Karoline Magalhães Ferreira Lubiana

Taxonomia e filogenia molecular de Cryptophyceae (Cryptophyta) em culturas

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Orientadora: Dra. Mariana Cabral de Oliveira

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Prof.a Dra. Mariana Cabral de Oliveira
Orientadora

À minha Helena, pelas horas ceifadas de convivência, paciência e por me ensinar que o maior amor do mundo é o de ser mãe!

"Não me iludo
Tudo permanecerá do jeito que tem sido
Transcorrendo
Transformando
Tempo e espaço navegando todos os sentidos
Pães de Açúcar
Corcovados
Fustigados pela chuva e pelo eterno vento
Água mole
Pedra dura
Tanto bate que não restará nem pensamento

Tempo rei, ó, tempo rei, ó, tempo rei
Transformai as velhas formas do viver
Ensinai-me, ó, pai, o que eu ainda não sei
Mãe Senhora do Perpétuo, socorrei

Pensamento
Mesmo o fundamento singular do ser humano
De um momento
Para o outro
Poderá não mais fundar nem gregos nem baianos
Mães zelosas
Pais corujas
Vejam como as águas de repente ficam sujas
Não se iludam
Não me iludo
Tudo agora mesmo pode estar por um segundo

Tempo rei, ó, tempo rei, ó, tempo rei
Transformai as velhas formas do viver
Ensinai-me, ó, pai, o que eu ainda não sei
Mãe Senhora do Perpétuo, socorrei"

(Gilberto Gil, Tempo Rei)

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SUMMARY

RESUMO

Criptofíceas são organismos biflagelados, majoritariamente fotossintetizantes, que habitam ambientes aquáticos marinhos, dulcícolas ou salobros. Embora facilmente diagnosticáveis por meio de microscópio de luz devido à sua forma assimétrica e aos movimentos giratórios, a identificação em nível específico exige uma ampla abordagem morfológica associada a dados moleculares. A taxonomia do grupo é confusa e os trabalhos se contradizem a respeito dos caracteres diacríticos para identificação dos táxons, e até mesmo da validade de alguns deles. Tanta discussão advém do fato das células de criptofíceas serem diminutas (menor que 20µm) e não possuírem cobertura rígida, que aumentam o grau de dificuldade de distinção morfológica. Consequentemente, o conhecimento da biodiversidade do grupo é bastante incompleto e baseado em amostragens limitadas. Dessa forma, o presente estudo visou ampliar o conhecimento da diversidade de espécies de criptofíceas mantidas em culturas. Foram utilizadas 53 cepas para estudo da diversidade filogenética por meio dos marcadores nucleares (SSU, ITS2) e nucleomorfo SSU rDNA. Seis cepas foram investigadas morfologicamente, usando microscópios de luz, confocal, e eletrônico de varredura e transmissão. Quatro espécies novas advindas de regiões costeiras do Brasil foram descritas, uma de *Hemiselmis* e três de *Rhodomonas*. A nova espécie de *Hemiselmis* tem um novo tipo de ficobiliproteína, Cr-PC564, e as demais linhagens de *Rhodomonas* possuem Cr-PE545. As espécies novas de criptofíceas são, até o momento, as primeiras descritas para o litoral do Brasil. Consequentemente, este estudo contribuiu para o conhecimento inicial da composição das criptofíceas no Atlântico Sul. Complementarmente, uma cepa de *Nephroselmis viridis* (Nephroselmidophyceae, Chlorophyta) foi isolada e caracterizada, visto que se tratava de um novo registro para o Oceano Atlântico (Anexo 1).

Palavras-chave: algas; Atlântico Sul; criptofíceas; filogenia; fitoplâncton; marinho; SSU rDNA.

ABSTRACT

Cryptophytes are in majority photosynthetic biflagellate organisms, living in aquatic environments, which can be marine, brackish and freshwater. Although cells are easily reckonable at class level due to the asymmetrical shape and the gyrating swimming motion, the identification of species needs a broad morphological approach associated to molecular data. The taxonomy of the group is confusing and controversial due to disagreements of the authors concerning the diacritic features for taxa identification, and even the validity of the taxa. Furthermore, cryptophytes cells are diminutive (less than 20µm) and do not possess rigid cover, which implies difficulties in morphological distinction. Consequently, the knowledge of the biodiversity of the group is very incomplete and based on limited sampling. The present study aimed to enhance the knowledge of the cryptophytes species diversity in cultures. We used 53 strains for the study of the phylogenetic diversity, using sequences of nuclear (SSU and ITS2), and nucleomorph SSU rDNA. Six strains had their morphologies investigated by the use of light, confocal, scanning and transmission electron microscopies. Four new species from coastal regions of Brazil were described, one for the genus *Hemiselmis* and three for *Rhodomonas*. The new *Hemiselmis* species has a new type of phycobiliprotein, Cr-PC-564, and the *Rhodomonas* strains have Cr-PE 545. The newly described species were also the first cryptophytes described for the marine environment of Brazil until this moment. Consequently, the present study enhanced the knowledge of cryptophytes composition in the South Atlantic. Furthermore, we isolated and characterized a *Nephroselmis viridis* (Nephroselmidophyceae, Chlorophyta), which was a new record for the Atlantic Ocean (Annex 1).

Keywords: algae; cryptophytes, cryptomonads; marine; phylogeny; phytoplankton; South Atlantic; SSU rDNA.

1 INTRODUCTION

EVOLUTIONARY HISTORY

The biological evolutionary process of endosymbiosis allowed the origin and diversification of eukaryotes. Mitochondria and chloroplast were both acquired through primary endosymbiosis, where a prokaryote was taken as an endosymbiont by a eukaryote host (FIGURE 1). It seems that a unique primary endosymbiosis between a photosynthetic cyanobacteria and a heterotrophic eukaryote originated the current lineage of Archaeplastida (FIGURE 1), composed by glaucophytes, red algae, and green algae, including land plants (BATTACHARYA; MEDLIN, 1995; GRAY, 1999; ARCHIBALD; KEELING, 2002; HAGOPIAN et al., 2004; KEELING, 2010). Thereafter, secondary endosymbiosis, which involves two eukaryotes, one heterotrophic and another photosynthetic, became possible. Indeed, secondary endosymbiosis involving red or green algae originated most of the current algal lineages (ARCHIBALD; KEELING, 2002; KEELING, 2010). The different origin of secondary plastids from green algae ancestor in euglenid and chlorarachniophyte appears to be well established (ROGERS et al., 2007). However, the origin and evolution of secondary red-plastids lineages is still controversial (STILLER et al., 2014). Lineages with secondary red-plastids, heterokonts, cryptophytes, haptophytes and dinoflagellates, are the main primary producers in the ocean where they have an important ecological role (FALKOWSKI et al., 2004).

Cryptophyceae is a monophyletic lineage of mostly photosynthetic organisms. Some colorless forms can occur with leucoplasts, such as *Cryptomonas paramecium*, with a reduced plastid genome. Moreover, heterotrophic *Goniomonas* species that lack plastids are also included in the group (DONAHER et al., 2009; HOEF-ENDEN, 2008; HOEF-EMDEN; MELKONIAN, 2003; MARIN; KLINGBERG; MELKONIAN, 1998; MCFADDEN; GILSON; HILL, 1994). Cryptophytes is pointed out to be close to other heterotrophic eukaryotes, such as katableparids, telonemids and palpitia, forming the group Cryptista (CAVALIER-SMITH; CHAO; LEWIS, 2015; NISHIMURA et al., 2016; YABUKI et al., 2014). The plastid bearing groups had acquired this organelle through secondary endosymbiosis involving a red algae ancestor. However, the relationship of cryptophytes with other photosynthetic eukaryotes is controversial. The

theories of endosymbiosis of secondary red-plastids lineages varies according to the he number of times in which endosymbiotic events occurred.

