**Faculty of Biological Sciences**

**University of South Bohemia in České Budějovice**


# Molecular characterization of novel symbiotic alveolate isolated from corals

Master Thesis


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Annotation

Novel alveolate RM12 isolated from stony coral *Plesiastrea versipora* was investigated via tools of molecular biology in order to infer its phylogenetic position. According to the data, RM12 is the closest photosynthetic relative of apicomplexan parasites.

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In České Budějovice, 23th of April, 2007 ……..

signature

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# Contents

# Introduction

## Very brief introduction to alveolates

Based on molecular and morphological markers apicomplexans, dinoflagellates and ciliates form well-supported group called Alveolata (Gajadhar et al. 1991, Wolters 1991, Fast et al. 2002, Saldarriaga et al. 2003). Within alveolates apicomplexans appear more closely related to the dinoflagellates rather than to the ciliates (Fast et al. 2002). Alveolates all share a system of sacs underneath their cell membranes. These closely packed sacs are termed alveoli.

## Phylum Ciliophora

Phylum Ciliophora Doflein, 1901 comprises unicellular organisms found in various environments. From ecological point of view they constitute good indicators of the health of environments they occupy (Lynn and Gilron 1992). Nearly all ciliates are phagotrophs using cytostome to ingest their food. Cytostome may be surrounded by ciliar structures called oral kinetids. Cilia are motile rod-shaped parts of the cell found on the surface. They consist of microtubules originating from the basal body known as kinetosome and are surrounded by the cell membrane. All members of the phylum have cilia in some time in their live cycle. Ciliates also contain one or more contractile vacuoles to regulate water content and ion concentrations in the cell. The cell membrane is supported by sacs-like membrane-bound organelles termed alveoli. Another feature that joins ciliates is presence of two nuclei. The bigger one called macronucleus is employed in the cell metabolism and the smaller one is involved in sexual reproduction (Perkins et al 2001a). However, micronucleus has been lost in some lineages (Raikov 1969, 1982). While cell division cycle, nuclei divide endomitotically i.e. the nuclear envelope do not dissolve and persists through cell division (Perkins et al 2001a).

## Phylum Dinoflagellata

Dinoflagellata Bütschli, 1885 is extremely diverse group of unicellular organisms. They inhabit various nutritious niches such as autotrophs, mixotrophs, osmotrophs, phagotrophs and parasites. Dinoflagellata contains more than 4000 sepecies in 550 genera and can be found in both marine and fresh water environments, as symbionts or parasites of various vertebrates and invertebrates, or even in the snow. The name

of this group came from the Greek word *dineo* which means to whirl. Most dinoflagellates have two distinctive flagella and their position creates characteristic rotatory motion when a dinoflagellate moves. This movement is quite fast achieving rates of 200-500 μm/s. The size of dinoflagellate cells varies from 2 to 2000 μm (Graham and Wilcox 2000). Some dinoflagellates produce toxins and can be dangerous for marine animals and humans (Van Dolah 2000). According to their cell wall there are two types of dinoflagellates described. The first group comprises armored dinoflagellates, which have cellulose plates arranged in distinct patterns, which are used to infer their taxonomy. The second group comprises naked dinoflagellates which lack these plates at all (see Fensome 1993).

Nuclear genome and its arrangement is one of the most unusual features of dinoflagellates. Their genomes are extremely large that is 3000 – 216 000 Mbps with high number of morphologically similar chromosomes (Loeblich 1976) which have permanently condensed chromatin. Despite the genome size, content of unique sequences is interestingly 50% (Allen et al. 1975, Hinnebush et al. 1980). Very unusual is also occurrence of the nucleotide hydroxymethyluracil in the DNA because it can not be found in any other eukaryote (Rae 1976). For a long time dinoflagellates have been thought to lack histones (Rizzo 2003, Moreno Diaz de la Espina et al 2005); in spite of the fact that putative histone H3 was annotated from *Pyrocystis lunula.* However this sequence was not further analyzed (Okamoto and Hasting 2003). Then Hackett et al (2005) found weakly expressed histone H2A in *Alexandrium tamarense*. So far histones H2A and H4 from *Crypthecodinium cohni* (Sanchez-Puerta et al 2007), and histone H3 from *Pyrocystis lunula* (Okamoto and Hastings 2003) have been sequenced. Except this rare histone expression found in several species, dinoflagellates contain basic DNA binding proteins similar to bacterial histone-like proteins (Rizzo 1981, Wong et al. 2003).

About one half of all dinoflagellates bear a chloroplast. Majority of these species contain secondary peridinin plastid, however, the rest of them has tertiary chloroplasts or kleptoplastids (Schnepf and Elbrächter 1999) (see paragraph Endosymbiotic theory and the origin of plastids). Plastid genome is reduced in peridinin plastid containing dinoflagellates. Individual genes are encoded on

plasmid-like minicircles 2-3 kbps in size (see Zhang et al 1999, Barbrook and Howe 2000, Zhang et al 2001, Barbrook et al 2001, Hiller 2001).

## Phylum Apicomplexa

Phylum Apicomplexa Levine, 1970 is a large group of parasitic unicellular organisms containing more than 4600 named species and about 180 genera (Levine 1988). A lot of apicomplexan species are of high medical or veterinary importance. Particularly, species of the genus *Plasmodium* cause different forms of malaria and infect 500 millions people every year and 1-2 million people dies (Kahl 2003).

The most characteristic feature of this group is the presence of apical complex in invasive life stages. Apical complex consists of polar ring, cortical microtubules, rhoptries and two apical rings associated with conoid apparatus. Apical complex is a sophisticated tool that allows apicomplexans penetrate host cell (Blackman & Bannister 2001). The second feature that joins almost all apicomplexans is presence of non-photosynthetic secondary plastid called the apicoplast suggesting that apicomplexans evolved from a photosynthetic ancestor. The loss of plastid occurred in some lineages such as colpodellids (free living phagotrops) (Williams and Keeling 2003), *Cryptosporidium* (Zhu et al. 2000) and in some gregarines (Obornik et al 2002, Tosso and Omoto 2007). Although the particular function of the organelle is not known, several important metabolic pathways such as fatty acid synthesis, non-mevalonate pathway for isoprenoid synthesis, or part of the heme pathway, take place in the apicoplast (McFaden and Roos 1999; Sullivan et al 2000; Maréchal 2001; Gornicky 2003). The apicoplast genome is circular and reduced to about 35 kB (Wilson et al. 1996). The copy number of plDNA has been poorly studied however it was found that there are 1 or 2 copies in case of *Plasmodium falciparum* and cca. 8 copies in case of *Toxoplasma gondi* present within the apicoplast (Wilson and Williamson 1997). Plastid of Apicomplexa is supposed to be monophyletic in origin (McFadden et al. 1997, Denny et al. 1998, Lang-Unash et al. 1998, Obornik et al. 2002).

## Endosymbiotic theory and the origin of plastids

Plastids are photosynthetic organelles found in plant and algae. In some cases plastid lost its photosynthetic function, however, essential metabolic pathways take place in it. The diversity of plastid forms is amazing as well as their various functions.

Primary plastids arose as a product of endosymbiosis of a cyanobacteria and a eukaryotic host around 1.6 billion years ago ( Hedges et al 2004, Yoon et al 2004). It is thought that phagotrophic eukaryote swallowed its cyanobacterial prey which it was unable to digest. The cyanobacterium lost some of its genes and many others were transferred to the host nucleus (Gray and Spencer 1996, Martin and Herrmann 1998). Descendants of this particular endosymbiosis are green plants, red algae and glaucophytes. Primary plastid is bound by two membranes, which correspond to the inner and outer membrane of the domesticated cyanobacteria (Jarvis and Soll 2001).

After the event of the primary endosymbiosis the secondary endosymbiosis occurred. An alga bearing primary plastid was engulfed by a eukaryotic phagotroph and became an organelle (Archibald and Keeling 2002). From this event originated green (green alga) and the red (red alga) plastid lineages respectively. It was inferred that event of secondary endosymbiosis of the red lineage occurred shortly after the primary endosymbiosis between 1.3 billion years ago (Yoon et al 2004). While establishing of the secondary endosymbiosis a huge number of genes were lost or transferred and a novel protein-targeting system was developed (McFadden 1999). Proteins encoded by genes that were transferred to the nucleus had to be somehow returned back to the complex plastid and overcome two additional membranes. Secondary plastids of the green algal origin are found in Euglenids and Chlorarachniophytes. In spite of that these two groups are not closely related (Perkins et al 2001b, Archibald et al. 2002) their plastids are monophyletic (Cavalier-Smith 1999). Secondary plastids of the red algal origin are found in Cryptomonads, Stramenopiles, Haptophytes, some Dinoflagellates and Apicomplexans. These taxa together with ciliates form a group called Chromalveolata (Cavalier-Smith 1999) and are postulated to share a single red algal endosymbiosis in their common ancestor. This suggestion is widely known as the chromalveolate hypothesis (Cavalier-Smith 1999). The major problem with the chromalveolate theory is the existence of

Ciliates, which are heterotrophs having no trace of plastid. Alternative theories assume that particular taxa of Chromalveolates gained their plastid independently (see Bodyl 2005, Falkowski et al 2004, Grzebyk et al 2003,).Despite of recent findings of Fast et al. 2001 and Harper and Keeling 2003, which support the existence of Chromalveolates, whole hypothesis remains unproven. Moreover the big challenge for chromalveolate hypothesis became the work of Bodyl (2005). He critically revisited this hypothesis in the light of recent phylogenetic, cytological and genomic data and suggested that plastids of Alveolata and Chromista evolved in array of independent endosymbiotic events and that majority of them may result from tertiary endosymbioses.

To make it even more complex dinoflagellates acquired their plastids not only from the secondary endosymbiosis (peridinin plastids). Plastid gain or loss is quite common in dinoflagellates. Such tertiary endosymbioses occurred in multiple occasions (Saldarriaga et al. 2001). Some dinoflagellates have tertiary plastid of the cryptomonad (Schnepf and Elbrächter 1988, Vesk et al. 1996), diatom (Chesnick et al. 1997) and prasinophyte origin (Watanabe et al. 1987). Recent discoveries of plastid-lacking alveolates (*Perkinsus, Oxyrrhis*) which fit to the base of dinoflagellate clade suggests a possibility that even peridinin plastids arose from the tertiary endosymbiosis (Hackett et al 2004, Bodyl 2005).

The special forms of plastids are kleptoplastids. Kleptoplastids means that these incorporated plastids were stolen from various algae by their new host (Schnepf and Elbrächter 1999). They are of limited survival and represent the way how a heterotrophic organism can obtain capacities of an autotroph. Such relationship provides no advantages for the plastid but it represent selective advantage for heterotrophic thieves (Graham and Wilcox 2000).

## Fundaments of discussion on apicomplexan plastid - green versus red

Since there was no known photosynthetic apicomplexan researchers argued whether is the apicoplast of the “red” or of the “green” origin. It is known that AT content of apicoplast genome is extremely high creating bias in phylogenetic analyses (Lockhart et al. 1992). Sequences encoding ribosomal RNA represent widely used

genes of choice when inferring phylogeny. In 1995 Egea and Lang-Unnash showed their analyses based on plastid 16S rDNA gene suggesting relationship of apicomlexans and euglenoids which belongs to the green lineage (Delwiche, 1999). Unfortunately there were no sequences from dinoflagellates available. The green origin was also inferred by Köhler et al. (1997) however the support for this branching was poor. They chose *tufA* gene encoding elongation factor TU. Wilson (1993), Williamson et al. (1994) and Yap et al (1997) suggested that the presence of the *sufB* gene previously known as ORF 470 or *ycf24* in the apicoplast shows the red algal origin. *sufB* is a homologue of a protein only found in the cyanobacteria and in the red algae. In the apicoplast *sufB* is responsible for iron cluster synthesis and oxidative stress resistance (Ellis et al. 2001). Blanchard and Hicks (1999) focused on the gene losses and rearrangements in sequenced plastid genomes and found „the green scenario" improbable. Mc. Fadden et al. (1997) and Stoebe and Kowalik (1999) found similarity in organization of the ribosomal protein gene cluster between apicomplexans and plastids from the red lineage. Zhang et al. (1999) and (2000) published plastid sequences of 16S and 23S rDNA genes from dinoflagellates and their phylogenetic analyses clustered together dinoflagellates and apicomplexans. They also encountered clustering of apicomplexans with euglenoids in case of 16S rDNA (like Egea and Lang-Unnash in 1995), however this branching was artificial. Another work that very strongly supported the red algal origin of the apicoplast appeared in 2001. Fast et al. (2001) used GAPDH (glyceraldehyde-3-phosphate dehydrogenase) in their study. There are two copies of GAPDH in plastid bearing organisms. The first of them is employed in the cytosol and the second one in the chloroplast. The cytoplasmatic version of GAPDH in the red algae, green algae and euglenoids is related to the other eukaryotic homologues. The plastid version of GAPDH is related to the cyanobacterial homologues. Surprisingly, this is not true in dinoflagellates, cryptomonads and apicomplexans. Both versions of GAPDH in these taxa are homologous to the cytoplasmatic versions! Fast et al. (2001) not only shown close relationship between dinoflagellates and apicomplexans but their work also supported the chromalveolate theory (Cavalier-Smith, 1999). Findings of Funes et al. (2002) were the last big challenges for the red algal origin of the apicoplast. In chlamydomonad algae the *cox2* gene was transferred from the mitochondrion to the nucleus and split into two subunits (Perez-Martinez et al. 2001). Funes et al. (2002) demonstrated that *cox2* encoded in the *Plasmodium* and *Toxoplasma* nuclear

genomes is split in the same way suggesting it was transferred from the green alga derived plastid. However this evidence for the green origin appeared much weaker than it might look. Except this possible gene transfer genome of *Plasmodium* contains no genes of the green algal origin (Gardner et al. 2002). Recently Waller and Keeling (2006) found out that curious *cox 2* rearrangement in alveolates and chlorophytes were two independent events, so that there was no lateral gene transfer as previously mentioned by Funes et al (2002).

