essential, so the two organelles coexist but share mitochondrial functions between them. In this case, the host mitochondria may be limited in number and importance, explaining their absence from our observations with electron microscopy. At the extreme, we cannot exclude the possibility, however unlikely it may seem, that the host mitochondria may be in the process of being replaced by those of the endosymbiont. Indeed, many characteristics of the endosymbiont are not what we have come to expect from such a situation: it retains a large nucleus, many mitochondria, and a large volume of cytoplasm relative to the host. Overall, it raises the interesting possibility that both cells, or perhaps only the host, may be reducing as this partnership progressively integrates.

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