# MITOCHONDRIA IN A TERTIARY ENDOSYMBIONT

by

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B.S., University of British Columbia, 2002

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Genetics)

THE UNIVERSITY OF BRITISH COLUMBIA

November 2006

## 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, I 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. I 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 that the endosymbiont mitochondria retain normal tubular cristae. Overall, these data suggest the endosymbiont mitochondria have not reduced at the genomic or functional level.

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## ACKNOWLEDGEMENTS

I would like to thank many people here, without whom this work would not have been possible. I want to thank Dr. Patrick J. Keeling, my supervisor, who gave me the opportunity to work in his lab, and provided me with the essential financial and academic support, and my committee members, Dr. Brian S. Leander and Dr. Philippe Tortell, who were always supportive and encouraging. I want to thank Dr. Hugh Brock, the director of the Genetics Graduate Program and the chair of the examining committee, and all my committee members, Patrick, Brian, and Philippe, and Dr. Keith Adams, the external examiner, for their time and effort in preparation for my defense.

I would also like to thank Dr. Marie-Pierre Oudot-Le Secq and Chris Bowler for providing the sequence data for the mitochondrial genes of *Phaeodactylum tricornutum* prior to publication, Dr. Claudio Slamovits for his friendship and for providing the starting amount of total RNA of *Kryptoperidinium foliaceum*, and Dr. Kevin J. Carpenter, who did most of the preparations for transmission electron microscopy (TEM) results presented here.

Many thanks are due to my good friends and colleagues in Keeling lab:

To Dr. Audrey de Koning for teaching me the useful practical details of genetic work, to Dr. Mathew Rogers, Dr. Bryony A. P. Williams, and Dr. Juan Saldarriaga for their warm friendship and daily support, and the rest of my lab mates and my friends in Fast lab and in the Department of Botany and Oceanography.

Finally, I would like to express my deepest gratitude toward my wife, Netsanet Tsegay, who has been a boundless source of encouragement, sympathy, and love.

## DEDICATION

I dedicate this work to my mother, Shokofeh Jahanbakhsh, whose love for life and for me has been inspirational to me since I have known myself, to my sister, Banafsheh Imanian, for her love and consistent support, and to the memory of my father, Iman Imanian, whose integrity was, and remains, exemplary for me.

## CO-AUTHORSHIP STATEMENT

A version of Chapter 2 has been accepted for publication in the *Journal of Eukaryotic Microbiology*. Dr. Kevin J. Carpenter and Dr. Patrick Keeling are listed as co-authors on this paper. Dr. Kevin J. Carpenter did all the preparatory work for transmission electron microscopy (TEM) on *Kryptoperidinium foliaceum*, and helped with the editing of the paper. Dr. Patrick J. Keeling was instrumental in guiding the approach of this project, and extraordinarily resourceful in the process of writing and editing this paper. I performed all the molecular work and data analyses, and wrote the first draft of the paper, which was revised by both Dr. Carpenter and Dr. Keeling, and further by me.

## CHAPTER 1: INTRODUCTION

The competition of all living things for the limited resources is one of the strong forces of evolution. This competitive struggle to live and to fit better for living, however, does not exclude or undermine the symbiotic association of living organisms. In the course of evolution and through symbiosis, living organisms have taken giant steps forward, and overcome tremendous obstacles. Providing novel adaptive forms and strategies, symbiosis has played a major role in speciation and diversification of life. This is through symbiosis that ancestors of modern plants could colonize the land, and furthermore, the original rise of eukaryotic cells is indebted a great deal to endosymbiosis.

Now, it is widely accepted that the eukaryotic organelles, mitochondria and plastids, are the descendents of the prokaryotic cells that also gave rise to modern α-proterobacteria and cyanobacteria, respectively, through the long-term endosymbiosis. On one hand, this process has increased the complexity of the host cell, and expanded its capability to perform, among other tasks, vitally important functions of oxidative phosphorylation with mitochondria and photosynthesis with the aid of plastids. On the other hand, it has reduced the engulfed bacterium into an organelle, eliminating its autonomy, many of its morphological traits, along with most of its genes, which have been either transferred to the nucleus of the host cell or lost completely. The new chimeric cells evolved to cope with the rising oxygen levels in the atmosphere, and those that harbored photosynthetic bacteria started harnessing the energy of the sunlight as well. The evolutionary history of mitochondria and plastids share striking similarities,

and reveal some differences. A closer look will reveal what is shared, and what is not, in the history of these two organelles.

### 1.1: The Evolution of Mitochondria

A survey among the mitochondrial genomes of eukaryotic organisms demonstrates that there is an astonishing variety of sizes, conformations, gene contents, gene orders, and gene expressions among the examined mitochondria (Clark-Walker 1992; Cummings 1992; Gray et al. 1998; Hanson and Folkerts 1992; Stuart and Feagin 1992; Wolstenholme 1992). For instance, although most mitochondrial genomes are circular, the linear form of the genome also exists in different taxonomical groups including ciliates, apicomplexans, algae, slime molds, fungi, and yeasts (Coleman et al. 1991; Nosek et al. 1998). The mitochondrial genome size varies widely among different organisms from about 6 kb in *Plasmodium falciparum* to more than 200 kb in many plants (some as large as 2400 kb), and so does the percentage of their coding sequences: a little less than 10% of mitochondrial genomes in protists are non-coding, as opposed to more than 80% non-coding regions in the mitochondrial genome of *Arabidopsis thaliana* (Gray et al. 1999; Leblanc et al. 1997). The number of mitochondrial protein-encoding genes varies widely across existing eukaryotes as well from 3 to 67; and from 0 to 27 tRNA genes are found in different mitochondrial genomes (Adams and Palmer 2003).