## Mitochondria

Mitochondria are cellular organelles which perform plenty of fundamental functions. They are employed in the ATP synthesis (Saraste 1999), cell death (Jones 2000, Youle and Karbowski 2005), cell signaling (Vandecasteele et al 2001, Logan and Knight 2003), biosynthesis of amino acids, vitamin cofactors, fatty acids, iron clusters (Mackenzie and McIntosh 1999, Bowsher and Tobin 2001).

| **group** | **cristae** |
|---|---|
| Glaucophytes | flattened |
| Cryptophytes | flattened |
| Euglenoids | disk shaped |
| Chlorarachniophytes | tubular |
| Red algae | flattened |
| Green algae | flattened |
| Haptophytes | tubular |
| Ochrophytes | tubular |
| Dinoflagellates | tubular |
| Apicomplexans | tubular |

*Table 1: Cristae morphology of major algal groups*

Mitochondrion comprises four distinct compartments: the outer membrane, the intermembrane space, the inner membrane and the matrix. The inner membrane is folded into cristae, which contain protein machinery producing ATP. The fold of the inner membrane is characteristic for certain taxa (for example see the table 1).

From the phylogenetic point of view mitochondria constitute monophyletic group sharing common ancestor with α-proteobacteria (Lang et al 1997, Andersson et al 1998, Gray et al 1999, Gabaldon and Huynen 2003, Esser et al 2004, Lang et al 2005). There are three major theories of the mitochondrial origin (Hackstein et al 2006). The first of them is the serial endosymbiotic theory. It assumes that

mitochondrion-lacking eukaryote hosted a bacterium that became an ancestor of current mitochondria. A eukaryotic cell and nucleus was formed via fusion of an archaebacterium and a proteobacterium followed by α-proteobacterium acquisition (Zillig et al 1989). Hydrogen theory postulated by Martin and Müller (1998) assumes simultaneous origin of the eukaryotic cell and its mitochondrion. A symbiosis established between a hydrogen producing α-proteobacterium and hydrogen requiring methanogenic archaebacterium. This theory can further explain the single origin of anaerobic and aerobic versions of mitochondria. Very similar is the hypothesis of Moreira and Garcia (1998) employing a δ-proteobacterium instead of α-proteobacterium.

## Mitochondrial genome of Apicomplexans

Mitochondrial genome of apicomplexan parasites is rather small and even gene content is reduced. It is linear molecule 5.9 kbps in size encoding only three proteins of respiratory chain Cytochrome C oxidase subunit I (*cox 1*), Cytochrome C oxidase subunit III (*cox 3*) and Cytochrome b (*cob*). Except these proteins it encodes truncated ribosomal RNAs LSU and SSU respectively. Surprisingly there are no genes for tRNAs, all required tRNAs are nuclear encoded and imported to the mitochondrion. In *Plasmodium* species mitochondrial genome consists of tandemly repeated 6 kbps units however Preiser et al (1996) detected circular molecules in total amount less than 1% of mtDNA mass. In *Theileria parva* the genom consist of coding elements flanked by inverted repeats (Faegin 2000). Ribosomal RNAs are fragmented (Faegin 2000) in apicomplexan mitochondria into twenty fragments encoded on both strands in *Plasmodium falciparum* (Faegin et al. 1997) and some of these fragments are oligoadenylated (Gillespie et al 1999). Transcription is driven by T3/T7 phage-like RNA polymerase (Li et al 2001) producing polycistronic product (Ji et al 1997) which is cleaved and mature mRNAs and rRNAs are adenylated (Redkopf et al 2000). There is no evidence that mRNA editing occurs in apicomplexans (Faegin 1992, McIntosh et al 1998, Redkopf et al 2000).

## Mitochondrial genome of Dinoflagellates

It is known and proven fact that mtDNA of dinoflagellates is extremely hard to study therefore only few works appeared on this theme enlightening small pieces of this

puzzle. So far mtDNA was only characterized from *Crypthecodinium cohnii*. It is a mixture of linear molecules smaller than 10 kbps (Norman 2000, Norman and Gray 2001). In *Crypthecodinium cohnii cox 1* coding sequence is present in two versions which differ mainly in C terminal region. Central repeat unit is flanked by 2 different upstream and 2 different downstream flanking regions. These flanking domains contain short inverted repeats (Norman 2000, Norman and Gray 2001). Very similar arrangement can be found in plant mitochondrial genomes (Hanson et al. 1992). Chaput et al (2002) reported occurrence of poolyadenylated transcripts similar to *cox 1*, *cox 3* and *cob* genes in the dinoflagellate *Gonyaulax polyhedra*. Lin et al. (2002) discovered widespread and extensive substitutional editing of mRNA for *cox 1* and *cob* respectively in the same species. Nucleotide substitutions were predominantly in the first or in the second positions changing the amino acid content. Because of the character, pattern and multiplicity of changes Gray (2003) suggested that this is a novel type of editing. Mitochondrion encoded rRNAs are fragmented and oligoadenylated (Norman 2000).

## Endosymbionts of corals

A lot of various animals and plants established symbiotic relationship with microorganisms obtaining large amount of benefit (Douglas, 1998). Many marine invertebrates (corals, anemones, jellyfish etc.) host endosymbiotic algae allowing them to inhabit nutrient poor environments (Muscatine and Porter. 1977; Miller D. J. and Yellowlees, 1989). Majority of these endosymbionts are dinoflagellates of the genus *Symbiodinium.*

Endosymbionts at least enable their hosts to get the great advantage of autotrophy. They were reported to release photosynthesate (Muscatine 1990). Moreover thanks to their endosymbionts cnidarians can recycle waste nitrogen, which is returned back in the form of aminoacids (Muscatine and Porter. 1977; Falkowski et al. 1993). Biosynthesis of essential aminoacids undermines the value of these symbiotic algae. Fitzgerald and Szmant (1997) reported synthesis of essential amino acids in scleractenian corals which commonly host various *Symbiodinium*. In the sea anemone *Aiptasia pulchella* Wang and Douglas (1999) found that symbiotic

*Symbiodinium* sp. synthesize and transfer to the host 7 aminoacids (histidine, isoleucine, leucine, lysine, phenylalanine, tyrosine and valine).

Endosymbionts enter the host cell via phagocytosis. In a host cell a symbiont is surrounded by symbiosome membrane derived from the phagocytic membrane. Using this membrane a host can control flow of nutrients (phosphorous, nitrogen…) to symbiont (Rees 1991, Rands et al. 1993) eg. rate of symbionts grow. *Symbiodinium* spp. can double its population *in vitro* of around three days (Fitt and Trench 1983, Domotor and D´Elia 1984) while *in situ* is its growth much slower (Miller and Yellowlees 1989).

Genus *Symbiodinium* plays crucial role in a host life. Loss of symbionts or degradation of their pigments in benthic animals such as corals is called bleaching. This negative event causes depressed growth and increased mortality of corals. Moreover bleaching events occur more frequently and are more severe than it used to be (Wilkinson 1999). Extent of bleaching events in last twenty years suggests that this phenomenon is something unnatural (e.g. Rosenberg and Ben-Haim 2002). Coral bleaching can be triggered in laboratory by extreme temperatures (cold/warm), high irradiance, prolonged darkness, heavy metals and pathogenic microorganisms (Hoegh-Guldberg 1999, Brown 2000, Ben-Haim and Rosenberg 2002). It is very probably linked with global warming and pollution of the sea (Harvell et al. 1999).

Coral bleaching represents a deleterious trait so why it persists in *Symbiodinium* symbioses? According to the adaptive bleaching hypothesis proposed by Buddemeier and Fautine (1993) it may represent a mechanism how to deal with bad conditions. It is risky adaptive strategy when animal partner tries to replace an old symbiont by an alternative one. Most of corals hosting *Symbiodinium* live in shallow water 1-2 ˚C bellow the temperature that triggers coral bleaching. Therefore global warming represents the real threat which can cause death of coral reefs and collapse of this unique ecosystem (Douglas 2003).

# Aims of the study

- Molecular phylogeny based on protein coding genes of the novel alveolate RM12 isolated from corals.
- Finding of unique features typical for particular groups of Alveolata in RM12.
- Estimation of mitochondrial genome size of RM12.

# Material and methods

## RM12 culturing

RM12 was cultured in f2 medium (see appendix) in plastic tubes. Tubes were placed in TS 606 CZ/2 – VAR (Schoeller) thermostat tempered to 18 ºC. Photoperiod was set to 14 hours of the light and 10 hours of the dark.

## DNA isolation

Cells were scraped from the wall of a tube and suspension was centrifuged at 13000 rpm (g = 20000 $m/s^2$) for 5 minutes. Supernatant was discarded and 96% ethanol was added to the pellet. Ethanol fixed cells were stored at -20 ºC for later use. Before DNA isolation fixed cells were peletted down and ethanol was discarded. Cells were kept at 37 ºC overnight to let remaining ethanol evaporate. Dried pellet in 1.5 ml eppendorf tube was covered with 400 µl of lysation buffer (Wattier et al 2000) (see appendix) and 100 µl proteinase K (20 mg/ml) was added. Lysis was carried out at 56 ºC overnight. Than reaction was transferred on ice and let stand for 5 minutes. Then it was spin down at 13000 rpm (g = 20000 $m/s^2$) for 5 minutes. Supernatant was transferred to the new tube and pellet discarded. Volume of the lysate was filled to 600 µl by dd$H_2O$ and the same volume of phenol was added and the tube was mixed gently, but thoroughly by inverting for 5 minutes. Then sample was centrifuged at 13000 rpm (g = 20000 $m/s^2$) for 5 minutes. Two phases appeared in the tube. Upper phase was transferred to the new tube and equal volume of phenol was added. Tube was mixed for 5 minutes by inverting followed by a new centrifugation. Water phase was purified by phenol, until interface appeared clear. Then phenol and chloroform (1 : 1) were added in equal volume to water phase and reaction was mixed and centrifuged again. Upper phase which arose from this step was mixed with pure chloroform (1 : 1) and mixed and centrifuged in the same way like in cycles before. The upper phase was transferred to the new tube, mixed with room temperature absolute (96 %) ethanol 1 : 3 and incubated at - 20 ºC for 3 hours. Tube was centrifuged at 4 ºC and 14000 rpm (g = 20600 $m/s^2$) for 15 minutes. Supernatant was discarded and 1000 µl of 70% ethanol were added. This step was followed by centrifugation at 4 ºC and 14000 rpm (g = 20600 $m/s^2$) for 10 minutes. Supernatant was discarded again and pellet dried at room temperature for 30 minutes.

Then 50 µl of $ddH_2O$ were added and pellet was resuspended by pipeting up and down. DNA was stored at -20 ºC.