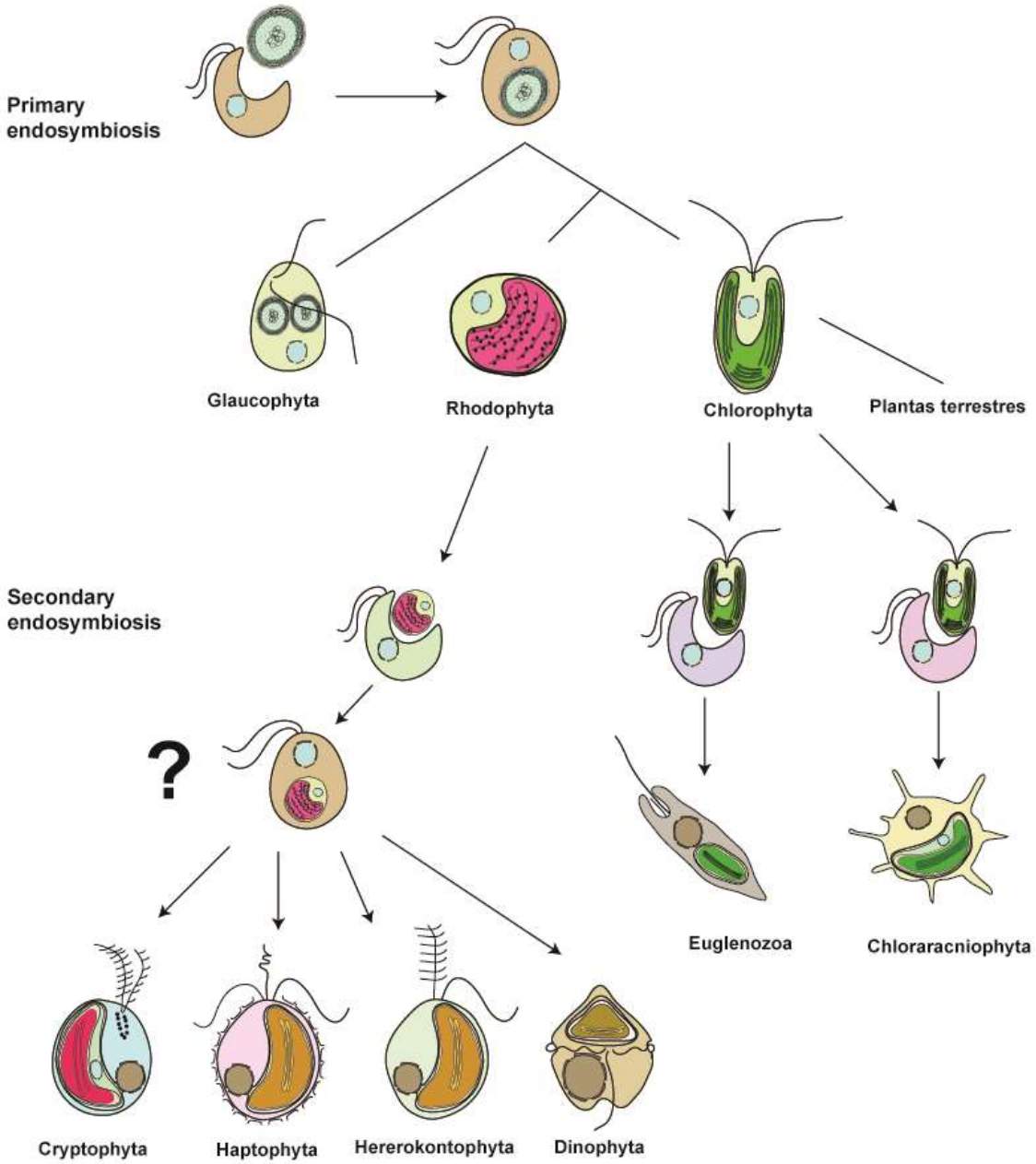

FIGURE 1: Representation of the lateral transference of plastids in different algal lineages.

Originally, based on evidences such a tubular cristae, plastid endoplasmic reticulum and/ or tubular mastigonemes, the Chromista kingdom was suggested for cryptophytes,

heterokonts and haptophytes (CAVALIER-SMITH, 1981). Other evidences used to delimit the Chromista were the storage of β1-3glycans in the cytoplasm, presence of chlorophyll *c* in the thylakoids, and the plastid envelope surrounded by four membranes (two extra). Afterwards, the hypothesis of Chromalveolata emerged, suggesting that the Alveolata (dinoflagellates and apicomplexa) last ancestor was a photosynthetic organism, and all secondary red plastids arose in a single endosymbiosis event (CAVALIER-SMITH, 1999) (FIGURE 2 and 3). Some studies performed with plastid genes and genomes agree with the phylogenetic relationship of cryptomonads, haptophytes and heterokontes, *i.e*. Chromista (YOON et al., 2002; HAGOPIAN et al., 2004; KHAN et al., 2007). However, these works did not include the strange plastid sequences of apicomplexa and dinoflagellates. Moreover, other phylogenetic inferences performed with a nuclear multigene data did not corroborate with chromoalveolates monophyly (BAURAIN et al., 2010; BURKI et al., 2016; BURKI; SHALCHIAN-TABRIZI; PAWLOWSKI, 2008; KIM et al., 2018; STILLER et al., 2014; YOON et al., 2008).

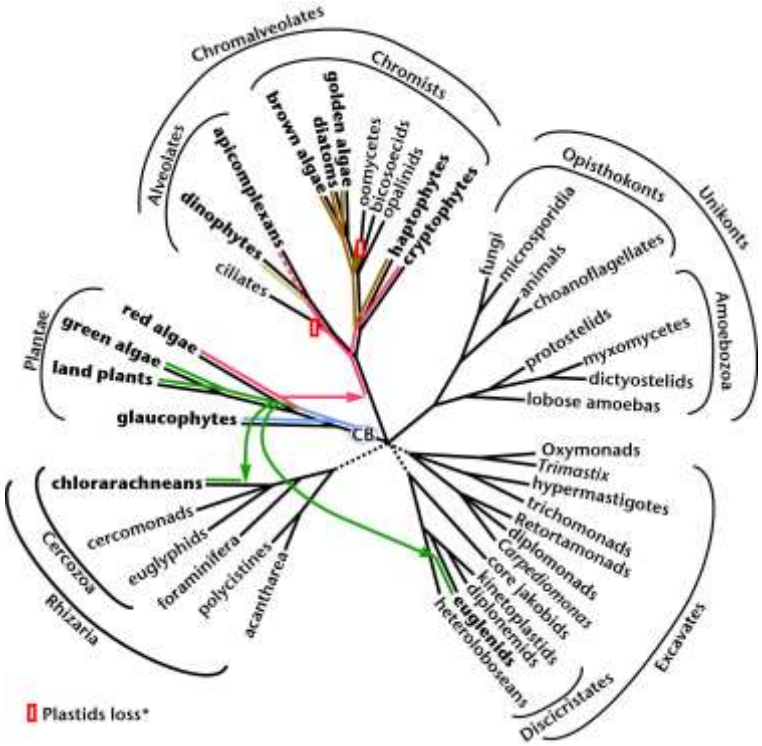

FIGURE 2: An overview of the eukaryotes phylogeny and the plastid acquisition in different lineages. CB represents the first gain of plastids by one ancient Plantae, which acquired a chloroplast by taking a cyanobacterium as endosymbiont. Arrows indicates events of secondary endosymbiosis. Green

arrows indicates two independents acquisition of secondary plastids from green algae, while the red ones represents red algae lateral transfer plastids in a single event, as argued to Chromoalveolata hypothesis. Source: (BELLORIN; OLIVEIRA, 2006)