Despite such variations, almost all the phylogenetic analyses based on the mitochondrial genes indicate that all the known extant mitochondria have descended from a prokaryote that is also an ancestor to modern α-proteobactria (Gray et al. 1999). It was once thought that several eukaryotic groups, collectively called Archezoa,

primitively lacked mitochondria, and therefore, evolved prior to the evolution of mitochondria. However, several recent studies have found either mitochondrial relict organelles or mitochondrial-derived genes within the genomes of the 'Archezoa' (Bui et al. 1996; Roger 1999; Williams et al. 2002; Williams and Keeling 2003). In some cases, the relict organelle has lost its genome and characteristic mitochondrial functions such as electron transport and oxidative phosphorylation, but it carries out other functions, such as iron-sulfur cluster assembly (Tachezy et al. 2001; Van der Giezen et al. 2005; Williams and Keeling 2003). In the light of these studies the hypothetical Archezoa has lost many members, and retortamonads and oxymonads are the last two remaining eukaryotic groups for which, to date, there has not been any evidence of a mitochondrion. In other words, the mitochondrial endosymbiosis has, most likely, occurred only once and very early in the evolution of all eukaryotic cells with mitochondria, and this is the most important unifying fact about all mitochondria.

In the course of the transition from a bacterium to an organelle, the bacterium has gone through extensive reduction processes, and in certain lineages such as parabasilids, diplomonads, entamoebae, and microsporidia, mitochondria have been nearly lost (Roger 1999). The mitochondrion has been lost entirely only during the secondary or tertiary endosymbioses that resulted in the movement of plastids between lineages (see section 1.2), where only the plastids of the eukaryotic endosymbiont are retained and its nucleus and mitochondria are discarded. This is a special case, since the eukaryotic endosymbiont is housed in an already mitochondriate host. Even among the other eukaryotes that retain their mitochondria, the ancestral prokaryote has suffered extensive losses. At genetic level, for instance, a modern free-living α-proteobacterium has about

3600 protein-encoding genes in its genome (Nierman et al. 2001), whereas the known mitochondrial genomes carry only 98 protein-encoding genes at most (Gray et al. 2004). Most of the ancestral genes have been lost entirely, and many genes have been transferred to the nucleus of the host cell, and retargeted back to mitochondria through the evolution of a novel targeting system. Similar processes of reduction and integration can be seen in the evolution of plastids.

**1.2: Plastid Evolution**

With one possible exception, *Paulinella chromatophora* (Keeling 2004; Martin et al. 2005), all extant plastids share a common ancestor, which is also the 'mother' to modern cyanobacteria (Bryant 1992; Cavalier-Smith 1982; Delwiche and Palmer 1997; Douglas and Penny 1999; Moreira et al. 2000). The first endosymbiotic event that involved this cyanobacteria-like organism and gave rise to contemporary plastids has become known as the primary plastid endosymbiosis. All the glaucophytes, red algae, green algae and plants are thought to have evolved from this event (Bhattacharya et al. 2004; McFadden 2001). The transition from a cyanobacteria-like ancestor to a plastid invovled reductive evolution similar to that seen in the mitochondrial endosymbiosis. The genome of a modern cyanobacterium contains 3229 genes (Kaneko et al. 1996), whereas most plastids contain only up to a few hundred genes. Some of the genes missing from the plastid's genome have been found in the nucleus of the host, but others seem to have been lost completely. The products of the nuclear plastid-targeted genes are sent to the plastid through a similar targeting system seen in the nuclear mitochondrial targeted genes. However, the extent of reduction varies considerably among the plastids.

Some protists, for example apicomplexans, have plastids with much reduced genome sizes, about 35 kilo base-pair (kb) in total, and they do not show any photosynthetic activity, living as obligate intracellular parasites (Wilson 2002). Ciliates are believed to have lost their plastids altogether, although the possibility of retention of cryptic plastids in ciliates has not been overruled (Harper and Keeling 2003).

Although all the extant plastids, in both photosynthetic and non-photosynthetic organisms, have a common ancestor, not all of them are acquired through primary endosymbiosis. The endosymbiotic acquisition of plastids has involved not only prokaryotic preys but also the eukaryotic ones with primary plastids. At least one secondary endosymbiosis with a red alga, and one or two separate endosymbiotic events with green algae (Keeling 2004) are hypothesized, the red giving rise to the plastid of a diverse group known as chromalveolates, and the green to euglenids and chlorarachniophytes. Typically, the eukaryotic prey with primary plastid becomes reduced to its plastid plus one or two extra membranes (Archibald and Keeling 2002), losing its nucleus and its mitochondria, as mentioned in section 1.1. In most cases, the extent of reduction in these endosymbionts is comparable to that of the primary or prokaryotic endosymbionts, which were once free-living, but were turned into obligate organelles.