## RNA isolation and cDNA synthesis

Trizol® Reagent (Invitrogen) was added to the pelleted cells (1 ml/50-100 mg of material), then glass beats was added and whole reaction was shook in Mini-Beadbeater™ (Biospec Products) for 5 minutes at 5000 rpm. Thorough shaking was the crucial step of whole isolation because the cell wall of RM12 is thick and is difficult to disrupt it. RNA was then isolated according to manufacturers instructions. At the end RNA was precipitated using 96% ethanol, air dried and then dissolved in 100 µl RNase free $ddH_2O$. Quality of isolated RNA was evaluated using electrophoresis. For this purpose 1.2% (w/v) agarose gel and 1 x TBE buffer was used. Single strand cDNA was synthesized using RevertAid™ H Minus Strand cDNA Synthesis Kit (MBI Fermentas) and random hexamers according to manufacturer's instructions. Concentration of RNA and cDNA was measured in cuvettes (UVette® 220-1600 nm; Eppendorf) at 260 nm using BioPhotometer (Eppendorf). Then cDNA was dilluted to the concentration of 100-150 ng/µl.

## Polymerase Chain Reaction (PCR)

PCR was carried out to obtain desired genes in useful number of copies. In spite of different PCR programs and different primer pairs the reaction mixture was always the same. The mixture contained 2.5 µl of 10x Taq PCR buffer (Top - Bio), 2 µl of 2.5 mM dNTP's, 1 µl (25 pmol) each primer, 1 unit of Taq purple DNA polymerase (Top - Bio), 1 µl (50-200 ng/ µl) of total DNA (cDNA), and 17 µl of $ddH_2O$. Amplification was made using T3 Thermocycler (Biometra).

Histone H2A was amplified from cDNA using H2AF/H2AR primer pair. For primer sequences see table 2. The amplification program consisted of pre-denaturation at 95 ºC for 5 minutes and 30 cycles consisted of: 94 ºC for 1 minute, 59 ºC for 1 minute, and 72 ºC for 1 minute. Program was terminated by final elongation at 72 ºC for 10 minutes.

Histon H2B was amplified from cDNA using H2B1/H2B2 primer pair. For primer sequences see table 2. The amplification program consisted of pre-denaturation at 95 ºC for 5 minutes and 30 cycles consisted of: 94 ºC for 1 minute, 60 ºC for 1 minute, 72 ºC for 1 minute. Program was terminated by final elongation at 72 ºC for 10 minutes.

GAPDH was amplified from cDNA using GAPDH1/ GAPDH2 primer pair (Harper and Keeling 2003). For primer sequences see table 2. The amplification program consisted of pre-denaturation at 95 ºC for 5 minutes and 30 cycles consisted of: 94 ºC for 1 minute, 56 ºC for 1 minute, 72 ºC for 2 minutes. Program was terminated by final elongation at 72 ºC for 10 minutes.

*Cox 1* gene was amplified from total DNA using BROWNKOX1f/KOX1R2 primer pair and sequence was extended using Cox1codingR, Cox1codingF, KOXBRF3NEST, LEXTEPLCOX1BR, ADDKX1BRF2, ADDKX1BRR1, RAND1, COXDSNOCDVR2F2, COXDSNOCODVR2F1, COXDSNOCODVR1F2, COXDSNOCODVR1F1, COXDSNOCODRES, KOXDS9BPOVERLAP, ZACKOX1FOR, KONECKOX1REF primers. For primer sequences see table 2. Moreover, sequences surrounding the gene were investigated too. It was performed using so called uneven PCR method developed by (Chen and Wu 1997). Uneven PCR uses 2 long specific (as much as possible) primers annealing to the target region on one strand of the DNA one next to other and 1 short arbitrary oligonucleotide which anneals randomly on the second strand. PCR is performed in 2 runs. In the first run, the first specific and the arbitrary primer are used. Product of this reaction serves as the template for the second run, when the second specific (nested) and the arbitrary oligonucleotide are used. Uneven PCR uses special cycling program with 2 distinct annealing temperatures within one cycle. The first temperature is high and only specific oligonucleotide anneals, after elongation and denaturation both primers anneal when the second annealing temperature occurs. This design of cyclic conditions favors specificity of the reaction by synthesis of specific ssDNA after the first annealing temperature. In this case following program was used. Pre-denaturation was at 95 ºC for 5 minutes followed by 20 cycles consisted of: 94 ºC for 1 minute, 50 ºC for 1 minute, 72 ºC for 3 minutes, 94 ºC for 1 minute, 40 ºC for 1

minute, 72 ºC for 3 minutes. Program was terminated by final elongation at 72 ºC for 10 minutes. Then 0.5 µl of PCR product was used as the template for the second run.

| name | gene | Sequence 5´-3´ |
|---|---|---|
| H2B1 | Histone H2B | GTBYTSAARCARGTNCAYCCNGANACKGG |
| H2B2 | Histone H2B | ACKGCRTGYTTNGCNARTTCKCCKGG |
| H2AR | Histone H2A | AAYTTGNWKARTTCYTGCTCRTTNCNNACNKC |
| H2AF | Histone H2A | AANTCTGCNAARGCNGGNYTNCARTTCCC |
| GAPDH1 | GAPDH | CCAAGGTCGGNATHAAYGGNTTYGG |
| GAPDH2 | GAPDH | CGAGTAGCCCCAYTCRTTRTCRTACCA |
| M13F | sequencing | GTAAAACGACGGCCAG |
| M13R | sequencing | CAGGAAACAGCTATGAC |
| Cox1codingR | *cox 1* | CTATTCATCGATGTTAATTATTG |
| Cox1codingF | *cox 1* | ATGGAAATATCCCTTCCGAGA |
| BROWNKOX1f | *cox 1* | TRTTCTGGTTTTTTGGNCA |
| KOX1R2 | *cox 1* | GCWAYWACATAATATGTRTCATG |
| KOXBRF3NEST | *cox 1* | CATTCTTATTAGGTGGAGTTACCGG |
| LEXTEPLCOX1BR | *cox 1* | GTGGGTATTATAATCATAAGTTTAAA |
| ADDKX1BRF2 | *cox 1* | TGGGAATTCATTAGTAGAT |
| ADDKX1BRR1 | *cox 1* | CCAAATGCTGGTAATATAA |
| RAND1 | *cox 1* | ANNAWATTCCA |
| COXDSNOCDVR2F2 | *cox 1* | CADATAATCTTACCGGTAACTCTACCTA |
| COXDSNOCODVR2F1 | *cox 1* | CCGTACAACGACTTCGATTCATAACCA |
| COXDSNOCODVR1F2 | *cox 1* | AGACAGGAAGGGTAAATAGAGTCTACA |
| COXDSNOCODVR1F1 | *cox 1* | TATGTAATAGGGATAGCGGTAGCGGGGT |
| COXDSNOCODRES | *cox 1* | TCTATAGCTATATGTACTGTGTGTGTGG |
| KOXDS9BPOVERLAP | *cox 1* | TTAACATCGATGAATAGTTGCTGGGTT |
| ZACKOX1FOR | *cox 1* | CGACAGACCTATTAATATTATCTAA |
| KONECKOX1REF | *cox 1* | ACCAAATCACTAGCTAACCCAGCAA |

Table 2: List of primer sequences

## Electrophoresis

Electrophoresis was used to separate DNA fragments obtained by PCR and later by plasmid restrictions. All samples were examined on 1% (w/v) agarose gel containing ethidium bromide (Sigma) in amount of 0.02 mg/35 ml of agarose gel. To determine the size of characterized DNA fragments 1 kb DNA ladder (New England Biolabs, Inc.) was used as a molecular marker. Products were detected on UV transiluminator (TFX-35.M Vilber Lourmat). Photos of gels were taken by digital camera (Kodak) and processed by software Kodak Digital science 1D version 3.0.2.

## PCR product isolation

Pieces of gel containing PCR product were transferred on the PARAFILM (which is in fact only plastic foil). They were wrapped and these foil-wrapped "parcels" were squeezed. Effluent fluid was collected by pipette and transferred to the clean tube. This fluid contained highly concentrated PCR product and it was used for cloning directly.

## Molecular cloning, transformation and plasmid isolation

PCR products were cloned into pCR®2.1-TOPO® and pDRIVE vectors using TOPO TA Cloning® Kit (Invitrogen) and QIAGEN® PCR Cloning Kit (QUIAGEN) respectively according to manufacturer instructions. Then DH5α chemically competent cells were added to the mixture and reaction was incubated on ice for 30 minutes. Then cells were heat-shocked (30 seconds at 42 °C) and cooled on ice immediately (for 2 minutes). 150 µl of tempered SOC medium (see appendix) was added to the each tube and tubes were shaken (200 rpm) at 37 °C for 1 hour. Competent cells were spread on preheated (37 °C) agar plates (see appendix) coated with 40 µl of X-gal (40mg/ml) (5 – bromo – 4 – cloro – 3 – inodyl – beta – D – galactosidase, Serva) and 4 µl of IPTG (isopropyl thiogalctoside). Petri dishes were incubated at 37 °C for 16 hours. After incubation white colored and white colored bacterial colonies appeared on agar plates. There was a high probability of presence of vector with bond PCR product in white colored colonies. The principle of white/blue selection is that vector contains *lacZ* gene encoding beta-galactosidase. PCR product is inserted into this gene making protein atelic. When PCR product is

not present in *lacZ* gene, functional protein is produced. Beta-galactosidase metabolizes X-gal sugar and blue product appears. White colored colonies were transferred to the tube containing 3 ml of LB medium (see appendix) and 12 µl of ampiciline (12.5 µg/ml) by sterile toothpick. Tubes were incubated in a shaker for 12-14 hours at 37 °C at 200 rpm). Bacterial plasmid containing PCR insert was isolated using QIAprep®Spin Miniprep Kit (QIAGEN). Concentration of plasmid DNA was measured in cuvettes (UVette® 220-1600 nm; Eppendorf) at 260 nm using BioPhotometer (Eppendorf). Isolated bacterial plasmid was analyzed by restriction analysis. 1 µl of ECO RI, 1 µl of Multi Core buffer (Promega) and 3 µl of $ddH_2O$ were mixed with 5 µl of plasmid DNA and tube was incubated at 37 °C for 60-90 minutes. Hydrolyzed plasmids were separated on 1% agarose gel (see electrophoresis).

## Sequencing

Sequencing reaction was carried out using Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to manufacturers instructions and contained 200-300 ng of plasmid DNA and 3.2 pmol of sequencing primer (M13F or M13R). Amplification was made using T3 Thermocycler (Biometra). Sequencing reaction was performed using this program> Pre-denaturation 96 °C for 1 minute followed by 30cycles of: 96 °C for 1 minute, 50 °C for 30 seconds, 60 °C for 4 minutes. The reaction mixture was transferred to the 0.5 ml tube containing 2 µl of 100 mM EDTA, 2 µl of 3 M sodium acetate and 1 µl of glycogen. Then 60 µl of 96% ice cold (-20 °C) ethanol was added and tube was centrifuged at 4 °C at 140 000 rpm (g = 20600 $m/s^2$) for 15 minutes. Small white pellet appeared after centrifugation. Supernatant was removed carefully and 200 µl of 70% ice cold (-20 °C) ethanol was added. Tube was centrifuged again at 4 °C at 140000 rpm (g = 20600 $m/s^2$) for 10 minutes. Supernatant was discarded and pellet dried at room temperature until ethanol evaporated (30 – 60 minutes). Samples were stored at -20 °C until analyzed in ABI 3130 (Applied Biosystems).

## PFGE (pulse field gel electrophoresis)

To separate large DNA molecules pulse field gel electrophoresis (PFGE) was used. This technique enables to separates even whole chromosomes. In this case PFGE was

employed in determination of size of the mitochondrial genome. PFGE was carried out using CHEF-DR® III (BioRad). After optimalisation, following conditions were chosen: initial switchtime = 0.1 s, final switchtime = 1.5 s, current = 6 V/cm, runtime = 13.5 h, temperature = 14 ºC. Living cells were embedded in 1.5% low-melting agarose (Sigma). Mixture was transferred to plug molds and allowed to solidify. Plugs were transferred to PFGE lysis buffer (see appendix) and incubated at 50 °C overnight. Digested molds were stored in SET buffer (see appendix) in 4 °C until use. Electrophoresis was run at 1% (w/v) agarose gel in 0.5x TBE buffer, 2-log ladder (New England Biolabs) and 48 kb ladder (BioRad) were chosen as molecular markers. At these conditions DNA of the size ranging from 4 kb to 50 kb could be detected.