Further publications concerning phylogenetic and phylogenomic inferences with chloroplast genes, as well as the hypothesis of lateral transfer of genes between cryptophytes and haptophytes, suggested that they are a monophyletic group (RICE; PALMER, 2006; HACKETT et al., 2007; KIM et al., 2017; PATRON; INAGAKI; KEELING, 2007; OKAMOTO et al., 2009). The clade Hacrobia was created based on phylogeny of multigene-data, to accommodate cryptomonads, haptophytes and some other heterotrophic groups, such as katablepharids, telonemids and centrohelids (OKAMOTO et al., 2009). However, studies based on the molecular phylogeny of nuclear genes pointed out that haptophytes and cryptophytes acquired their plastids in different events (BAURAIN et al., 2010; BURKI et al., 2012, 2016; STILLER et al., 2014; MILLER; DELWICHE, 2015). Recently, phylogenomic analyses of nuclear and mitochondrial genomes contradict all the last theories proposed to the evolutionary history of cryptophytes. These works suggested that cryptophytes are sister to Archaeplastida instead to haptophytes or others chromists (BURKI et al., 2012, 2016; KIM et al., 2018; STILLER et al., 2014). Some authors suggested that the acquisition of photosynthesis occurred through serial endosymbiosis, starting with the secondary endosymbiosis with red algae, which originated cryptophytes (FIGURE 3). Posteriorly, a heterotrophic heterokont ancestor engulfed a cryptomonad in a tertiary endosymbiotic event. Lastly, the ancestor of haptophytes engulfed an heterokont algae in a quaternary endosymbiosis event (STILLER et al., 2014; BURKI et al., 2016). As discussed above, the phylogenetic affinities of cryptomonads are not well solved and remain as one of the main questions in the understanding of eukaryotic lineages evolution.

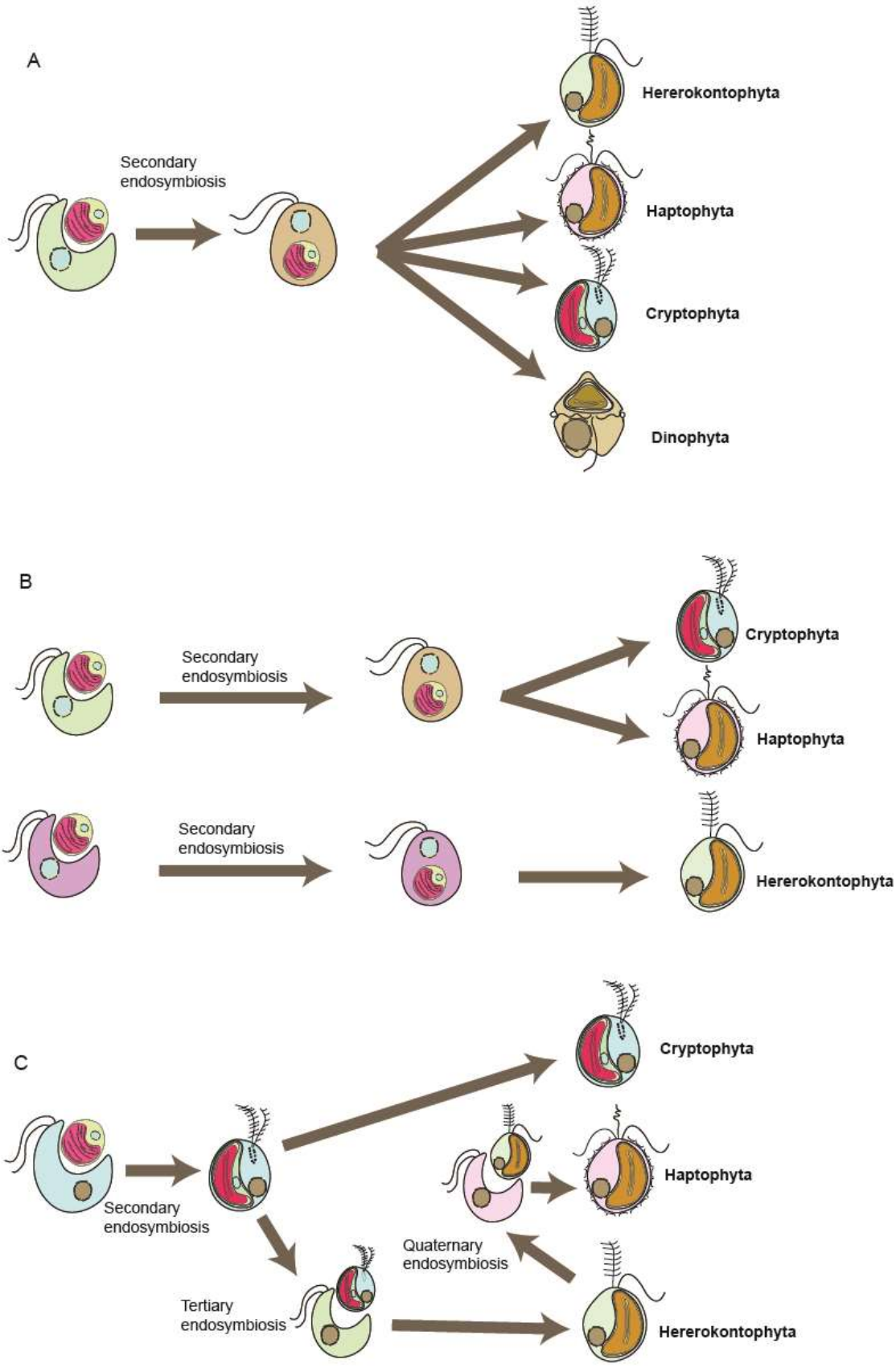

FIGURE 3: Representation of the hypothesis concerning the evolution of red-lineages plastids. (A) Chromoalveolata hypothesis: single endosymbiosis event for all secondary red plastids lineages. (B) Hacrobia hypothesis: two separated

endosymbiosis events diversified in the current secondary red plastids lineages. (C) Serial endosymbiosis hypothesis: a cryptophyte acquire it plastid through a secondary endosymbiosis with a red algae ancestor. A heterokont ancestor engulfed a cryptophyte in a tertiary endosymbiosis. Posteriorly, an ancestor of haptophytes retained a heterokont as endosymbiont.

CRYPTOPHYTE CELL BIOLOGY AND MORPHOLOGY

Cryptophytes are unicellular organisms, biflagellate, mainly photosynthetic. Cells are asymmetrical, due to the subapical insertion of an unequal pair of flagella (FIGURE 4). The longer flagellum (dorsal) is usually arranged with two rows of mastigonemes, while the smaller (ventral) one with one row (NOVARINO, 2003). Close to the flagellar region, a gullet extends to the cell inner. Some taxa can exhibit an aperture of the gullet, called furrow, which can be partial or total. Large ejectisomes are found lining the furrow/gullet system and smaller ones are disposed along the periplast, among their plates. Ejectisomes are explosive organelles related to mechanisms of scape or defense against injuries (LEE, 2008).

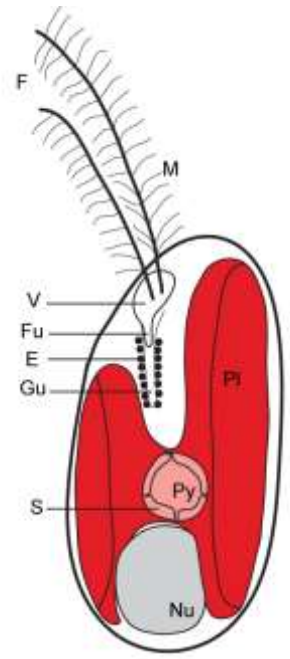

FIGURE 4: General cell morphology of photosynthetic cryptophytes. (E) Ejectosomes, (F) flagellum, (Fu) furrow, (Gu) gullet, (M) mastigonemes, (Nu) nucleus, (Pl) plastid, (Py) pyrenoid, (S) starch.