Sometimes, the reduction processes go to such extent that the plastid, just like the mitochondrion, becomes a relict in a cell either without any known function or with a newly adopted function as seen in some apicomplexans that use their non-photosynthetic plastid for the biosynthesis of isoprenoid and fatty acids (Gleeson 2000). Amusingly, the process of secondary endosymbiosis can occur many times within a species lineage. This

serial secondary endosymbiosis may result in the replacement of the old secondary plastid (Ishida et al. 1997). For instance, some dinoflagellate lineages have lost their peridinin-containing plastid, acquired through secondary endosymbiosis, replacing it with a new one (Saldarriaga et al. 2001). From among the lineages with secondary plastids, two groups stand out: cryptophytes and chlorarachniophytes. The endosymbionts of these two groups are related to red algae and green algae, respectively (Douglas et al. 1991; McFadden et al. 1995; Van de Peer et al. 1996), and unlike the other lineages, they still keep, within a small remaining volume of the endosymbiotic cytoplasm, a miniature eukaryotic nucleus, called nucleomorph, which is highly compacted, and severely reduced in size and in its genome content (Gibbs 1992; Stibitz et al. 2000). Because of the presence of this persevering nucleus, these endosymbionts are more readily recognizable as remnants of eukaryotic cells, and are considered at earlier stages of endosymbiosis.

There is also evidence that some dinoflagellates have gained their plastids through tertiary endosymbiosis by engulfing another protist with a secondary plastid, for example, a haptophyte (Tengs et al. 2000), a cryptophyte (Hackett et al. 2003; Hewes et al. 1998; Schnepf and Elbraechter 1988), or a diatom (Chesnick et al. 1997; Tamura et al. 2005). Some of the diatom endosymbionts in this group, such as that of *Kryptoperidinium foliaceum*, have lost their cell wall and motility, but still have, in addition to their secondary plastid(s), their own prominent nucleus and even their own mitochondria, and they are the least reduced forms of such permanent endosymbionts. Other cases, like *Hatena* and its prey/symbiotic partner are more transient (Okamoto and Inouye 2005), but the dinoflagellate host and its diatom endosymbiont seem to be inseparably fused

throughout all the stages of cell cycle, including cell division and sexual life as is well documented in the dinoflagellate *Durinskia baltica* (Chesnick and Cox 1989; Tippit and Pickett-Heaps 1976). What makes these endosymbionts so exceptional is that in spite of irrevocable integration within their hosts after evolutionary time, they have retained more cellular structures than any other endosymbiont. *Kryptoperidinium foliaceum*, a close relative of *D. baltica*, is a faithful representative of these extraordinary tertiary endosymbionts, and what follows is intended to summarize what is known about this organism.

### 1.3: *Kryptoperidinium foliaceum*

*Kryptoperidinium foliaceum* is a thecate dinoflagellate with a complicated taxonomic history: It was first described in 1883 by Stein, and it was classified under the genus *Glenodinium*; then it was placed in the genus *Cryptoperidinium* by Lindemann in 1924; Van Goor in the same year called this organism *Peridinium cuneatum*; in 1926, it was re-described under a completely different genus as *Phyllodinium scutelaris*; later, Stein renamed the organism *Peridinium foliaceum* (Biecheler 1952); more than a decade later, it was re-classified under genus *Glenodinium* (Dodge and Crawford 1969; Prager 1963).

*Kryptoperidinium foliaceum* was once thought of as a heterotrophic dinoflagellate (Withers et al. 1977); however, the results of a study on the effects of light in the growth of *K. foliaceum* and its response to an inhibitor of photosynthesis, a drug called 3-(3,4-dichlorophenyl)1,1-dimethylurea (DCMU), indicate that the growth is strongly inhibited in the dark and by DCMU (Morrill and Loeblich 1977; Morrill and Loeblich 1979). Also

I have successfully grown cultures of *K. foliaceum* for months in the presence of four different antibiotics, which significantly reduce the number of bacterial prey in the culture. Therefore, this organism seems to be able to sustain its life by photosynthesis alone, and should be considered as one of the ocean's primary producers. In addition, it has been reported that *K. foliaceum* forms recurring blooms in a wide geographic range from estuaries in Ireland and Spain (Jenkinson 1990; Trigueros et al. 2000), to South Carolina, sometimes with harmful effects on the local animal populations (Kempton et al. 2002).

*Kryptoperidinium foliaceum* received extra attention when it was discovered that it contained two nuclei instead of one. The two nuclei of this organism are dissimilar: one nucleus is the typical dinoflagellate nucleus with large amount of DNA, without nucleosomes, but with permanently condensed chromosomes (Taylor 2004), and the other is a generally normal eukaryotic nucleus with its dispersed chromatin and its DNA in association with histones (Kite et al. 1988; Morris, et al. 1993). Apparently the chromosomes of the endosymbiont's nucleus do not condense, the mitotic spindle does not form, microtubules have not been observed, and the nucleus seems to divide amitotically as is the case in *Durinskia baltica*, which bears a similar endosymbiont (Jeffrey and Vesk 1976; Tippit and Pickett-Heaps 1976).

The ultrastructural studies revealed that in addition to a three-membrane bound eyespot (Dodge 1983), *K. foliaceum* has an endosymbiont with plastids. Several ultrastructural, biochemical, and genetic studies have suggested a diatom ancestry for the endosymbiont in *K. foliaceum* (Chesnick et al. 1997; Chesnick et al. 1996; Kite and Dodge 1985; Schnepf and Elbraechter 1999). The diatom endosymbiont has lost its cell

wall and motility, and the nuclear division seems to occur amitotically in concert with the nuclear division of the host, suggesting that the relationship between the host and the endosymbiont is obligatory.