## Southern blot and hybridization

After the PFGE agarose gel was stained using ethidium bromide (0.5µg/ml) in 0.5x TBE buffer for 30 minutes. Then DNA was visualized on UV transiluminator (TFX-35.M Vilber Lourmat). Photos of gels were taken by digital camera (Kodak) and processed by software Kodak Digital science 1D version 3.0.2. Pieces of the gel corresponding to size markers bands were cut out to be able to detect marker later on the membrane. Then DNA was blotted on the BioBond™-Plus NYLON MEMBRANE (Sigma) via alkalic transfer (Sambrook and Russell 2001). *Cox1* gene DNA was radioactively labeled using HexaLabel™ DNA Labelling Kit (Fermentas) according to manufacturer's instructions and probe was purified on MicroSpin™ G-25 columns (Amersham). Hybridization was carried out according to (Sambrook and Russell 2001) in phosphate buffers at 65°C. Results were visualized using TYPHOON 9410 (Amersham Biosciences)

## Primer design

Except of primers for GAPDH gene adopted from (Harper and Keeling 2003), all primers are original. They were designed according to aminoacid/nucleotide alignments aligned in Meg Align (DNASTAR, Inc.).

## Processing of sequences

Raw sequence data were viewed by Sequence Scanner 1.0 (Applied Biosystems) and later processed by DNASTAR programs 5.06 (DNASTAR, Inc.) namely EditSeq and SeqMan II. Sequence identification was carried out by NCBI BlastSearch (http://www.ncbi.nlm.nih.gov/BLAST/).

## Sequence alignment

Aminoacid sequences were aligned in MAFFT (Katoh et al. 2002, 2005) using L-INS-i algorithm at default conditions. L-INS-i is iterative refinement method incorporating local pairwise alignment information and is designed to align a set of sequences containing flanking sequences around one alignable domain. Nucleotide datasets were aligned in BioEdit (Hall 1999) using clustal W algorithm at default conditions as aminoacids and translated back to nucleotides. Alignments were manually edited in BioEdit (Hall 1999), unaligned and ambiguously aligned positions were discarded from the dataset.

## Phylogenetic analyses

Datasets were analyzed by maximum parsimony (MP) using PAUP* 4.10b (Swofford 2001) and by maximum likelihood (ML) using PHYML 2.4.2 (Guindon and Gascuel 2003). Datasets in nucleotides were computed using LogDet-paralinear distances (LogDet) method in PAUP* 4.10b (Swofford 2001). To cope with saturation on the amino acid level, AsaturA (Van de Peer et al 2002) was also employed. Amino acid substitution models were inferred using ProtTest 1.3 (Abascal et al 2005). Substitution models for nucleotide data were inferred using Modeltest3.7 (Posada and Crandall 1998).

For MP analyses heuristic searches were carried out using TBR (Tree Bisection Reconnection) branch swapping and nodes supports were assessed by 100 bootstrap replicates per dataset. Gaps were treated as missing and transversion/transition ratio was default 1:1. ML analyses were performed with appropriate models (cpREV for *cox 1,* WAG for GAPDH) and GTR+Γ+I with gamma distribution in 6 categories was used for all nucleotide datasets and bootstrap analysis was run at 100 replicates. ASA analyses were performed with appropriate models with four distinct cutoff

values also run at 100 bootstrap replicates. Changes in tree topologies were then searched among different methods and datasets.

# Resuts

## *cox 1* gene

Full length *cox 1* gene was amplified and sequenced from total DNA. Coding sequence was 1182 bp long (for nucleotide frequencies see table 3 bellow).

| Species | %A | %G | %T | %C | %A+T | %C+G |
|---|---|---|---|---|---|---|
| RM12 | 27.75 | 13.28 | 44.67 | 14.30 | 72.42 | 27.58 |
| Coccidia | 25.85 | 17.66 | 38.96 | 17.50 | 64.82 | 35.16 |
| Haemosporidia | 30.14 | 14.86 | 42.16 | 12.84 | 72.29 | 27.70 |
| Piroplasmida | 26.89 | 18.71 | 41.67 | 12.74 | 68.55 | 31.45 |
| Dinoflagellata | 26.88 | 15.03 | 40.03 | 18.04 | 66.91 | 33.07 |
| Oomycetes | 25.59 | 18.28 | 44.32 | 11.76 | 69.91 | 30.09 |
| Haptophytes | 23.42 | 19.50 | 40.13 | 16.95 | 63.55 | 36.45 |
| Cryptophytes | 24.45 | 19.82 | 40.34 | 15.38 | 64.79 | 35.21 |
| Brown algae | 22.17 | 20.61 | 39.98 | 17.24 | 62.15 | 37.85 |
| Green algae | 25.52 | 19.51 | 36.91 | 18.06 | 62.43 | 37.57 |
| Red algae | 27.44 | 18.30 | 39.85 | 14.41 | 67.29 | 32.71 |

**Table 3**: Nucleotide frequencies in *cox 1* gene of RM12 and groups of organisms used in phylogenetic analyses

Moreover, regions downstream and upstream were sequenced too. 127 bp downstream from the end of the coding sequence 2 different regions were found suggesting that *cox1* gene is present at least at two different molecules within mitochondrial genome (see figure1).


**Figure. 1**: *cox1* gene of RM12 and its flanking regions

## Phylogenetic analyses of cox 1 gene

Phylogenetic analyses were preformed using methods described in the chapter material and methods. Since Piroplasmida, Ciliates and unfortunately RM12 created long branches, several datasets excluding some of these taxa were examined to infer topology behavior (see table 4). Coccidia were also included in this analysis because they tend to jump at the base of dinoflagellate and apicomplexan clades. Heamosporidia were not excluded from the dataset because if excluded, too few apicomplexans would remain in dataset. Phylogenetic trees were computed from amino acids sequences. Phylogeny was inferred from nucleotide data only from dataset 6.

| DATASET | Haemosporidia | Piroplasmida | Coccidia | Ciliata | Dinoflagellata | RM12 |
|---|---|---|---|---|---|---|
| 1 | YES | YES | YES | YES | YES | NO |
| 2 | YES | NO | YES | YES | YES | NO |
| 3 | YES | NO | YES | YES | YES | YES |
| 4 | YES | YES | YES | NO | YES | NO |
| 5 | YES | NO | YES | NO | YES | NO |
| 6 | YES | NO | YES | NO | YES | YES |
| 7 | YES | YES | YES | YES | YES | YES |
| 8 | YES | YES | YES | NO | YES | YES |
| 9 | YES | NO | NO | YES | YES | YES |

**Table 4**: This table shows occurrence of particular taxa in computed datasets

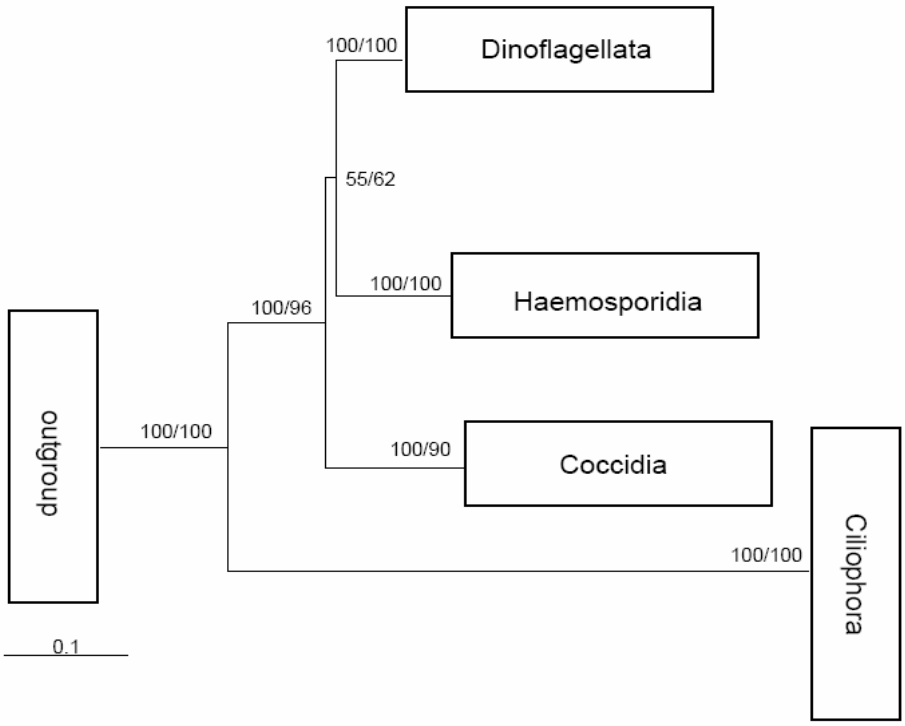


**Figure 2**: Phylogenetic tree constructed using AsaturA inferred from the dataset 2 of *cox 1* gene. Bootstrap supports represent values from AsaturA and MP respectively.

Topologies were inferred from amino acids using ML, MP and AsaturA (five distinct cutoff values), for nucleotide data (i. e. dataset 6) ML, MP, and LogDet were used.

Amino acid trees: ML trees supported monophyly of dinoflagellates and apicomplexans and also supported alveolates (e. g. datasets 1, 2, 3, 7, 9). However, MP and AsaturA found that apicomplexans do not form sister clade to dinoflagellates (figure2). In ML trees monophyly of apicomplexans was supported. RM12 when included in the dataset decreased bootstrap supports between apicomplexans and dinoflagellates (figures 3 and 4) in ML trees and apicomplexan clade in fact collapsed. When changing content of datasets, RM12 appeared basal either to apicomplexans or to dinoflagellates however these positions were not supported. Datasets 3 and 6 analyzed using AsaturA showed branching of RM12 with Coccidia (figure 5) assessed by bootstrap values of 57 and 73 respectively.

Nucleotide trees: Topology inferred from nucleotides was similar to that from amino acid data. It was computed only from sequences of the dataset 6, to avoid long branching artifact. In ML and MP trees position of RM12 could not been inferred

due too low bootstrap support. LogDet distance method revealed strong clustering of RM12 with apicomplexans placing RM12 at the root of Coccidia. Moreover, using LogDet distances, artificial polyphyly of apicomplexans disappeared and they formed supported monophyletic clade (figure 6). Topologies were inferred from amino acids using ML, MP and AsaturA (five distinct cutoff values), for nucleotide data (i. e. dataset 6) ML, MP, and LogDet were used.

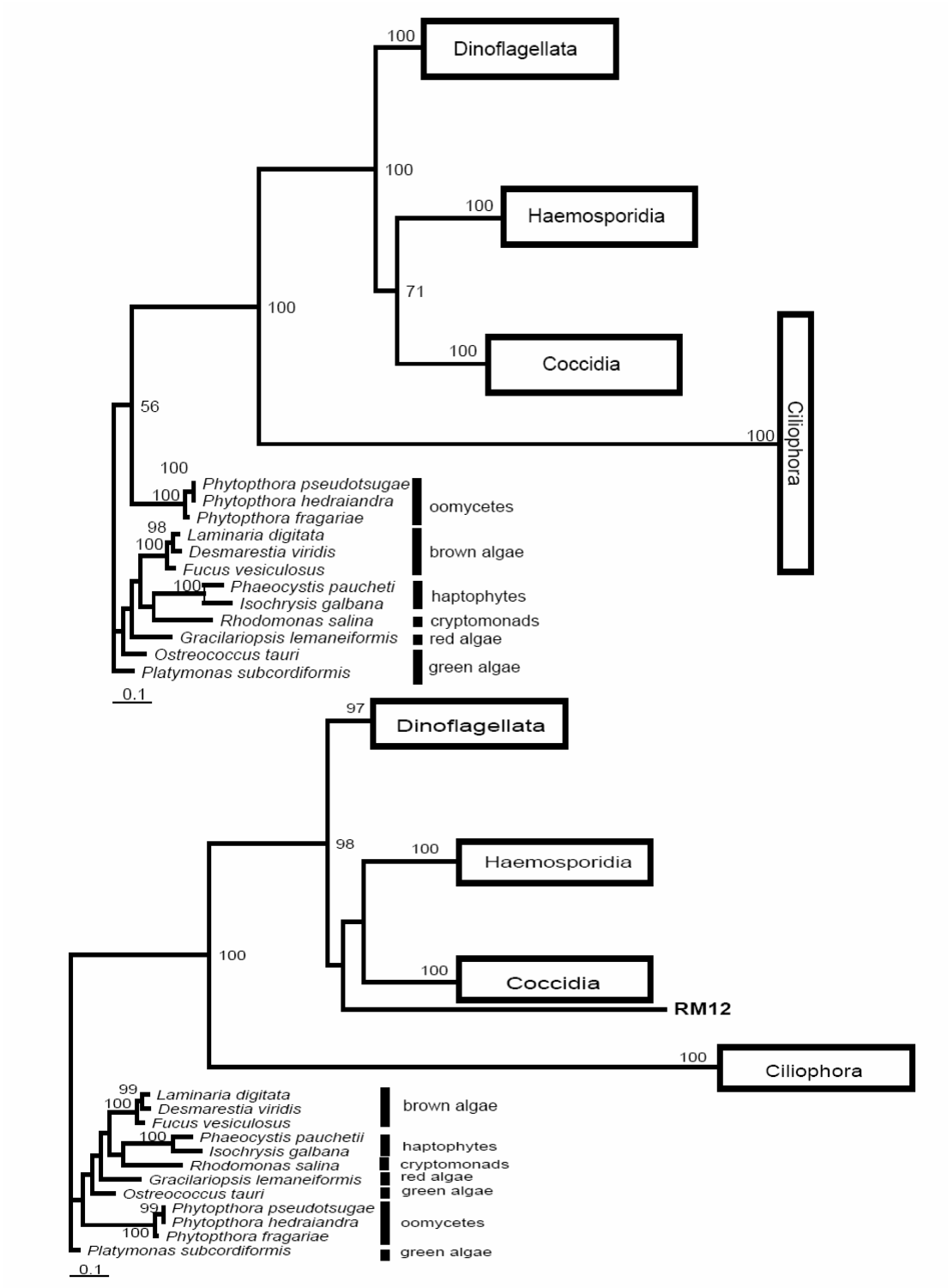


**Figure 3**: Phylogenetic trees inferred using ML method from *cox 1* gene. The tree above displays supported branching of apicomplexan parasites. The tree bellow shows influence of RM12 on the topology and bootstrap support