The periplast covers the cryptomonad cell, which is organized in two proteinaceous layers covering the plasma membrane, one outside (SPC-surface periplast component) and another inside (IPC-inner periplast component). The forms of periplast plates vary among cryptomonads genera and within some of them. The mitochondria is unitary and the morphology of the cristae is flattened finger-like. The mitochondria can have variable shapes in some genera, such as unbranched vermiform-like, while others have branched complex forms (SANTORE; GREENWOOD, 1977). This organelle is mostly disposed longitudinally in the cell passing close to other organelles. Mitochondrial genome in cryptophyte species are rich in repetitive non-coding region and can varies in size from ~37Kbp, in *Cryptomonas curvata*, to ~54Kbp, in *Storeatula* sp. CCMP1868, keeping 42-conserved protein genes. Moreover, although there exists differences in the synteny degree among species, the DNA gene content is essentially identical (KIM et al., 2018). The main nucleus is situated at the basal half of the cell. *Pyrenomonas salina* was pointed out to harbor at least 20 nuclear chromosomes (ESCHBACH et al., 1991). *Guillardia theta* was the first nucleus of cryptophytes sequenced and it has 87 Mbp in size. More than 21000 protein genes were found and these genes are intron rich (CURTIS et al., 2012).

Cryptophytes are a group that arose by secondary endosymbiosis, where a heterotrophic eukaryote host encompassed a red alga as a plastid. Accordingly, the plastids of cryptomonads are complex and have some unique features. Four membranes surround their plastids, the two external are from the endoplasmic reticulum, and the two internal are from the chloroplast envelope (FIGURE 5). The original vacuole membrane formed during the engulfment of the endosymbiont seems to have merged with the nuclear envelope (DOUGLAS et al., 2001).

Most of cryptomonads have one lobed parietal chloroplast, which can have a big pyrenoid (FIGURE 4 and 5). The chloroplast have a lamellar arrangement of the thylakoids, and there is no connection between the thylakoids (HOEF-EMDEN; ARCHIBALD, 2010). The photosynthetic pigments are chlorophylls *a* and c_2, phycobiliprotein and carotenoids. However, phycobiliprotein is not arranged in phycobilisomes, as in cyanobacteria and red algae. Instead, the biliprotein is located inside the thylakoids (GANTT; EDWARDS; PROVASOLI, 1971). All the cryptomonads' biliproteins are not found in red algae and cyanobacteria (HILL; ROWAN, 1989).

The plastids of cryptomonads still present a relict vestigial eukaryotic nucleus, called nucleomorph (DOUGLAS et al., 2001; LEE, 2008; HOEF-EMDEN, 2012). This organelle location varies according to the cryptomonads taxa, and can be used as diagnostic. Moreover, the cryptomonads nucleomorph have three chromosomes, but the size of the genome varies according to the taxon (DOUGLAS et al., 2001; LANE; ARCHIBALD, 2006; TANIFUJI; ONODERA; HARA, 2010; MOORE et al., 2012).

The process of evolutionary compactness of the nucleomorph genetic material have begun millions of years ago, and over the time it eliminated and transferred to the nucleus of the host cell almost all genes from the nucleomorph (DOUGLAS et al., 2001; LANE et al., 2007). The reduction of the nucleomorph genome size occurred in different rates of evolution, and there is no obvious correlation among phylogeny and the nucleomorph genome size (TANIFUJI; ONODERA; HARA, 2010). Moreover, this organelle had been considered to be very interesting concerning the endosymbiosis origin of plastids, because it is regarded as an intermediary state of the endosymbiont nucleus reduction (TANIFUJI; ONODERA; HARA, 2010).

In the cryptomonads plastids, the space between the two outer membranes and the two inner is termed periplastidial space. In this space are located starch grains, 80S ribosomes, and the nucleomorph (HOEF-EMDEN; ARCHIBALD, 2010). Therefore, cryptomonads cells have two citoplasmatic compartments and four genomes, two eukaryotic (nuclear, nucleomorph), and two prokaryotic (plastid and mitochondria). While the nuclear and mitochondrial genomes came from the host, the nucleomorph and plastid genomes came from the endosymbiont. Each genome commands the protein synthesis in different cellular compartments and exchange molecules, which requires a complex subcellular protein-targeting mechanisms and coordination of the compartments of the cell (DOUGLAS et al., 2001; ARCHIBALD, 2007; CURTIS et al., 2012).

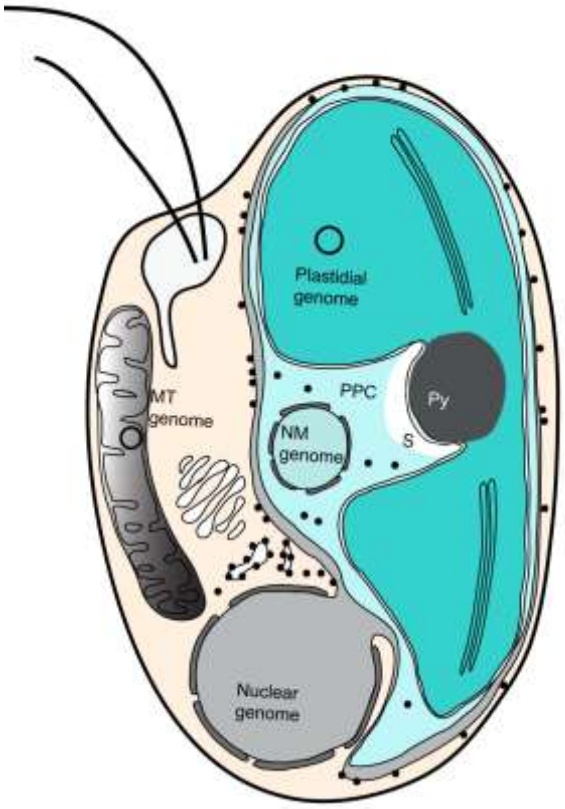

FIGURE 5: Representation of cryptophyte cell biology. Cells display one complex plastid surrounded by four membranes, four genomes, and two cytosolic compartments. (PPC) periplastidial compartment, (Py) pyrenoid, (S) starch grain.

HABITAT, ECOLOGY AND BIOLOGICAL INTERACTIONS

Cryptophytes are found in marine, brackish and freshwater environments, from tropical to polar areas. They are frequently reported in planktonic communities of aquatic environments, although one species was described from soil and another from ice (BICUDO, 1966; JAVORNICKÝ; HINDÁK, 1970). Occasionally, some species populations can multiply very fast and produce blooms (MENEZES; NOVARINO, 2003; LAZA-MARTÍNEZ, 2012; KRASNOVA et al., 2014).

Cryptophytes are important food source for some animals, such as juvenile oysters and ciliates (PEDRÓS-ALIÓ et al., 1995; DUNSTAN; BROWN; VOLKMAN, 2005). Accordingly, to reduce grazing, *Cryptomonas* species were reported living close to a chemocline of a dimictic, meso-eutrophic lake. These *Cryptomonas* species dominated the deep chlorophyll maximum in this lake near the summer thermocline for 6 years.

These populations showed a regular diel vertical migration. Daytime, due to light supply, the populations moved upward in the water column, and nighttime, downward towards the chemocline. Besides enhancing photosynthesis, the migration seems to be correlated to reducing grazing pressure, because *Cryptomonas* populations maintained themselves in anoxic hydrogen sulphide layer. (GERVAIS, 1997). The presence of sulphide limited other primary producers and grazing losses enhancing the cryptomonads populations in this water layer (GERVAIS, 1998). Previous studies in other lake, with similar conditions, showed that *Cryptomonas* populations reduced the predation loses by 38 % (PEDRÓS-ALIÓ et al., 1995).

Some organisms from marine plankton can harbor cryptomonads plastids permanently or as kleptoplastids for period of time, such as the ciliate *Myrionecta rubra*, and some species of dinoflagellates (GUSTAFSON et al., 2000; JANSON, 2004; PARK; KIM; KANG, 2013; PARK; KIM; KIM, 2014). These cryptomonads chloroplasts hosts bloomed in Brazilian and worldwide marine waters (OWEN; GIANESELLA-GALVÃO; KUTNER, 1992; WILLIAMS, 1996; PROENÇA, 2004). Among the cryptomonads kleptoplastids hosts are toxic species of dinoflagellates that can cause human poisoning (LEWITUS; GLASGOW; BURKHOLDER, 1999; TAKISHITA et al., 2002).