Despite its transformation, the endosymbiont of *Kryptoperidinium foliaceum* is one of the rare endosymbionts that are morphologically much less reduced than all the other endosymbionts. This endosymbiont resides within the cytoplasm of its dinoflagellate host, and separated from it by a single membrane. It has a two-membraned large multi-lobed nucleus, several plastids, all sacked within the endoplasmic reticulum (ER), which is continuous with the outer membrane of the nucleus, and most interestingly has many mitochondria within its cytoplasm (Dodge 1971; Dodge 1983; Eschbach et al. 1990; Jeffrey 1975; Kite et al. 1988; McEwan and Keeling 2004; Rizzo and Cox 1976). In addition, the endosymbiont of *K. foliaceum* occupies a large volume of the host's cytoplasm, much larger than that observed and reported in other organisms with endosymbionts. This is in contrast, for example, with the endosymbionts found in cryptophytes and chlorarachniophytes, which are located in the endomembrane system of the host (Gilson 2001), and their nuclei have been termed nucleomorphs to connote their reduced genome sizes.

The retention of mitochondria is the unique characteristic of the endosymbionts of *K. foliaceum* and a few of its close relatives: all other known tertiary endosymbionts, with haptophyte or cryptophyte origins, and all secondary endosymbionts, with green or red algal ancestry, have lost their mitochondria, suggesting that these organelles are among the first losses of the endosymbionts. This makes *K. foliaceum* one of the very few organisms that harbor two distinct types of mitochondria, one from the dinoflagellate host

and the other from the diatom endosymbiont. Therefore, *K. foliaceum* is an excellent system for investigating the reduction processes that occur at early stages in endosymbiosis, which remain, for the most part, unknown. For instance, we know very little about the environmental and organismal requirements for the endosymbiotic event, the principles and the order of the endosymbiont's genetic and morphological reduction, the pace and rates of these losses or gene transfer events. Genetically speaking, we also know very little about *K. foliaceum*: only sixteen protein-encoding and three ribosomal RNA (rRNA) genes from the complex genome of *K. foliaceum* have been sequenced, and there has been no genetic information from either the host's or the endosymbiont's mitochondria.

Here, I present the first molecular data from the mitochondria of *K. foliaceum*, and discuss the origin of the recovered genes. I also discuss the implications of these data, and the reasons for the retention of the endosymbiont's mitochondria and their genome.

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# CHAPTER 2: MITOCHONDRIAL GENES OF A TERTIARY ENDOSYMBIONT

## 2.1: Introduction*

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 et al. 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 et al. 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

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* A version of this chapter has been accepted by the Journal of Eukaryotic Microbiology. Imanian, B., Carpenter, K. J. & Keeling, P. J. 2007. The Mitochondrial Genome of a Tertiary Endosymbiont Retains Genes for Electron Transport Proteins. *Journal of Eukaryotic Microbiology.*

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 et al. 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); and *Kryptoperidinium* and several related genera have a plastid derived from a diatom (Chesnick et al. 1997; Inagaki et al. 2000; Tamura et al. 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., (Lewitus 1999; Fields and Rhodes 1991; 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 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 eye-spot (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 ten 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, since 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 prior to the endosymbiosis from which mitochondria originated, relic organelles have now been found (Bui and Johnson 1996; Roger 1999; 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-sulfur cluster assembly (Tachezy et al. 2001; van der Giezen et al. 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).

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.

## 2.2: Materials and Methods

### 2.2.1: 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, USA) 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 et al. 1988). Cultures for electron

microscopy 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). PCR was carried out using PuReTaq (Amersham Biosciences, Baie d'Urfé, QC) and long range PCR using Elongase Enzyme Mix (Invitrogen). 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*, 6 for *cob*, and 4 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.

**2.2.2: 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 et al. 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), since 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 ($\alpha$) with 8 variable rate categories were estimated from the data with PhyML 2.4.4 (Guindon and Gascuel 2003) under the Whelan and Goldman (WAG) model of substitution with the frequency of amino acid usage calculated from the data. The i and $\alpha$ 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 analyzed using PhyML. For distance trees, distances were calculated using TREE-PUZZLE 5.2 (Schmidt et al. 2002) with 8 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, et al. 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).

### 2.2.3: 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 at 80 kV.

## 2.3: Results and Discussion

### 2.3.1: 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, see below) resulted in fragments of the expected size, and sequencing multiple clones yielded a single copy of each gene (from DNA, 3 clones for *cob*, 3 clones for *cox3*, 2 clones for the intergenic space between *cob* and *cox3*, and 4 clones for *cox1*; from RNA, 1 clone for each of the *cob, cox3*, and the spacer between the two, and 4 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 et al. 2005; Van de Peer and De Wachter 1997), and a sister relationship between haptophytes and cryptophytes in *cox3* and *cob* trees (Fig. 3, 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.

### 2.3.2: Endosymbiont mitochondrial genes are expressed and organised 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 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 (see below). 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 the diatom endosymbiont mitochondrial genome is expressing genes for electron transport proteins.

### 2.3.3: Group II introns in the *K. foliaceum* endosymbiont *cox1*.

RT-PCR 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 et al. 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 et al. 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 were 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.*

**2.3.4: 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, 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 to 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).