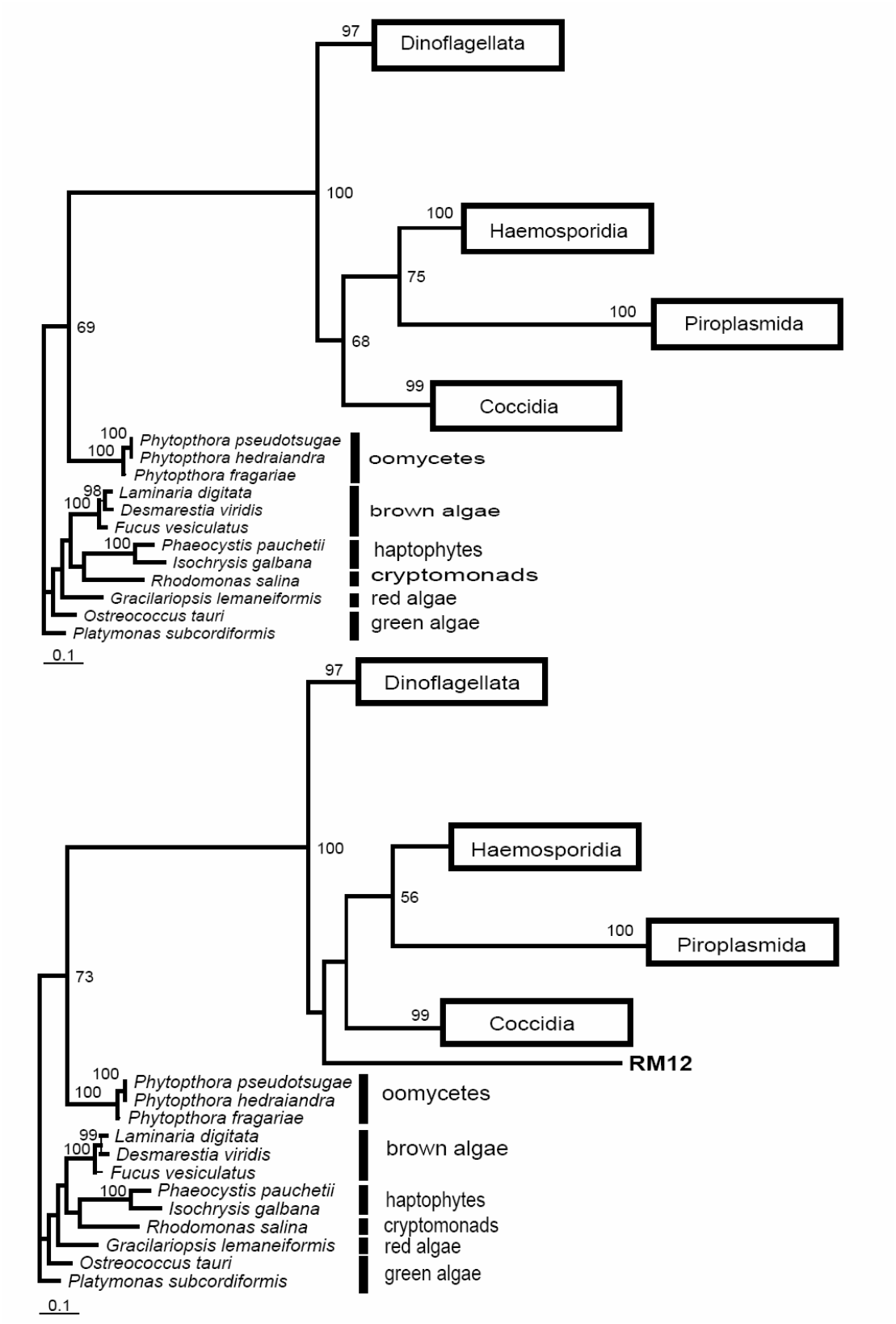


**Figure 4**: Phylogenetic trees inferred using ML method from *cox 1* gene. The tree above displays supported branching of apicomplexan parasites. The tree bellow shows influence of RM12 on the topology and bootstrap support.

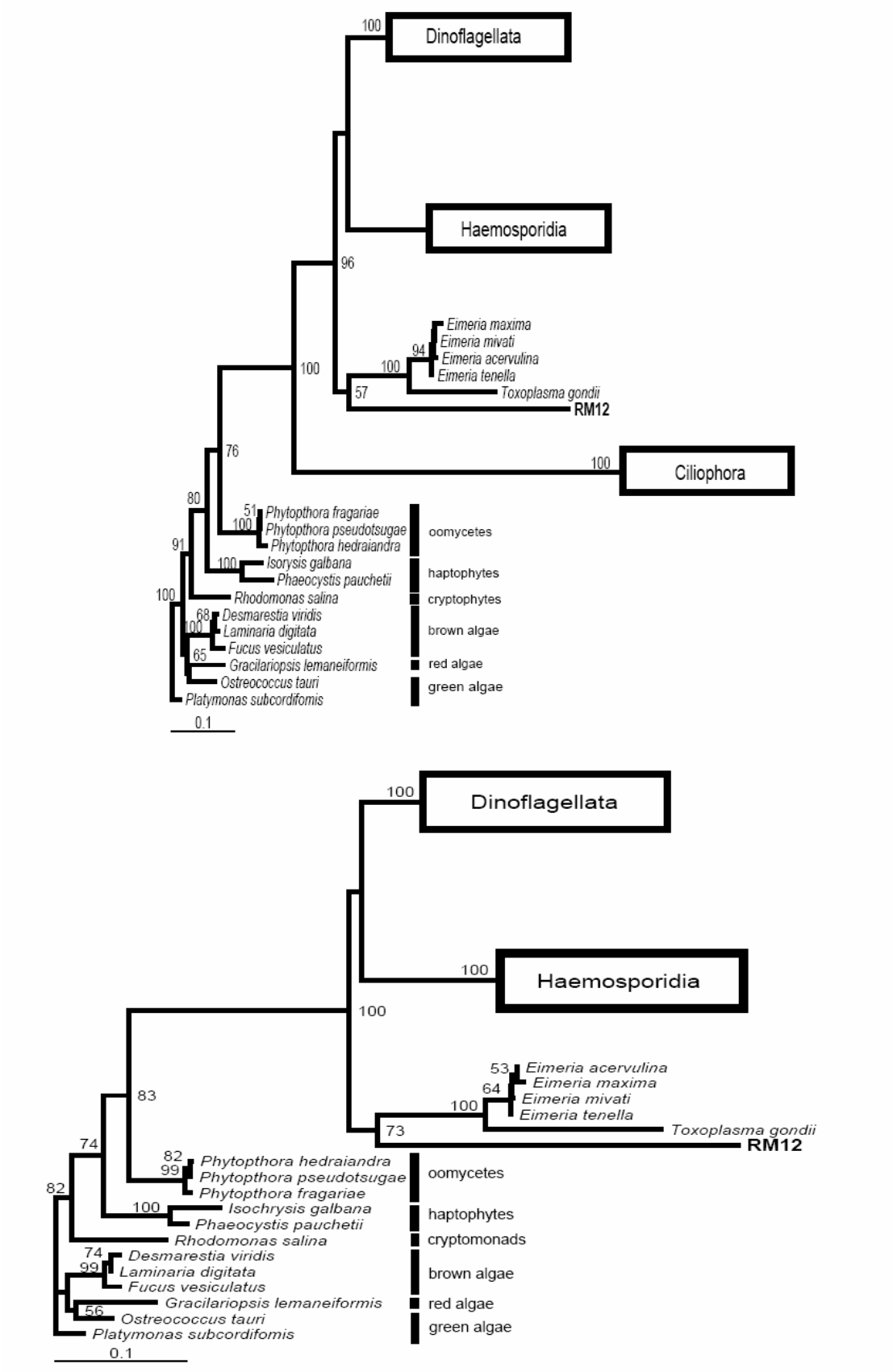


**Figure 5**: Phylogenetic trees constructed using AsaturA from *cox 1* gene, datasets 3 (above tree) and 6 (bellow tree). Cutoff values are 2871 and 2209 respectively.

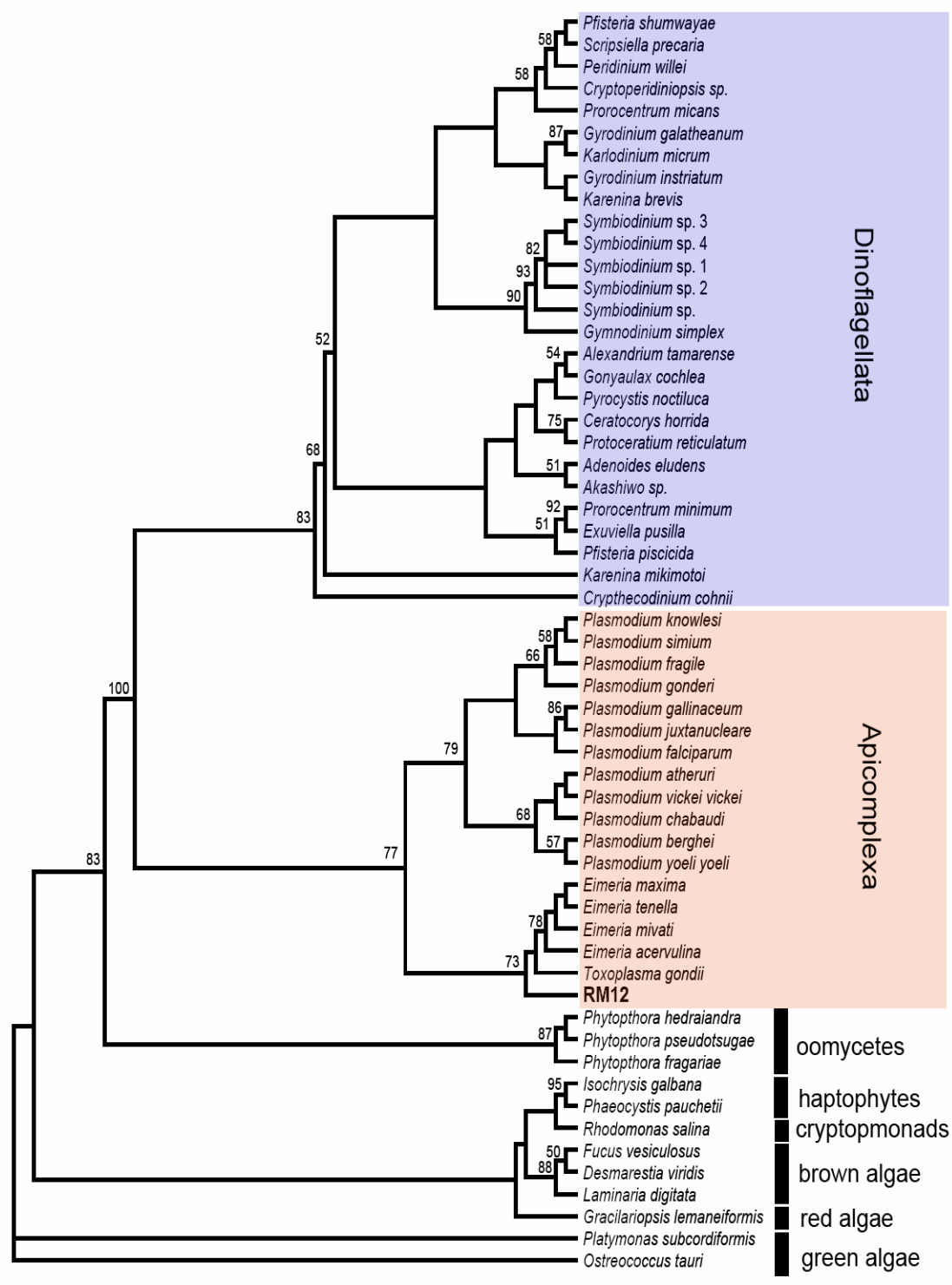


**Figure 6**: Phylogenetic tree constructed using LogDet distances from *cox 1* gene. It was computed from nucleotide data. Taxon sampling corresponded to dataset 6. Third codon positions were discarded from the dataset. The tree indicates affiliation of RM12 with Coccidia.