HISTORY OF CLASSIFICATION

Antonie van Leeuwenhoek (1632-1723) is universally regarded as the father of microbiology due to his work *Letter on the protozoa*, in 1677. He introduced detailed description of protists and bacteria form several environments (LANE, 2015). After almost two centuries, Georg A. Goldfuss (1782-1848), in 1820, created Protozoa for a class of organisms within Kingdom Animalia that comprised Infusoria (ciliates), Lithozoa (corals), Phytozoa (viz., flagellated unicellular photosynthetic organisms), and Medusinae (SCAMARDELLA, 1999). In this context, Ehrenberg (1795-1876) in 1831 described *Chilomonas paramaecium* and *Cryptomonas ovata* as members of Phytozoa, Polygastrica, Cryptomonadina (EHRENBERG, 1831). The prefix “crypto” in names of the group’s classification is based on the first species described, *Cryptomonas* (HOEF-EMDEN; ARCHIBALD, 2010).

Ehrenberg described other *Cryptomonas* species in1832 and 1856- *C. erosa*, *C. curvata*, and *C. concentrica*. Hansgirg, in 1885, erected a new genus of cryptomonad by the

typification of *Chroomonas nordstedtii*. A decade ahead, Karsten described *Rhodomonas baltica*, in 1898 (GUIRY; GUIRY, 2017). The number of species and genus of the group were increasing progressively due to new discoveries, but the phylogenetic relationships among them were poorly understood. Pascher, in 1913, described more *Cryptomonas* species and did some attempts towards cryptomonads classification. One of his major questions was regarding the cellular features suitable for the establishment of natural groups (PRINGSHEIM, 1944).

Fritsch named the class Cryptophyceae in 1927 (GUIRY; GUIRY, 2017), and few years later, in 1935, he published *The structure and reproduction of the algae*. In this book he pointed out two orders; Cryptomonadales, including the families Cryptomonadineae, Nephroselmidaceae and Phaeocapsineae, and the order Cryptococcales, with Cryptococcaceae (FRITSCH, 1935). Pringsheim, in 1944, proposed a new system of classification for cryptomonads. He recognized five families, three of Pascher (Cryptomonadaceae, Cryptochrysidaceae, Nephroselmidaceae), one of Skuja (Kathablepharidaceae), and created the taxon the Cyathomonadaceae (PRINGSHEIM, 1944). The furrow-gullet and the lining trichocysts were considered important morphological traits for the classification (FRITSCH, 1935; PRINGSHEIM, 1944). These authors pointed out that the presence of pyrenoid, eyespot, and number of plastids were also good traits to identify species. Furthermore, Pringsheim also used the type of biliprotein as important trait for taxonomy.

Due to the small sizes, few morphological structures can be observed under light microscopy. Consequently, misidentification of cryptophytes is relatively common. Historically, *Nephroselmis* Stein (1879) was regarded as Cryptophyceae. Pascher, in 1912, reported a species of *Hemiselmis* as *Nephroselmis olivacea* (BUTCHER, 1967). One year after he created the Nephroselmidaceae taxon. Due to Pacher's error, some authors confused the morphological traits of *Nephroselmis* and *Protochrysis* with *Hemiselmis*, as observed in figures and descriptions of Fritsch's publication in 1935. On the other hand, Scherffel, in 1902, stated that *Nephroselmis olivacea* must be member of Volvocales, a green algae, instead of Cryptophyta (PRINGSHEIM, 1944). *Nephroselmis* was posteriorly moved to green algae group, although Pringsheim (1944) and Fritsch (1935) still recognized it sensu Pascher. Butcher in 1967, recognized the need for replacement of Nephroselmidophyceae sensu Pascher, consequently he created

Hemiselmidaceae for the bean shaped forms of cryptophytes with two flagella inserted near a gullet, located across the cells' short axis (BUTCHER, 1967).

Butcher made important contributions to the description of cryptomonads diversity and systematics in his publication "*An introduction account of the smaller algae of British coastal waters"* from 1967. However, he did many of taxa descriptions without nomenclatural types. Moreover, he rejected the genus *Rhodomonas* and transferred their species to *Cryptomonas, Hillea* and *Chroomonas*. Previous author, considered that the genus *Rhodomonas* needed a taxonomical revision or should to be abandoned (PRINGSHEIM, 1944). Butcher described many new species, made combinations and created new families. He divided the cryptomonads in tree families: Hilleaceae, Hemiselmidaceae, and Cryptomonadaceae. For Butcher, the nature and structure of the furrow-gullet system form the only basis for primary classification (BUTCHER, 1967). Despite the fact that some species were examined with electron microscopy in Butcher's publication, actually, the species descriptions were based on observations under light microscope.

From the end of 60s until the middle 80s, many researches were published concerning the microanatomy of cryptophytes, presenting observations about micromorphology and establishing evolutionary considerations (DODGE, 1969; LUCAS, 1970; GANTT, 1971; GANTT; EDWARDS; PROVASOLI, 1971; SANTORE, 1977, 1982a, 1982b; SANTORE; GREENWOOD, 1977; MORRALL; GREENWOOD, 1982; MEYER; PIENAAR, 1984, and others). The electron microscopy opened a new horizon into the cryptophytes classification because it enhanced the number of visible features that can be compared among species, genera, and other taxa, in a group with few conspicuous morphological traits. New discoveries were performed with microanatomy studies, as the structure and morphology of periplast, the mastigonemes arrangement, the vestigial nucleus-nucleomorph and its location, the complex chloroplast arrangement, the lack of phycobilisome, the mitochondrial complex, and others.

In 1984, Santore published a revision concerning cryptomonads classification. He was optimistic about the advances of electron microscopy in cryptomonads classification, and was right that investigations of microanatomy provided necessary information for recognizing a primary division of the class. He recognized three past published genera, *Hemiselmis*, *Chroomonas*, *Cryptomonas,* and created a new one, *Pyrenomonas,* to

replace the subgenus of *Chroomonas, Cryptochrysis* sensu Butcher, and *Pyrenomonas salina (*Wislouch) Santore is the type species (SANTORE, 1984). The validity of the genera *Isoselmis, Plagioselmis, Chilomonas, Cyanothomonas, Phytollomitus* and *Rhodomononas* was taken into account. According to Santore, the genus *Rhodomonas* needs revision and previous authors had stated that the name *Rhodomonas* should be discontinued (PRINGSHEIM, 1944; BUTCHER, 1967, SANTORE, 1984).

Klaveness, in 1985, did a revision on the criteria for species determination of cryptophytes. The systematics at species level was still inadequate, reflecting an artificial, subjective, or situation-dependent species concept (KLAVENESS, 1985). According to Klaveness, the cell shape is the most important feature for the species determination, but he admitted that cells shape change due the cellular cycle even in clonal cultures. Other characters were also cited as significant for taxonomical purposes, such as the shape and size of the gullet, flagellar length, number and position of plastids, the pyrenoid presence and number, the Maupas ovals, and the structure of the external periplast component (KLAVENESS, 1985). The furrow-gullet relation was a point of disagreement between Santore and Klaveness. Santore believed that the furrow is an artifact, a rupture of the gullet, while Klaveness argue that it should be used for species identification (SANTORE, 1984; KLAVENESS, 1985).

The description of *Proteomonas sulcata* Hill & Wetherbee (1986) unveiled cryptophyte species that exhibit dimorphic forms in its life cycle. Although sexual reproduction was previously reported (KUGRENS; LEE, 1988), it was the first time that dimorphisms in a same species was confirmed presumable by sexual reproduction. The two forms, haplomorph and diplomorph, can be distinguished by size, periplast structure, configuration of the flagellar apparatus, and the quantity of nuclear DNA (HILL; WETHERBEE, 1986).