### 2.3.5: 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 it is not reduced in physical size (Fig. 7) like the nucleomorphs. However, since only a handful of its genes have been sequenced from the endosymbiont nucleus (Chesnick et al. 1997; Chesnick et al. 1996; 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, since 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 most likely comparable in content and size to 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 an apparently functional mitochondrion? 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 to 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.

Fig. 2.1: 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.

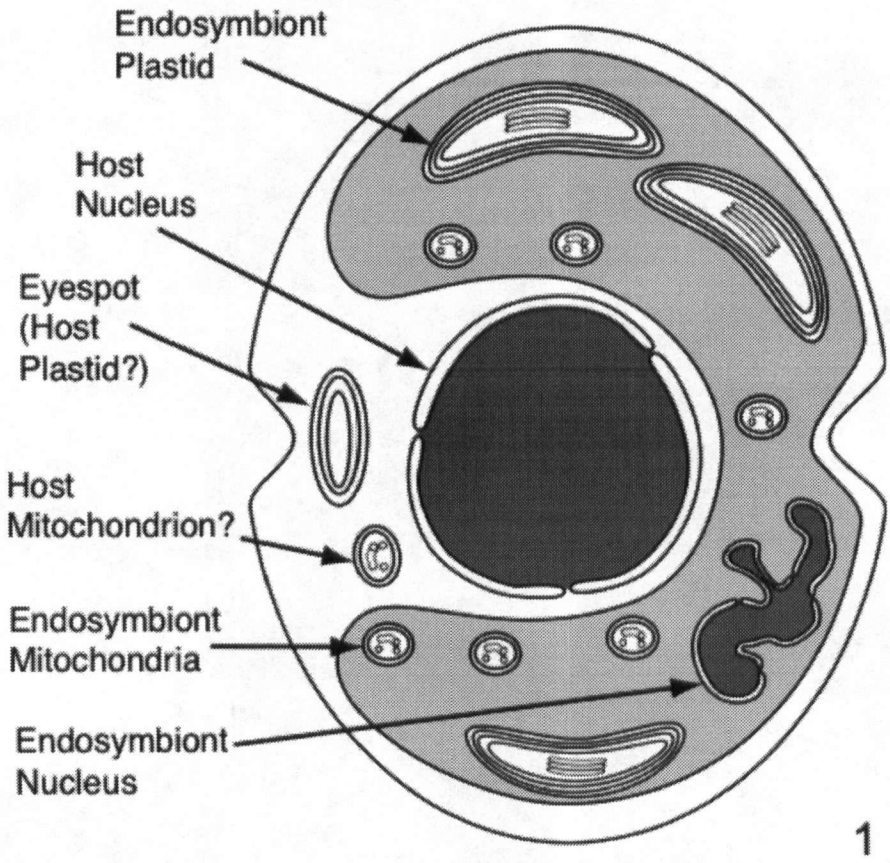


Fig. 2.2: Protein maximum likelihood phylogeny of cytochrome c oxidase 1 (cox1). Numbers at nodes indicate bootstrap support for major nodes over 50% from ML (top) and distance (bottom). A dash (-) indicates support less than 50%. Major groups are labeled to the right, with diatoms indicated by a box. The scale bar is proportional to the number of character change.