## Mitochondrial genome size

Size of the mitochondrial genome should have been estimated using radioactively labeled *cox 1* coding sequence. Hybridization resulted in smear ranging from 4 to 50 kbps with highest intensity about 16 kbps (Figure 7). This result suggests that there must be some kind of complex organization of the mitochondrial DNA in RM12.

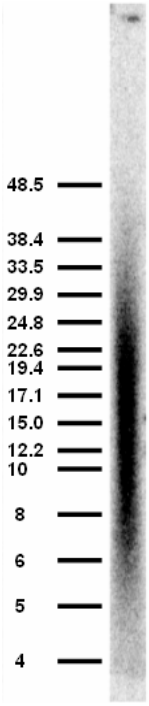


**Figure 7**: Result of Southern blot hybridization with radioactively labeled *cox1* probe

## Histone H2B

Partial sequence of the histone H2B was amplified from cDNA and was 197 bp long. Sequence was identified using blast search and aligned with H2B histone sequences from chromalveolates (figure 8).

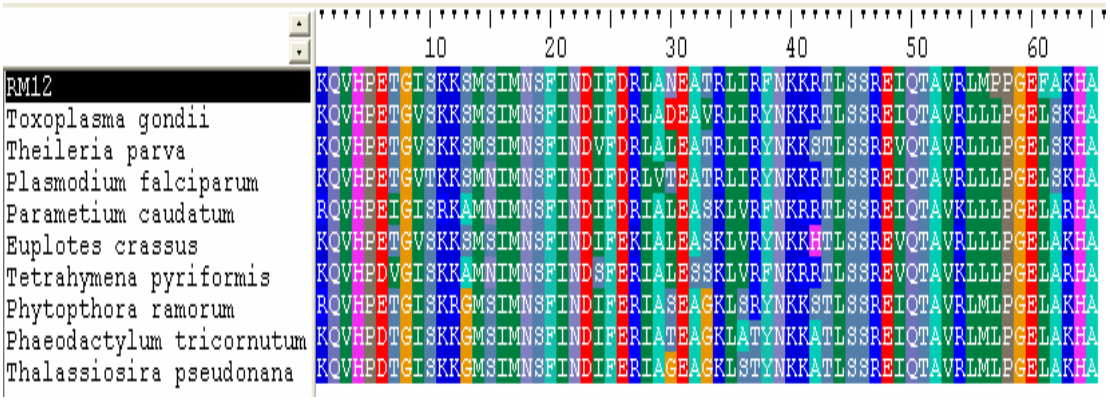


**Figure 8**: Multiple alignment of H2B histone sequences

## Histone H2A

Partial sequence of histone H2A was amplified from cDNA. It measured 177 bps. Histone sequence from dinoflagellates *Crypthecodinium cohnii* and *Alexandrium tamarense* evidently differed a lot from that from RM12 which rather affiliated with other alveolates.

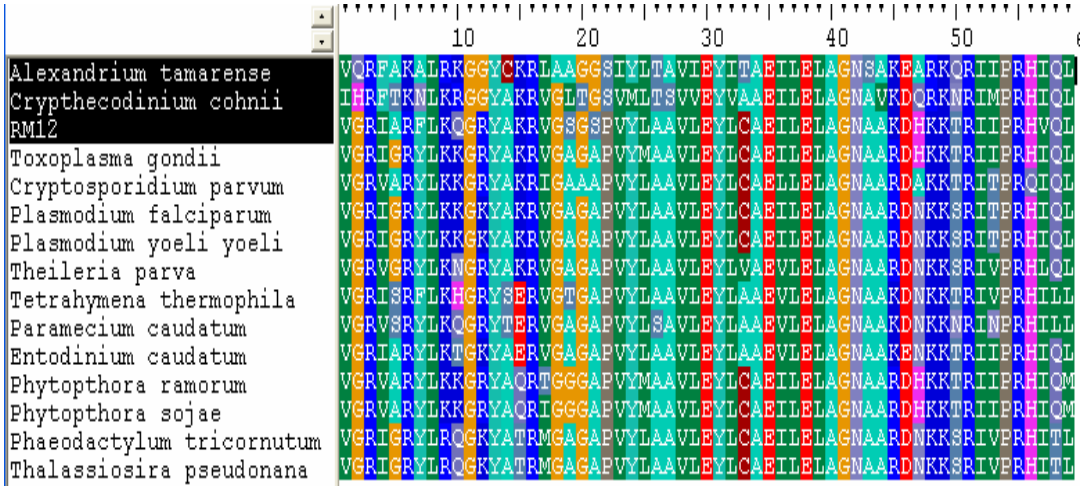


**Figure 9**: Multiple alignment of H2A histone sequences

## GAPDH (glyceraldehyde-3-phosphate dehydrogenase)

Partial sequence of GAPDH gene 996 bp in length was amplified from cDNA (For base frequencies see table 5). First, sequence was examined using MP and ML to infer whether is encoding enzyme employed in cytosol or in chloroplast. Phylogenetic tree was computed from cytosolic, plastid-targeted, mitochondrion-targeted and bacterial versions of GAPDH. However unsupported, both MP and ML placed this sequence within cytosolic versions of this gene (figure 10). Then, another dataset was made comprising only cytosolic forms of GAPDH from chromalveolates. In ML tree particular groups of Apicomplexa were not grouped together and RM12 and *Cryptosporidium* jumped at the base of dinoflagellate clade. Haemosporidans were affiliated with Ciliates (bootstrap value 55). MP grouped together species of Apicomplexa (excluding *Cryptosporidium*) with bootstrap support of 66. A SATUR A grouped together all apicomplexans (except *Cryptosporidium*) with RM12 at basal position, however, this topology was not supported.

| Species | %A | %G | %T | %C | % A+T | %C+G |
|---|---|---|---|---|---|---|
| Haemosporidia | 33.38 | 19.82 | 29.04 | 17.75 | 62.43 | 37.57 |
| Coccidia | 20.63 | 27.86 | 21.60 | 29.91 | 42.23 | 57.77 |
| Piroplasmida | 25.83 | 25.69 | 21.72 | 26.76 | 47.55 | 52.45 |
| Dinoflagellata | 21.92 | 30.01 | 20.47 | 27.60 | 42.39 | 57.61 |
| RM12 | 20.65 | 27.10 | 21.25 | 31.09 | 41.90 | 58.10 |
| Ciliophora | 27.50 | 22.48 | 25.94 | 24.08 | 53.44 | 46.56 |
| stramenopiles | 19.99 | 28.37 | 23.84 | 27.79 | 43.84 | 56.16 |

**Table 5**: Nucleotide rates of GAPDH gene among chromalveolates used for inferring phylogeny

When only Hematosporidia and RM12 were left in the dataset, RM12 jumped at the base of dinoflagellate clade with bootstrap support MP=74, AsaturA=90, ML=77 and Haematosporidia even strongly affiliated with Ciliates MP=74, AsaturA=65, ML=86 (figure 11). When on the one hand Heamosporidia were excluded and on the other hand other apicomplexan species were included, both in MP and ML trees species of Apicomplexa clustered together, however unsupported, and RM12 appeared either at the base of Dinoflagellates (MP) or as an ancestor of Dinoflagellates and Apicomplexa (ML). None of these positions were supported. AsaturA in this case did not supported apicomplexan clade and placed RM12 at the base of dinoflagellates. Trees computed from nucleotides (even when the third codon positions were removed) behaved similar e.g. strong affiliation of Heamatosporidia with ciliates was observed and position of RM12 could not be inferred. Then, LogDet was used to further investicate signal of this gene. Third codon positions were removed. In LogDet trees Hematosporidia still clustered with ciliates. When Haematozoa and *Cryptosporidium* were removed, RM12 created supported grouping with *Toxoplasma* and *Theileria* species (figure 12).

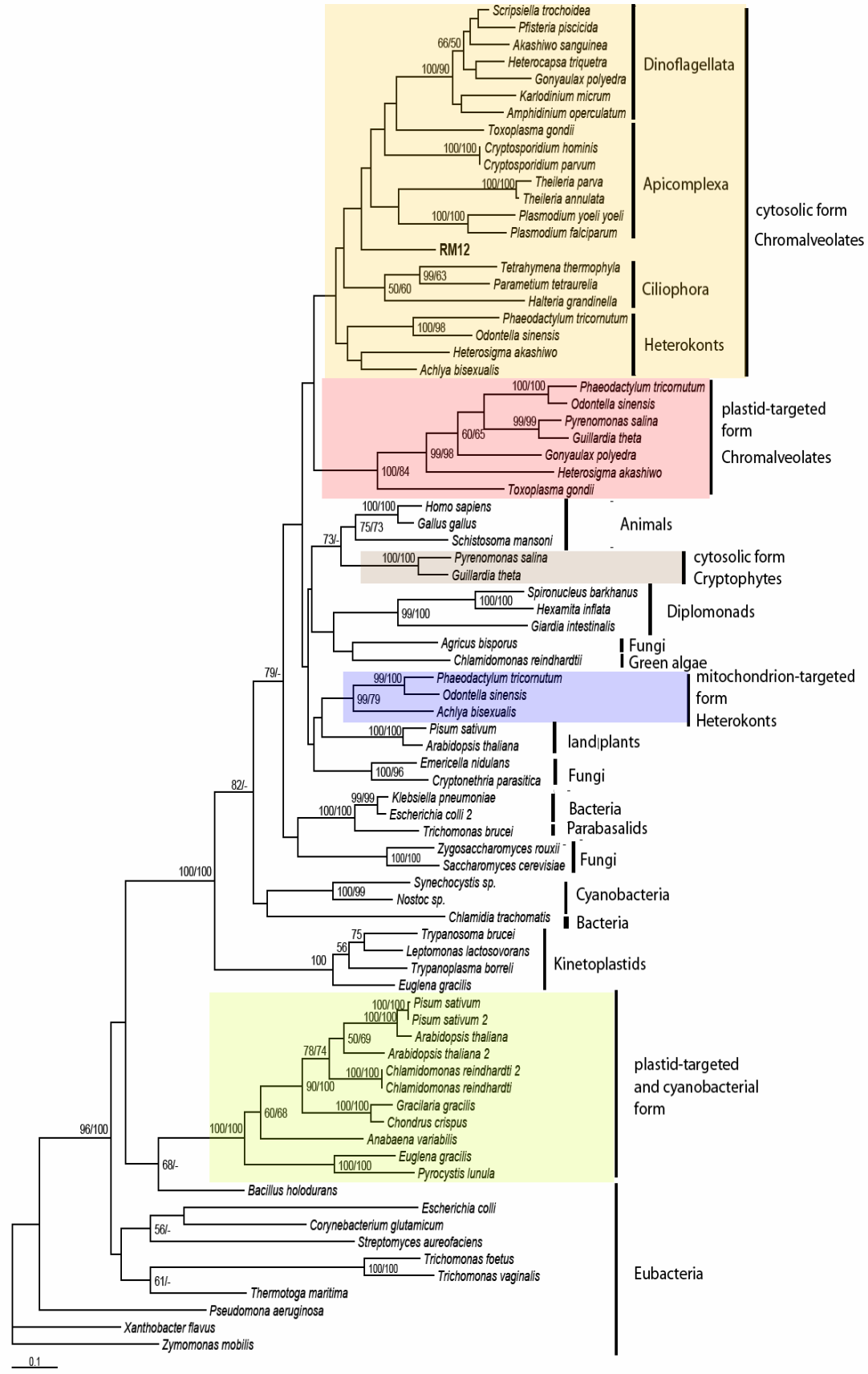


**Figure 10**: Phylogenetic tree inferred using ML method. It clusters together sequence acquired from RM12 with cytoplasmatic forms of GAPDH. Supports above branches are from ML and MP respectively.