Reexaminations of *Cryptochrysis* sensu Butcher by Hill and Wetherbee (1988) yielded new species descriptions, such as *Rhinomonas pauca.* In the publication of Butcher, he separated four species in *Chroomonas* species key based on their diminutive size, and these cells have an anterior rhinote- prominently protruding anterior (HILL; WETHERBEE, 1988). The authors also revised the diagnostic features of genera close to *Rhinomonas,* as the vestibulum furrow-gullet system, the periplast morphology, the

plastidial complex (form of chloroplast, pyrenoid, location of the nucleomorph and phycobiliprotein content).

In 1989, Hill and Wetherbee revised and emended the genus *Rhodomonas,* and designated *Pyrenomonas* Santore (1984) as heterotypic synonym of *Rhodomonas*. They described a new species, *Rhodomonas duplex*, and did new combinations to the genus *Cryptomonas* and *Chroomonas* sensu Butcher. Additionally, they designated the type for many species proposed by Butcher that were invalid (HILL; WETHERBEE, 1989).

In studies of cryptophytes, the phycobiliprotein type is also considered as a taxonomic useful trait. In 1979, Elisabeth Gant did a great revision about the cryptomonads phycobiliproteins and recognized five different types, two of phycocyanin and three of phycoerythrin (GANTT, 1979). Ten years after, Hill and Rowan (1989) studied the phycobiliproteins of 26 strains and found three types of phycoerythrin and four types for phycocyanin. Moreover, they debated a correlation among the type of phycobiliprotein and the genera (HILL; ROWAN, 1989).

Guillardia theta was described in 1990, a new species and genus (HILL; WETHERBEE, 1990). In two publications in 1991, Hill created six new genera by the reexamination by electron microscopy of species assigned previously as *Chroomonas* and *Cryptomonas.* The proposed new genera were *Komma, Falcomonas, Teleaulax, Storeatula, Geminigera,* and *Campylomonas* (HILL, 1991a, 1991b). According to the author, features such as the nature of the furrow-gullet system, structure of the periplast, structures of the plastidial complex, and organization of the flagellar root, are useful for providing delimitation of the genera (HILL, 1991a, 1991b)..

At the same time, Novarino published two papers describing four new variations and three new combinations of *Pyrenomonas* and *Rhinomonas*. Indeed, he rejected the name *Rhodomonas,* and used *Pyrenomonas* sensu Santore (NOVARINO, 1991a, 1991b). According to him, the genus *Pyrenomonas* has rectangular plates of external periplast, instead *Rhinomonas* has hexagonal ones (NOVARINO, 1991a). Concerning the systematics of cryptophytes, in 1993, Novarino and Lucas published a proposal for new classification system. They based the classification on literature's description of fine structure of the cell, allied to phycobiliprotein type, and consequently recognized seven pigmented genera (NOVARINO; LUCAS, 1993). They used the absence of plastids and nucleomorph, as a feature to separate the orders. If nucleomorph is present, its position

in the cell is also used as diagnostic trait of at order level. Finally, the location of flagella position is suitable to identify families. Three orders were recognized, Goniomonadales, Pyrenomonadales, and Cryptomonadales. They typified the last two. The authors documented four families, Goniomonadaceae, Pyrenomonadaceae, Cryptomonadaceaea and Hemiselmidaceae. However, they did not included the recent genera descriptions, viz., *Guillardia, Campylomonas, Geminigera, Storeatula, Teleaulax, Falcomonas* and *Komma.*

In the 90's, the use of DNA sequences in phylogenetic analysis, aiming to solve evolutionary relationships, became more common among many organisms. A pressing force in the molecular phylogeny works is to find out and understand what makes a molecule a reliable measure of the organism´s history, what is the dynamics of evolution and how is the best way to analyze it (OLSEN; WOESE, 1993). Accordingly, genes that perform main functions in the cell have been used for inferring molecular inferences, as rRNAs, RNApolimerases, and others (OLSEN; WOESE, 1993). This rely on the thinking that housekeeping genes tends to evolve less fast and being under high evolution pressure. The rRNA genes have being used as a valorous sequence for inferring phylogenies, (OLSEN; WOESE, 1993).

Although the endosymbiotic origin of algae and plants plastids was largely accepted at that moment, the evolutionary history regarding it was unclear. Cryptophytes were proven evolutionary chimaeras in 1991. One molecular phylogenetic study of *Guillardia* genes of SSU rDNA from nucleus and nucleomorph showed that these sequences tells different histories, and that the nucleomorph sequence was sister of red algae clade (DOUGLAS et al., 1991). Posterior studies of SSU rDNA phylogenies confirmed the red algal ancestry of cryptophyte plastids (CAVALIER-SMITH et al., 1996; HOEF-EMDEN; MARIN; MELKONIAN, 2002).

Molecular phylogenies corroborated some morphological groups of cryptophytes. That happened with Pyrenomonadales clade, which was based on the synapomophy of the nucleomorph position inside the pyrenoid (CAVALIER-SMITH et al., 1996). Some molecular phylogenetic studies of cryptophytes 18S rRNA divided the group into seven major lineages (DEANE et al., 2002; HOEF-EMDEN; MARIN; MELKONIAN, 2002). Since then, new species descriptions, new combinations or emends have been accompanied at least by genetic sequences of SSU rDNA (CLAY; KUGRENS, 1999;

HOEF-EMDEN; MELKONIAN, 2003; HOEF-EMDEN, 2007; LANE; ARCHIBALD, 2008; LAZA-MARTÍNEZ, 2012; MAJANEVA et al., 2014). Concerning phycobiliproteins evolution, studies with amino acids sequences of biliproteins showed that cryptomonads and red algae form a monophyletic clade (APT; COLLIER; GROSSMAN, 1995; GLAZER; WEDEMAYER, 1995). All the phycobiliproteins of cryptomonads are phylogenetically derived from a red algal phycoerythrins due to the loss of allophycocyanin and phycocyanin (GLAZER; WEDEMAYER, 1995). Moreover, the phycobiliprotein type was found out to be congruent with the molecular phylogenies of nuclear and nucleomorph SSU rDNA genes (MARIN; KLINGBERG; MELKONIAN, 1998; DEANE et al., 2002; HOEF-EMDEN; MARIN; MELKONIAN, 2002).

After all these evidences, the classification system of Novarino and Lucas (1993) needed a revision. Then, Clay, Kugrens and Lee published "*A revised classification of Cryptophyta"* in 1999. They provided complete ultrastructural descriptions for each genus based on literature information. Using ultrastructural traits, phycobiliprotein type and molecular information, they delineated 16 genera. Two classes were recognized, Goniomonadea and Cryptophyceae. Three orders were discussed and separated based on phycobiliprotein type, viz., Goniomonadales (colorless), Cryptomonadales (phycoerythrin 566), and Pyrenomonadales (diverse phycobiliprotein content). The first one, Goniomonadales contain single genus, which is aplastidial. Cryptomonadales comprised two families, Campylomonadaceae and Cryptomonadaceae, Finally, the order Pyrenomonadales, which was divided into four families. Three new families were described, Campylomonadaceae, Geminigeraceae, and Chroomonadaceae (CLAY; KUGRENS; LEE, 1999).

Although most of morphological features investigated with light and electron microscopies seemed to be congruent with molecular phylogeny, some concepts concerning the groups' classification were not. Hoef-Emden and Melkonian in 2003 revised the *Cryptomonas* genus using seventy-three strains. Using light microscopy, spectrophotometry, and electron microscopy, they found that characters previously used to distinguish *Cryptomonas* from *Campylomonas* occurred together in dimorphic strains, pointing out that a single taxon can have different forms of the periplast due to life history-stage. Furthermore, molecular phylogeny provided evidence that the genus *Campylomonas* is an alternate form of *Cryptomonas.* The combined approach of

characterization allowed identifying several cryptomorph/campylomorph pairs in *Cryptomonas* species. Moreover, the genus *Chilomonas* was also assigned as synonymous of *Cryptomonas* (HOEF-EMDEN; MELKONIAN, 2003). In other study, Hoef-Emden, using molecular phylogeny inferences of partial nucleomorph SSU rRNA and secondary structure comparisons, suggested that in freshwater *Cryptomonas* there are at least three independent losses of the photosynthetic ability (HOEF-EMDEN, 2005) .