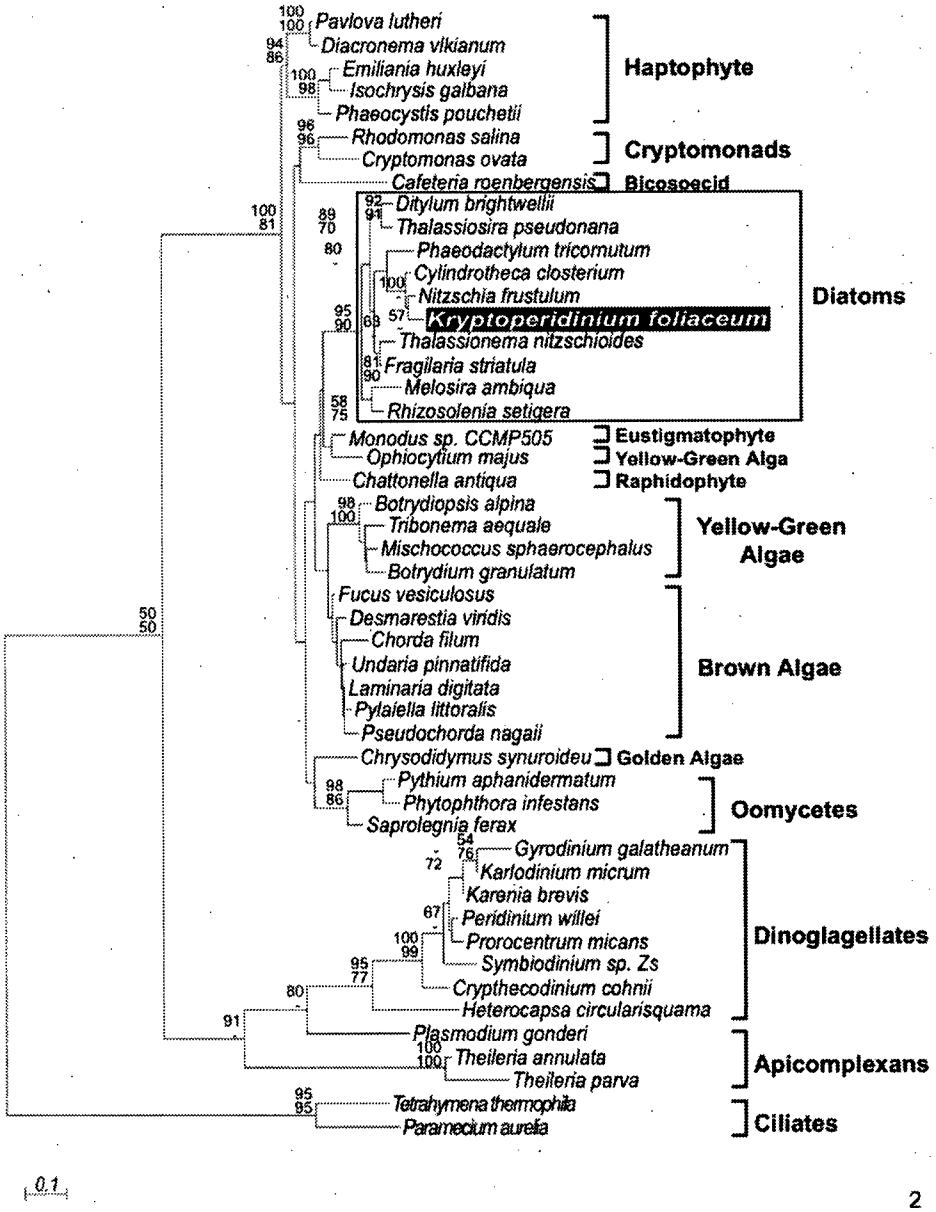


Fig. 2.3: Protein maximum likelihood phylogeny of cytochrome oxidase 3 (*cox3*). Numbers at nodes indicate bootstrap support for major nodes over 50% from ML (top) and distance (bottom). A dash (-) indicates support less than 50%. Major groups are labeled to the right, with diatoms indicated by a box. The scale bar is proportional to the number of character change.