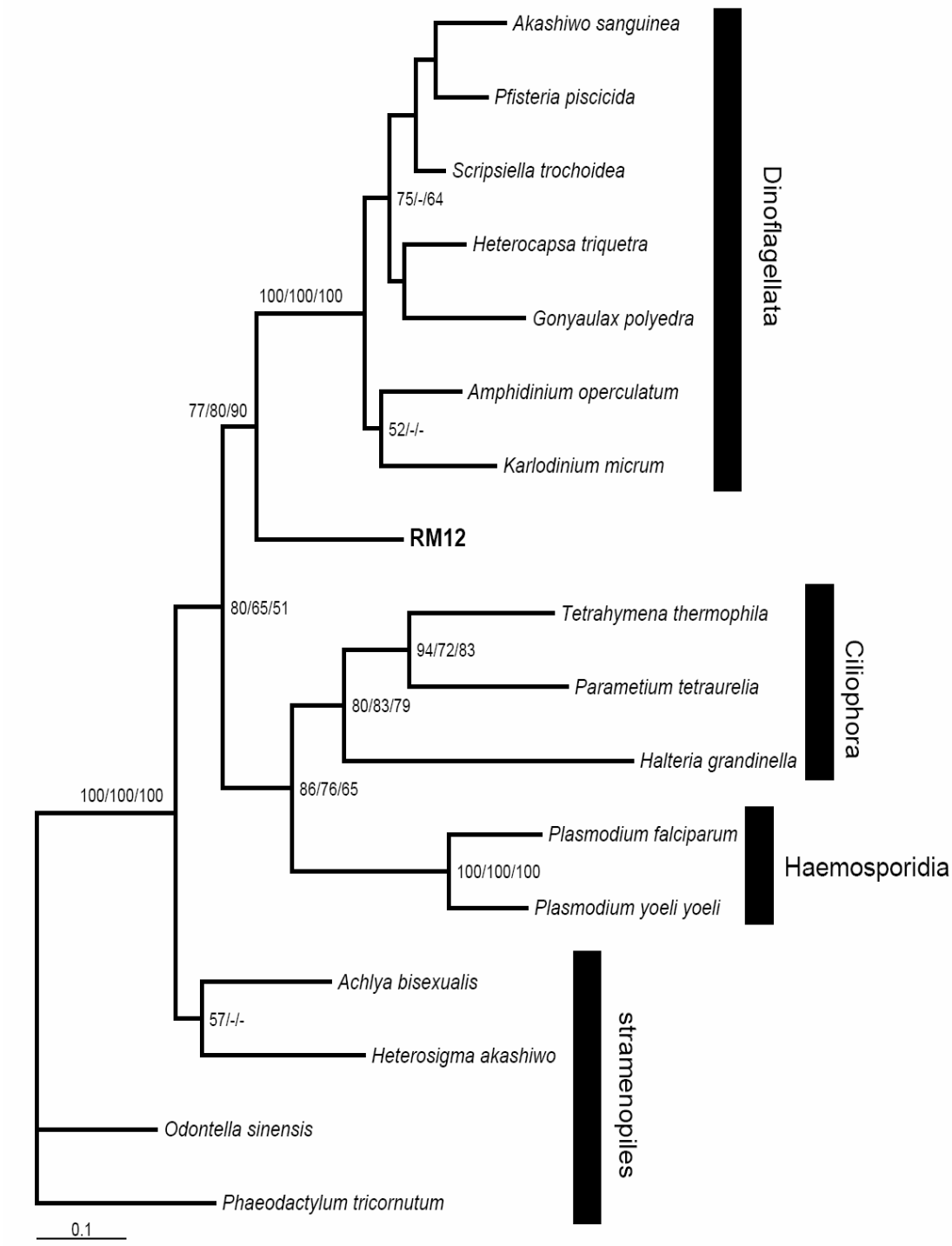


**Figure 11**: The phylogenetic tree constructed using ML from cytosolic versions of GAPDH sampled within chromalveolates. Bootstrap supports from MP and AsaturA (cutoff = 3.035) are also shown.

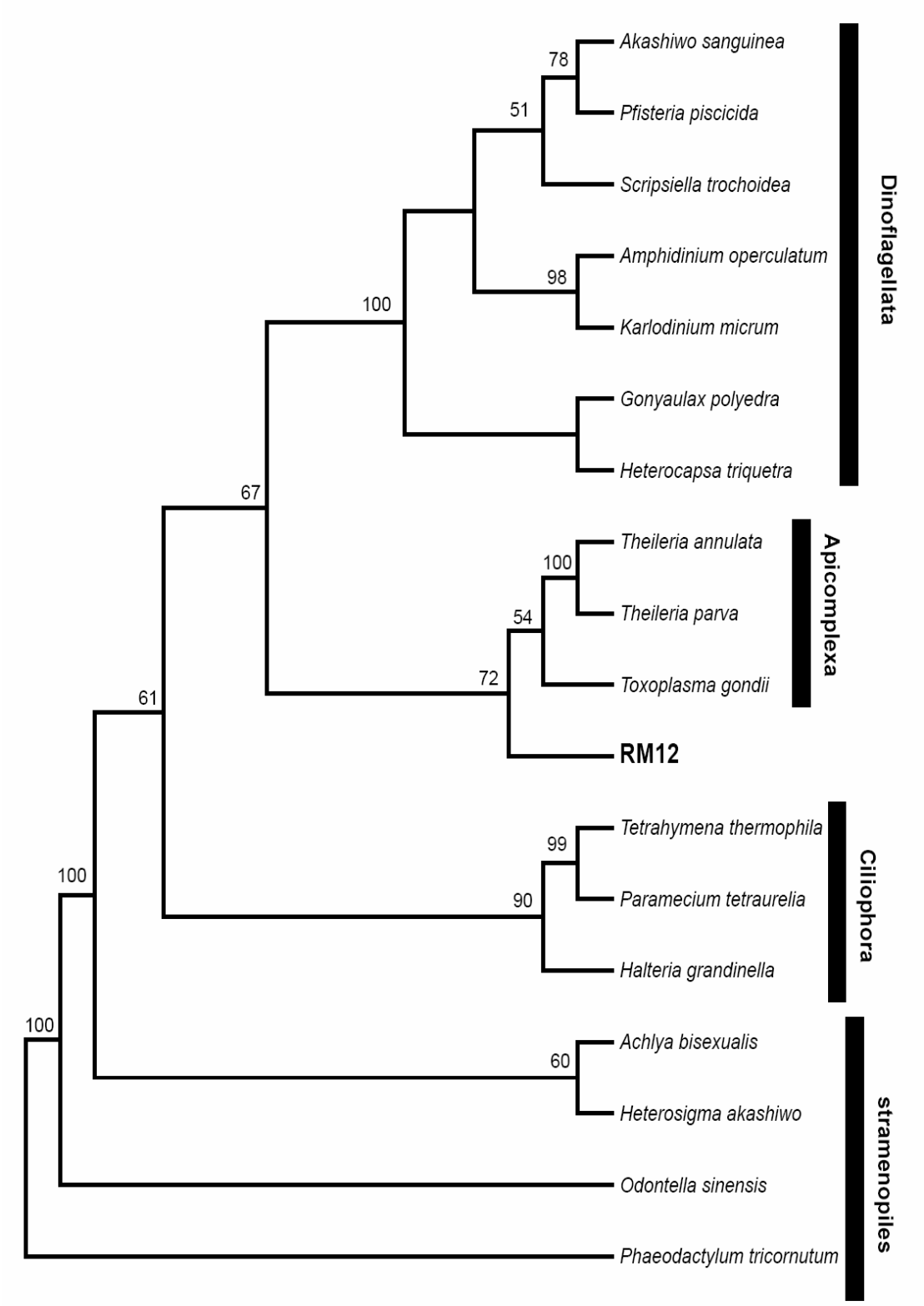


**Figure 12**: Phylogenetic tree constructed using LogDet distances from GAPDH translated to nucleotides. Third codon positions were removed. It shows RM 12 in basal position to *Toxoplasma* and *Theileria* species.

# Discussion

While studying symbionts of stony coral *Plesiastrea versipora* the novel photosynthetic alveolate RM12 was isolated and described. It may represent either a free living organism or a minor symbiont somehow associated with this coral. It contains typical ultrastructural features of alveolates such as alveoli, and a unique complex chloroplast surrounded by four membranes, and pigmented with chlorophyll-a, and a novel form of isofucoxanthin. Interestingly, it contains no chlorophyll-c (Moore et al. unpublished).

Phylogenetic analyses based on nuclear encoded rRNA genes and plastid encoded SSU rRNA gene placed this alveolate at the root of apicomplexans and colpodellids respectively, and its chloroplast appeared at the root of apicoplasts. It has been also demonstrated on the case of chloroplast encoded protein psbA that RM12 encodes tryptophan by UGA codon instead of UGG in its chloroplast. Such feature has been observed only in apicoplasts of coccidia so far. Based on the phylogenetic position, photosynthetic character of the alveolate as well as the chloroplast codon usage it was suggested that the alveolate RM12 represents the closest photosynthetic relative to apicomplexan parasites and that its chloroplast share common ancestor with apicoplasts (Moore et al unpublished). In this work RM12 was further investigated to confirm or disapprove postulated phylogenetic position.

Affiliation of RM12 to apicomplexan parasites should be reflected in many various molecular characters. Apicomplexa possess not only unusual non-photosynthetic chloroplast, but also one of the smallest mitochondrial genomes. Therefore I decided to estimate the size of the genome using PFGE and hybridization with specific probe. To obtain the probe and some mitochondrial genes usable for phylogenetic reconstruction mitochondrial *cox1* gene was primarily chosen. There is sufficient taxon sampling in *cox1* in public databases allowing computing reasonable phylogenetic trees. Since phylogenetic analysis based on chloroplast genes has already been performed (Moore et al. unpublished) I wanted to look also at the mitochondrial evolution. *Cox 1* was amplified using uneven PCR method which allowed investigating of flanking regions of this gene. Nucleic acid hybridization

revealed some kind of complex organization of the mitochondrial genome of RM12. However, this result is not as much surprising since both groups of organisms to which RM12 could belong, Apicomplexa and Dinophyta, contain species displaying complex smear as mtDNA hybridization result. Mitochondrial DNA of malarial parasite *Plasmodium* is arranged mainly as polydisperse linear concatemers, therefore nucleic acid hybridization of undigested DNA resulted in smear ranging from ranging from 6 to 30 kbps (Preiser et al 1996). The only known mitochondrial genome organization within dinoflagellates so far is that of *Crypthecodinium cohnii* (Norman 2000, Norman and Gray 2001). This genome consists of heterogenous mixture of molecules smaller than 10 kbps. If I compare my results with those from *Plasmodium* and *Crypthecodinium*, hybridization pattern of RM12 is completely different (figure 13) and requires further investigation. However, it is evident that *cox 1* gene is present at least at two different molecules (see results).

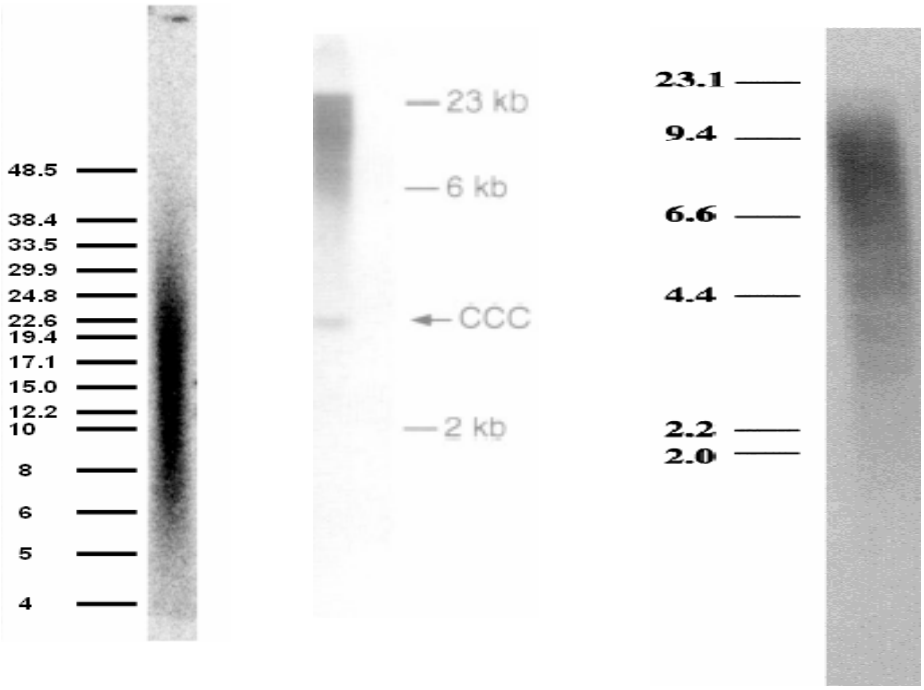


**Figure 13**: Comparison of three Southern blot hybridization results of RM12 (left), *Plasmodium falciparum* (in the middle) and *Crypthecodinium cohnii* (right). Photos were adopted from Preiser et al (1996) and Norman and Gray (2001).