In summary, the cells of cryptophytes are often small; most of species are between nano- and picoplankton size fractions (smaller than 40µm). Moreover, few morphological features are available to distinguish cells. Therefore, the species diagnosis based solely on light microscopy investigations is incomplete. Electron microscopy techniques provide a wider morphological view of the structures of the cells and enhance the resolution regarding the taxa identification. However, cryptophyte cells morphology can vary considerable, even in a clonal culture, due to dimorphic life cycle (HILL; WETHERBEE, 1986; HOEF-EMDEN; MELKONIAN, 2003; HOEF-EMDEN, 2007). Additionally, it seems that in some clades, such as *Cryptomonas*, the morphological species concept does not work for some species. Strains that were identical or almost in ITS2 rDNA nuclear spacer region displayed different cell morphologies to the point that they could be assigned to different morphological species. On the other hand, strains morphologically similar presented enough molecular divergence to represent different species. Additionally, some authors state that most of the putative biological species studied could just be identified with molecular sequences, and ITS2 secondary structure is likely a way to identify cryptophytes biological species (HOEF-EMDEN; MELKONIAN, 2003; HOEF-EMDEN, 2007).

Concerning the highest classification of cryptophytes, since the groups description, in 1831, for more than one century few things changed. Cryptophytes were classified as from Protista Kingdom and kept there until 1981, when Cavalier-Smith described Chromista for the photosynthetic lineages that contain chlorophyll *c* as accessory pigment as well as the chloroplasts surrounded by four membranes (CAVALIER-SMITH, 1981). Although this classification still controversial, especially after the last phylogenomic studies, as discussed above, the name is still used to separate these lineages with plastids from red algae (RUGGIERO et al., 2015).

BIODIVERSITY STATUS AND FINAL CONSIDERATIONS

Nowadays, the number of species described of cryptomonads is close to 200 (CERINO; ZINGONE, 2007; GUIRY; GUIRY, 2017). The most diverse order is Cryptomonadales, with 166 species, followed by Pyrenomonadales, with 40 taxa. *Cryptomonas* is the most diverse genus, with 54 species taxonomically recognized, followed by *Chroomonas, Rhodomonas,* and *Hemiselmis* (GUIRY; GUIRY, 2017). However, some of the taxa were poorly typified, accordingly needs further investigations.

Recently, a metagenomics study concerning the diversity of eukaryotes in sunlit ocean based on libraries of *V9* region of 18S rDNA showed that the number of cryptomonads operational taxonomic units (OTUs) was close to 150. Moreover, near 90% of the cryptomonads OTUs identified are in the picoplankton size fraction (DE VARGAS et al., 2015). However, the last study sampled very few coastal areas worldwide, thus the number of OTUs should even be higher. This represents more than half of the species number of marine cryptomonads diversity estimated by Sournia, Chrétiennot-Dinet & Ricard (1991).

In Brazil, the knowledge concerning cryptomonads biodiversity is incipient. All the species reports of cryptomonads were based on light microscopy surveys, which enhance the uncertainty of the taxa estimative. The scarcity of cryptophytes specialists, allied to problems concerning the lack of infrastructure to use electron microscopy and molecular biology techniques, are factors that could have influenced for the current biodiversity status. Until 2015, 35 taxa were recorded in Brazilian waters (MENEZES et a., 2015). Regarding the geographical distribution in Brazilian ecosystems, cryptomonads are highly reported in reservoirs and coastal lagoons at the southeastern of the country. Few reports of cryptomonads have been done in marine waters and the taxa are unidentified and/ or with unclear identification (MENEZES et al., 2015; MENEZES; NOVARINO, 2003)..

The planktonic cryptomonads have been specially understudied in tropical regions, such as in Brazil, and therefore these group deserve urgent attention (MENEZES; NOVARINO, 2003) Accordingly, the present study aims to expand the limited knowledge mainly regarding marine cryptomonads diversity in Brazilian coastal waters as well as worldwide. For this purpose, fourteen strains were isolated from coastal regions in Brazil and maintained in cultures to be studied as regards morphology,

phycobiliprotein type, and molecular phylogeny. Moreover, forty strains were brought from Roscoff Culture Collection (RCC), isolated from a wide variety of marine regions. Therefore, this work is expected to enhance the knowledge about publication cryptophytes biodiversity in marine environments, specially, in Brazilian coastal waters.

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2 *HEMISELMIS AQUAMARINA* SP. NOV. (CRYPTOMONADALES, CRYPTOPHYCEAE), A CRYPTOPHYTE WITH A NOVEL TYPE OF PHYCOBILIPROTEIN (CR-PC564)

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Abstract: Cryptophytes are a small group of photosynthetic biflagellate organisms distributed worldwide in both fresh and marine waters. Although members of this class are easily distinguished from other algae, species identification is difficult and studies concerning their diversity are scarce. Strains of an undescribed species were isolated from Brazil and Japan for which morphological and molecular phylogeny analyses were performed. Cells of *Hemiselmis aquamarina* sp. nov. are reniform and possess hexagonal plates of superficial periplast component, a basal middle band, a refractive body, one parietal boat-shaped plastid with a new type of cryptophyte phycocyanin. The plastid has a pyrenoid coated with starch. Molecular phylogeny of concatenated SSU (nuclear and nucleomorph) genes highly supports the relationship of *H. aquamarina* as a sister species of *H. tepida*. Most *Hemiselmis* species have been described for the subtropical region, and all of them are from North Hemisphere. *H. aquamarina* is the first species of this genus recorded in both the South Atlantic Ocean and East Pacific Oceans, suggesting that this species may be cosmopolitan.

Key index words: morphology; molecular phylogeny; phytoplankton; South Atlantic Ocean; Pacific Ocean.

Abbreviations: BA, Bayesian analysis BP, base pairs; Cr-PC, cryptophyte phycocyanin; Cr-PE, cryptophyte phycoerythrin; ITS2, internal transcribed spacer 2; nSSU, nuclear small ribosomal subunit; nmSSU, nucleomorph small ribosomal subunit; PP, posterior probability; RCC, Roscoff Culture Collection; SPC, superficial periplast

component; UTEX, The Culture Collection of Algae at the University of Texas at Austin.

INTRODUCTION

The Cryptophyceae form a group of unicellular organisms, mostly photosynthetic, widespread in the pelagic zones of brackish, marine, and freshwater environments (Klaveness 1985). The furrow gullet system, where a pair of flagella is inserted, is surrounded by ejectosomes. This system has a strong impact on cell morphology resulting in an asymmetrical shape. The photosynthetic pigments of cryptophytes include chlorophylls *a*, *c*, carotenoids, and phycobiliproteins, which give them a range of different colors, such as red, orange, blue-green, olive-green, and brown.

Cryptophyceae are monophyletic although their relationship with other eukaryotes remains still unclear (Baurain et al. 2010, Burki et al. 2008, 2016, Kim et al. 2017,Okamoto and Inouye 2005). The cryptophyte plastid is surrounded by four membranes, originating from secondary endosymbiosis from a red alga ancestor and still possesses a relict of the endosymbiont nucleus, the nucleomorph (Douglas et al. 2001, Douglas and Penny 1999, Keeling 2010). Phycobiliproteins are not located on the outer surface of the thylakoid (the stromal side) and are not arranged in a phycobilisome, as in cyanobacteria, glaucocystophytes, and red algae. Instead, the phycobiliproteins are located in the intra-thylakoidal spaces (Gantt et al. 1971, Glazer and Wedemayer 1995, Spear-Bernstein and Miller 1989) . Cryptophyte phycobiliproteins are composed of two subunits α and β, where four chromophores are attached, one to the- or α'- subunit and three to the β- subunit. The diversity of phycobiliprotein spectral signatures in cryptophytes is related to the nature, number, locations and also three-dimensional structure of the bilins (Overkamp et al. 2014, Wedemayer et al. 1996). Each species of cryptophyte has a single type of phycobiliprotein, either Cr-Phycocyanin (Cr-PC) or Cr-Phycoerythrin (Cr-PE).