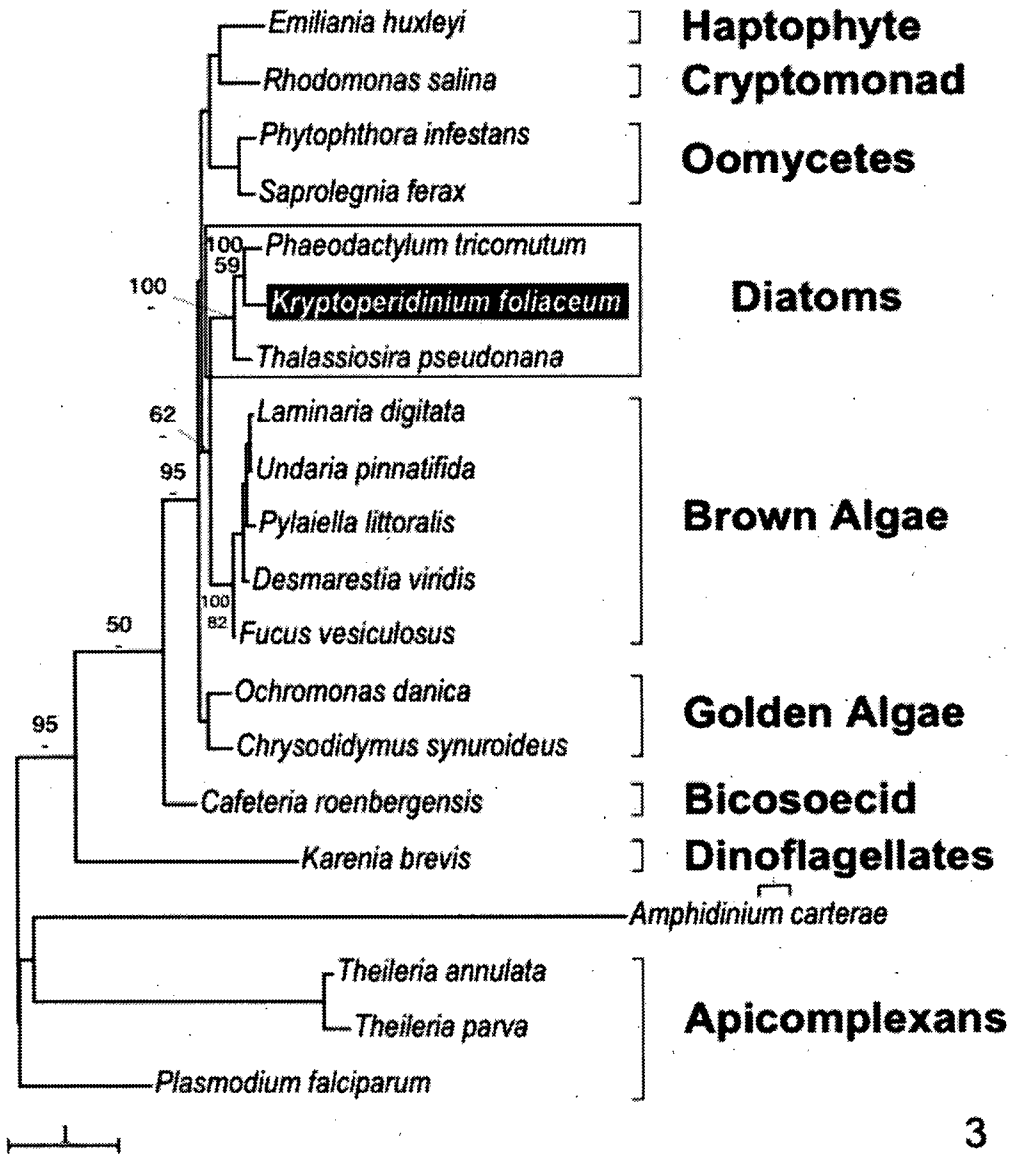
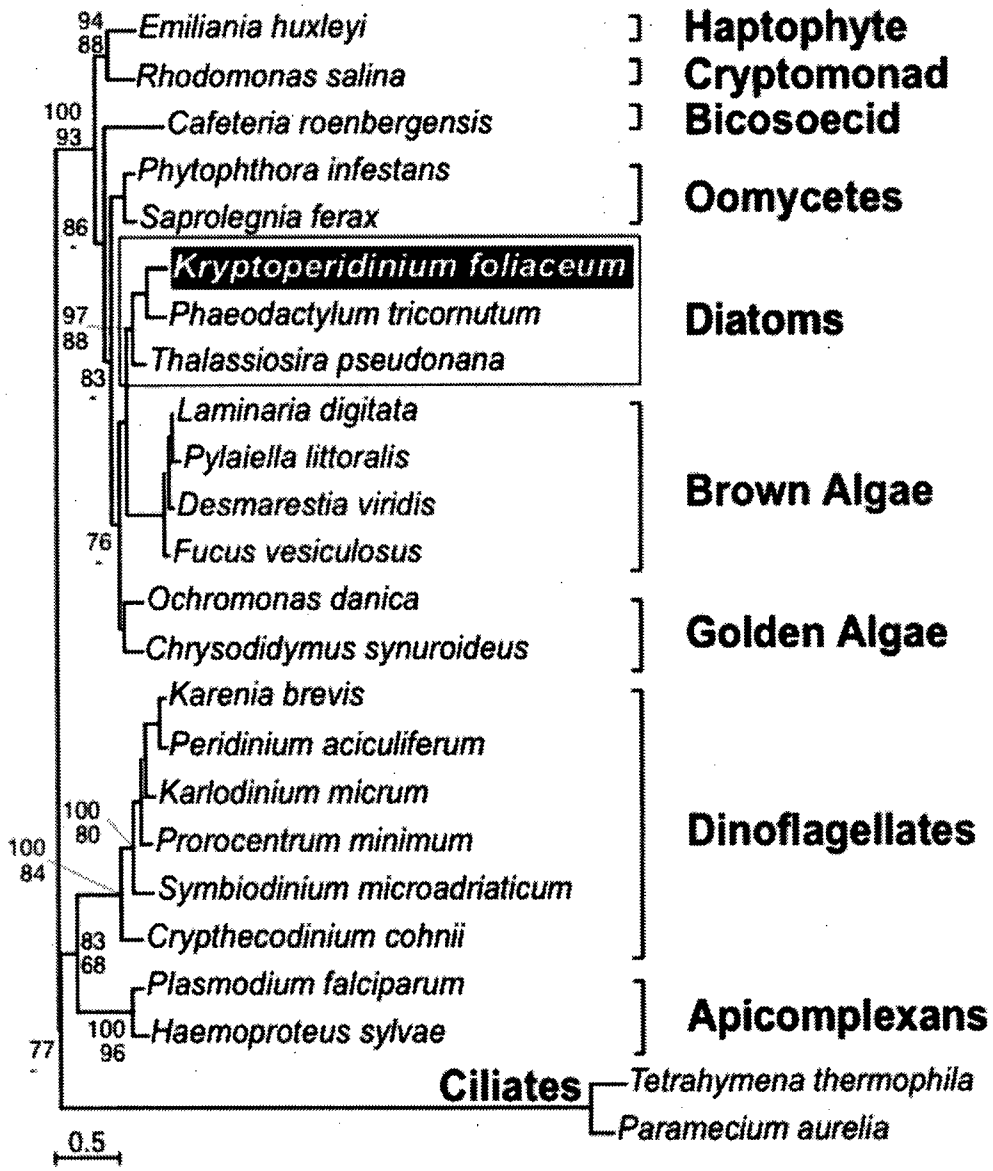
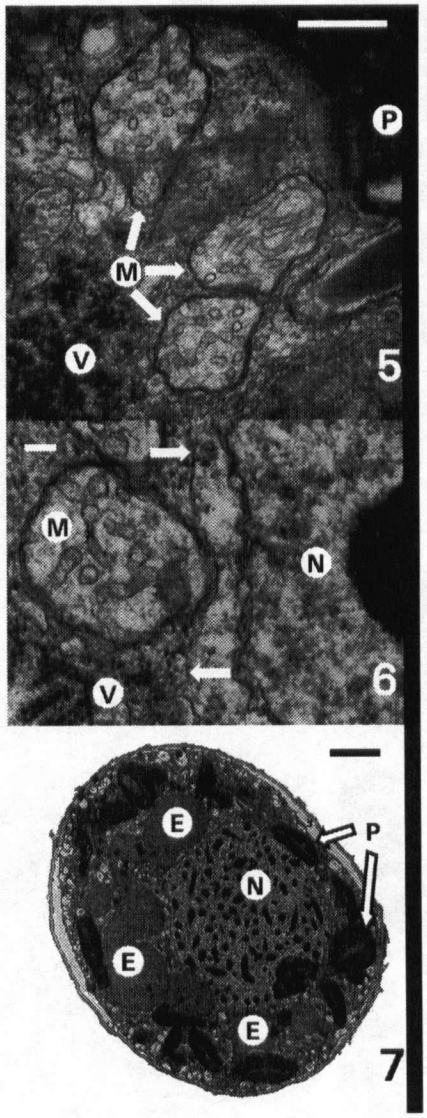