Phylogeny based on amino acid sequences of *cox1* did not to lead to any stable and supported phylogenetic position. Alveolate RM12 appeared to be related to both,

dinoflagellates and apicomplexans depending on the method of inference and taxon sampling used. The bootstrap support did not overcome 47% in both possible positions. The only acceptable supports were obtained only when nucleotide alignment was used and processed by LogDet-paralinear distances. In such a case RM12 branched together with Apicomplexa with bootstrap support of 77% for the apicomplexan clade. However, in this particular case, the branching order showed relation of RM12 mitochondrion to mitochondrion from coccidia with bootstrap 73%.

The second molecular feature, which is typical for dinoflagellates, is the absence of histones. Dinoflagellates were thought they lack histones at all, however, recent EST projects revealed their expression (see introduction). Within dinoflagellates only the H2A histone has been identified in ESTs from *Alexadrium tamarense* and *Crypthecodinium cohnii* (Hackett et al 2005, Sanchez-Puerta et al 2007). Therefore we could expect the lack of histones or presence of highly divergent homologues in RM12 in the case that RM12 is dinoflagellate. However, I was able to amplify histones H2A and H2B from cDNA of alveolate RM12 and those could be aligned to any eukaryotic homologues with very high similarity as usual for typical eukaryotic histones. At the same time, dinoflagellate H2A homologues show quite high diversity when compared to other eukaryotes including RM12. Moreover, histone H2B has not been identified in any of dinoflagellate so far. Based on amplification of the gene encoding H2B protein from RM12 and the level of identity H2A to other eukaryotic homologues, we can suggest that this species is not a dinoflagellate.

GAPDH is the protein coding gene of the first choice when inferring phylogenetic history of chromalveolates (eg. Fast et al 2001, Harper and Keeling 2003, Takishita et al 2004, 2005). It represents essential and rather conserved gene with very good taxon sampling in public databases, which was used by Fast et al. (2001) and Harper and Keeling (2003) to support Chromalveolate hypothesis. I decided to use at least one originally cytosolic gene (gene originating in host eukaryotic nucleus) for

searching the phylogenetic position of RM12. Four different methods for inferring phylogeny were used in this work. This approach minimizes impact of computing artifacts, because each method has its weak spots. Phylogenetic inference is always problematic if both short and long branches occur together in the underlying tree. ML and MP failed to resolve position of RM12 based on GAPDH sequences. Apicomplexan clade generally collapsed in ML trees when RM12 was implemented into the dataset. AsaturA (method designed to deal with saturation of amino acids) and MP trees showed artificial branching of particular orders within Apicomplexa. However, in the case of datasets 3 and 6 AsaturA revealed branching of RM12 with order Coccidia. This result was further confirmed by phylogenetic inference using LogDet-paralinear distances computed from nucleotides in the dataset 6. LogDet method was developed to treat dissimilar nucleotide frequencies, which bias phylogenetic analyses. Topology obtained by LogDet is similar to that computed using LogDet from plastid encoded SSU rRNA (see figure14). Similar branching order was found by AsaturA in some *cox1* trees. Although different genes were used to infer phylogeny of RM12 (plastid SSU rRNA gene, *cox1* gene, and cytosolic GAPDH) LogDet and AsaturA both designed to deal with unusual sequences agreed on the same topology.

Figure 14 contains secret data and is to be found only in original master thesis version stored at the Faculty of Biological Sciences University of South Bohemia in České Budějovice

**Figure 14**: The phylogenetic tree computed from plastid encoded SSU rRNA gene adopted from Moore et al (unpublished). Version A is the tree computed using Bayesian inference. Values above branches represent posterior probabilities and maximum likelihood bootstrap supports respectively. Asterisks demonstrate maximal support of both Bayesian inference and maximum likelihood. Version B shows branching of Apicomplexa computed using LogDet distances. Codon usage for tryptophan of particular apicomplexans is also showed.

Phylogenetic analysis assigned acquired GAPDH sequence to the cytosolic form of GAPDH. Clustering was not supported at all, however, this part of the tree was not supported even in the work of Harper and Keeling (2003). In trees computed from dataset reduced only to chromalveolates, strong artificial branching of *Plasmodium* spp. with ciliates appeared and this artifact was not broken even when using LogDet method. If I look at the base frequencies (table 5) extremely high AT content can be observed in case of Haemosporidia. That is usually reflected in the amino acid bias as well, which is even harder to eliminate when amino acids are use to infer phylogeny. Moreover, ciliates also have rather high AT content making their clustering with haemosporidians much more comprehensible and clearly artifactual. However, trees based on amino acid GAPDH sequences without haemosporidians forming long branches placed RM12 to be related to dinoflagellates with no support. On the other side, using nucleotide sequences LogDet method revealed clustering of RM12 with Coccidia and Piroplasmida. When I take into account also tree topologies obtained from other genes such as nuclear SSU and LSU rRNA sequences, the affiliation of RM12 with dinoflagellates is likely to be an artifact. Since nucleotide rates among taxa are different and probably caused clustering of Haemosporidians with Ciliates, tree topology acquired using LogDet appears most reliable.

RM12 represents a novel species which was placed at the root of the Apicomplexa by rDNA phylogenies (Moore et all. unpublished, Chrudimsky et al 2006). Affiliation of RM 12 to apicomplexan parasites was supported by presence of H2B histone in RM12 genome and similarity of its highly conserved H2A histone to that

from Apicomplexa. There are three possible phylogenetic positions of the RM12. The first of them is the basal position to Apicomplexa. As I have mentioned above, this branching was supported by nuclear SSU rRNA (RM12 branched at the root of colpodellids) and nuclear LSU rRNA trees. These trees strongly support this topology, which was also confirmed by Approximately Unbiased (Shimodaira and Hasegawa 2001, Shimodaira 2002) tests. The second possibility is that RM12 is basal to order Coccidia. This is congruent with LogDet trees computed from *cox1* gene and plastid SSU rRNA respectively. However, we must take into consideration that plastid evolution could be different from nuclear and that the same nuclear lineages may host various plastids. LogDet tree computed from GAPDH gene nucleotide sequences can not either confirm or deny this topology because haemosporidians had to be discarded from the dataset. It shows only that RM12 is related to apicomplexans rather then to dinoflagellates. The last possibilty is represented by possible close relationship between RM12 and dinoflagellates. However, all the other results characterizing RM12, such as presence or regular histones, codon usage in the chloroplast and LogDet based phylogenies contraindicate that this alveolate is dinoflagellate.

It seems that RM12 uses exclusively UGA codon to encode tryptophan in its chloroplast (as inferred from *psbA* and *tufA* gene, Moore at al. unpublished and Janouškovec et al. unpublished respectively) and shares this feature with coccidian parasites. However, it has to be noted that coccidian plastids may use both codons as shown in the case of *Toxoplasma gondii*. If the correct position of RM12 was at the root of the apicomplexan clade and we assumed monophyly of the apicoplast, it would require change in codon usage from UGA to UGG in orders Haemosporidia and Piroplasmida and reverse change in Coccidia representing derived group within Apicomplexa. Although this scenario does not seem to be parsimonious the usage of both codons in coccidian plastid makes this reversion possible. However, whole conundrum can be easily explained by polyphyly of the apicoplasts. Evidences for apicoplast monophyly are not so strong and the main arguments are the gene content and order within apicoplast genomes (Denny et al. 1998). If both endosymbionts would be of the same origin (red) but required independently, then we would probably get very similar results of gene content and order within genomes originating from closely related sources and passing through similar processes such

as lose of photosynthesis and adaptation to parasitic life style. The second way how to explain UGA codon usage of both RM12 and Coccidia is synapomorphy of this trait.

If the correct position of RM12 was basal to Coccidia, then there would be no need to assume polyphyly of apicoplasts and evolution of apicomplexans would correspond to that of their plastid. Primitive apicomplexan orders such as Haemosporidia and Piroplasmida would use UGG codons while derived Coccidia and RM12 UGA codons. Such scenario would necessarily require two independent losses of photosynthesis in ancestors of coccidia and haemosporidia respectively. Despite the fact that existence of photosynthetic coccidians may look funny, this possibility should not be overlooked. Though nuclear encoded SSU and LSU rRNA genes did not support this version, *cox1* did. The mitochondrion likely emerged nearly at the same time as nucleus (Grey 1999) and mitochondrial genes are appropriate for inferring phylogeny of the host cell despite their organellar origin (Lang et al 1998, 1999). Phylogenetic position of alveolate RM12 in this case would further mean that parasitism within Apicomplexa originated in multiple independent events. At this point neither of these two alternatives can not be responsibly rejected since evidence from nuclear rDNAs and mitochondrial *cox 1* are contradictory and simple conclusion from all observations we made does not exist.

# Conclusions

Phylogenetic analyses of the novel alveolate RM12 based on *cox1* and GAPDH sequences failed to favor any of all possible topologies, which came into question. Determining of the accurate phylogeny of this species would require investigation of much more genes.

Though phylogenies based on *cox1* and GAPDH genes did not resolved position of RM12 unambiguously, it can be inferred from supporting evidence such as the presence of regular histones and codon usage in chloroplast that RM12 is much closely related to apicomplexans than to dinoflagellates. It is the closest photosynthetic relative of apicomplexan parasites known so far. Existence of RM12 rise many question about apicomplexan phylogeny and mainly about origin of their plastid. Discovery of the close photosynthetic relative of apicomplexans represents long time seeking missing link, which will allow studying of the apicoplast evolution in much wider context then it has been possible so far.

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# Appendix

**Medium f2 (stock solution):** 1 ml $NaNO_3$ (7.5% w/v), 1 ml $NaH_2PO_4 \cdot H_2O$ (0.5% w/v), 1 ml $Na_2SiO_3 \cdot 9H_2O$ (3% w/v), 1 ml f/2 trace metal solution, 0.5 ml vitamin solution

**f/2 trace metal solution:** 1.3 g $FeCl_3 \cdot 6H_2O$, 8.7 g $Na_2EDTA \cdot 2H_2O$, 1 ml $CuSO_4 \cdot 5H_2O$ (9.8 mg/1 ml $dH_2O$), 1 ml $Na_2MoO_4 \cdot 2H_2O$ (6.3 mg/1 ml $dH_2O$), 1 ml $ZnSO_4 \cdot 7H_2O$ (2.2 g/100 ml $dH_2O$), 1 ml $CoCl_2 \cdot 6H_2O$ (1 g/100 ml $dH_2O$), 1 ml $MnCl_2 \cdot 4H_2O$ (18 g/10 ml $dH_2O$), pH 4.5; Make final volume up to 1 L with $dH_2O$

**Vitamin solution:** 1 ml vitamin $B_{12}$ (10 mg/10 ml $dH_2O$), 1 ml Biotin (10 mg/10 ml $dH_2O$), 200 mg Thiamine HCl; make final volume up to 1 L with $dH_2O$

**Lysation buffer (Wattier et al 2000):** 100 mM Tris-HCl, 50 mM EDTA, 500 mM NaCl + 0.4 µl SDS (20% w/v)

**1 x TBE buffer:** 2 mM $Na_2EDTA$, 89 mM boric acid, 89 mM Tris

**LB medium:** 1% bacto-tryptone, 0.5% bacto-yeast extract, 0.5% NACl, pH 7.0; sterile

**SOC medium:** 2% bacto-tryptone, 0.5% bacto-yeast extract, 0.05% NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM Mg $SO_4$, 20 mM glucose, pH 7.0; sterile

**Agar plates:** 1.5% bacto-agar in LB medium, 50 µg/ml ampicilline

**PFGE lysis buffer**: 0.5 M EDTA ( pH=9.5 ), N-Lauroylsarcosine 1% (V/V), Proteinase K (20 mg/ml).

**SET buffer**: 0.15 M NaCl, 0.1 M EDTA, 10 mM Tris (pH=8.0)