Fig. 2.4: Protein maximum likelihood phylogeny of cytochrome b (*cob*). Numbers at nodes indicate bootstrap support for major nodes over 50% from ML (top) and distance (bottom). A dash (-) indicates support less than 50%. Major groups are labeled to the right, with diatoms indicated by a box. The scale bar is proportional to the number of character change.




4

Fig. 2.5—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 = 5μm.



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# CHAPTER 3: CONCLUSIONS AND FUTURE DIRECTIONS

## 3.1: Conclusions

The results of this study indicate that the tertiary endosymbiont in *Kryptoperidinium foliaceum* has retained not only its mitochondria but also its genome. The presence of numerous mitochondria within the cytoplasm of the endosymbiont in *K. foliaceum*, and their well-defined mitochondrial cristae had already, long before this study, hinted at somehow functional mitochondria within the exceptional endosymbiont that is less reduced than most other endosymbionts. The discovery of three mitochondrial genes, *cox1*, *cox3*, and *cob* and their respective transcripts provided strong support, for the first time at molecular level, for the hypothesis of functional mitochondria within this unusual endosymbiont. Although all these genes and their transcripts were recovered from the total genomic DNA, phylogenetic analyses of these genes indicated that all three had a pennate diatom origin. In addition, the presence of two type II introns within the *cox1*, which is a feature of other *cox1* homologues found in some diatoms such as *Phaeodactylum tricornutum* and *Thalassiosira pseudonana*, and their complete absence from the mitochondrial genome of any known dinoflagellate, gave further support for the diatom origin of these genes. One last line of evidence supporting the diatom origin of the recovered genes here came from the fact that all three transcripts were faithful copies of these genes with no editing, given that the mitochondrial transcripts are heavily edited in known dinoflagellates (Lin et al. 2002).

That these mitochondria existed at all, that they contained a genome of their own, that one of these genes contained two large introns, and that the recovered genes are

transcribed, all showed, surprizingly, that there was no sign of reduction in the genome of these mitochondria. Considering that other such endosymbionts have yielded, one way or another, to the forces of reductive evolution, the question remains as to why and how the endosymbiont of *K. foliaceum* has been more resistant to these forces, especially in retaining its mitochondria and their genome.

### 3.2: Future Directions

Although this study provides answers to a few questions about the endosymbiont of *K. foliaceum*, it poses several more. The first is whether there is any sign of reduction in the complete genome of these mitochondria, and whether there is any novelty in the organization of this genome. This study has provided first anchorage points for linking these genes together, and sequencing the whole genome. The second question is whether the endosymbiont of *K. foliaceum* is the only endosymbiont whose mitochondria retain a genome, or if this is a feature of all or some of the endosymbionts found in the small group that includes *K. foliaceum*'s relatives. Sequencing the complete genomes of these rare mitochondria, if they exist, will offer more clues to answer a third question: did all these dinoflagellate species acquire their endosymbionts through one or more endosymbiotic events? If only one endosymbiotic event gave rise to these different dinoflagellates, as it seems to be the case for *K. foliaceum*, *D. baltica*, and *Galeidinium rugatum* (Inagaki et al. 2000; Tamura et al. 2005), a forth question is raised as to which dinoflagellate, phylogenetically speaking, acted as the host ancestor in taking up the pennate diatom ancestor of all these endosymbionts. The fifth question would be whether there is a shared trend in the organizations of these endosymbionts' mitochondrial

genomes. A follow-up question would be, to what degree these genomes have diverged, and why.

Another series of questions raised here involves the mitochondria of the dinoflagellate host and their genome: are they rare in *K. foliaceum*, and if so, why? Do they contain a genome? If so, why does *K. foliaceum* retain two redundant organelles from two distinct sources, and if not, are there enough reasons to believe that the host's mitochondria share function with, or are being replaced by, the endosymbiont's mitochondria? These questions can be asked about all the close relatives of *K. foliaceum.*

An interesting recent study about one of *K. foliaceum*'s relatives, a benthic, non-motile dinoflagellate called *Peridinium quinquecorne*, which suggests that this organism might have replaced its pennate diatom endosymbiont with a centric one (Horiguchi and Takano 2006), reveals also that the evolutionary history of this small group of dinoflagellates is much more eventful and complicated than one may think or wish.

*Kryptoperidinium foliaceum* is one of the most complex cells to date, and this study has just scratched the surface. This cell is a successful fusion of two distinct eukaryotic cells, representing the most beautiful endosymbiotic partnership, in which it is hard to determine which partner is the 'dominatrix' and which is the 'slave.' Studying this cell is as demanding as it is exciting, and I am optimistic that *K. foliaceum* draws even more attention than it already has.

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