Phylogeny analysis of the cryptophyte phycobiliprotein beta-subunit suggest that all cryptophyte phycobiliproteins are derived from red algae phycoerythrin (Apt et al. 1995, Glazer and Wedemayer 1995), due to the loss of phycocyanin and allophycocyanin in the cryptophyte ancestor. For that reason, the classification of phycobiliproteins in cryptophytes as Cr-PC or Cr-PE is based on the color of the protein extract, if bluish or reddish, respectively (Glazer and Wedemayer 1995, Overkamp et al. 2014,).

The genus *Hemiselmis* was first described in 1949 from British coastal waters by the typification of *Hemiselmis rufescens* Parke (1949) which has a reddish plastid. The next species described was *Hemiselmis virescens* Droop (1955), a blue-green member, also from British coastal waters. Droop based the species description on the differences in plastid color and slight morphological variances. In 1967, Butcher did a major review concerning cryptophytes classification and adopted as the main trait to separate the genera the number of visible ejectosomes rows. The subgenera were divided according to their color. He also assigned ten new species to *Hemiselmis* and divided the genus into two new genera, *Hemiselmis* and *Plagiomonas* (Butcher 1967). However, the taxonomic value of cell color is dubious (Santore 1982) and most of Butcher species were based solely on light microscopy investigations. Moreover, the absence of the cultures from which the species types were obtained resulted on invalidation of Butcher species descriptions of *Hemiselmis* (Lane and Archibald 2008).

In the last decades, five new species of *Hemiselmis* have been described. *H. amylosa* Clay & Kugrens was the first species described from a freshwater environment, from Colorado Lake, USA (Clay and Kugrens 1999). The other four species, *H. andersenii* Lane & Archibald, *H. cryptochromatica* Lane & Archibald, *H. pacifica* Lane &

Archibald, and *H. tepida* Lane & Archibald are from marine environments, and except for *H. pacifica* from the North Pacific Ocean, all others had the type locality attributed to the North Atlantic Ocean (Lane and Archibald 2008).

Despite the fact that cell color should not guide species description in cryptophytes, the phycobiliprotein type has been shown to be correlated with phylogeny (Deane et al. 2002, Hoef-Emden 2008, Lane and Archibald 2008, Marin et al. 1998). Most cryptophyte families have one single type of phycobiliprotein. One exception is the Hemilsemidaceae Butcher (1967). Currently, four types of biliproteins are found in *Hemiselmis*, one phycoerythrin, Cr-PE 555 in *H. andersenii* and *H. rufescens*, and three phycocyanin types, Cr-PC577 in *H. pacifica*, Cr-PC612 in *H. virescens, H. tepida* and *H. amylosa*, and Cr-PC630 in *H. cryptochromatica* (Clay and Kugrens 1999, Hoef-Emden 2008, Lane and Archibald 2008). The only known reversal from Cr-PC to Cr-PE in cryptophytes has been found in *H. rufescens* and *H. andersenii*, and this may be the reason why the Cr-PE555 is restricted to these species (Clay and Kugrens 1999, Lane and Archibald 2008).

At the genus level, *Hemiselmis* can be easily recognizable by light microscopy (Santore 1982). The cells are smaller than most of cryptophytes and their morphology is very different from the other genera, due to the ventral insertion of the flagella almost in the middle of the cell, the short gullet, the bean-shaped form, and the conspicuous refractive body. However, species lack distinguishing features and strains of the same species can differ morphologically, making an accurate diagnosis without molecular tools almost impossible (Hoef-Emden and Melkonian 2003, Lane and Archibald 2008) . One example is the description of *H. brunnescens*, which after DNA phylogenetic analysis was treated as synonymous of *H. rufescens* (Lane and Archibald 2008).

We investigated in detail two cryptophyte strains, BMAK265 and RCC4102. The first was isolated from the coast of Brazil and the other isolated from the coast of Japan. We used different sources of microscopes, phycobiliprotein spectral characterization and DNA sequences, aiming to uncover different sources of variations, which could justify a new species description. Accordingly, these strains correspond to a yet undescribed species of *Hemiselmis* with a unique type of phycobiliprotein.

MATERIAL AND METHODS

Sampling, strain isolation, cultivation conditions, and phycobiliprotein extraction

Strain BMAK265 was isolated in August 2011 from the coast of Brazil, close to the Anchieta Island, Ubatuba, São Paulo (23º 35.847'S, 45º 01.70' W). The water sample was collected at a depth of 40 m with a Nansen bottle and enriched with Erd-Schreiber medium diluted 10 times. After a few days, a single cell was selected by micropipetting and carefully washed. The culture is maintained in Erd-Schreiber medium (Throndsen 1997), 32-35 salinity, 20°C temperature, photoperiod of 12:12 L:D cycle at 50 µmol $m^{-2}.s^{-1}$. This strain is deposited in the Microorganisms Collection of Oceanographic Institute of University of São Paulo (Banco de Microorganismos Marinhos Aydar & Kutner) as BMAK265 and the Roscoff Culture Collection (http://roscoff-culture-collection.org) as RCC5634.

The strain from the coast of Japan was isolated in August of 2013, during the Oshoro-Maru Cruise. Four liters of surface water were collected from station S3 near Kurosaki, Iwate (39° 59' N, 142° 15'E) and concentrated by tangential flow filtration to 100 mL. Single cells were isolated in K medium at 20°C by micropipetting and then maintained under these conditions. This strain is deposited in Roscoff Culture Collection as RCC4102.

Although we wished to perform similar analyses on strain UTEX2000, which SSU rRNA sequence is very close to those of BMAK265 and RCC4102, we could obtain this

strain from the UTEX collection where it is cryopreserved only and not distributed (https://utex.org/products/utex-lb-2000).

Phycobiliprotein extraction was performed for strains BMAK265 and RCC4102 following Hill and Rowan (1989). Cultures were grown in 50 ml polycarbonate culture flasks for 2-3 weeks following the conditions described above, and harvested by centrifugation (300 g, 8 min). The pellet was frozen and kept at-80°C until processing. Cells were lysed by two rounds of melting-freezing after which phosphate buffer (pH 6.8) was added. To remove cellular debris, the samples were centrifuged at high speed (16,000 g, 30 min). The absorption spectra of the supernatant liquid containing the pigment extract were determined using an Epoch 2 microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA).

Morphological observations

2-3 weeks old BMAK265 cultures were used for morphological observations. Fixed (2% glutaraldehyde) and living cells were observed by light microscopy (bright field and phase contrast) using a Leica DM 4000 B (Leica Microsystems, Wentzler, Germany) and confocal microscopy using a Zeiss LSM 440 Axiovent 100 (Carl Zeiss, Jena, Germany). Morphometric values were estimated from 40 individuals measurements. For confocal microscopy, cells were excited with a laser at 543 nm and fluorescence emission was observed using a long pass filter 570 nm. For electron microscopy, cells were harvested by gentle centrifugation (3 min, 100-150 g), and then immediately fixed for 90 min with a solution containing glutaraldehyde (2%), sodium cacodylate trihydrate (0.1 M) and sucrose (0.8M), as described in Majaneva et al. (2014). The cells were washed using the latter solution (without glutaraldehyde) and after they were post-fixed with a solution containing osmium tetroxide (1 %) and cacodylate trihydrate (0.1 M) for 60 min. Cells were then washed twice in cacodylate buffer (0.1M). For scanning electron microscopy (SEM), a sample was dehydrated in a series of increasing ethanol concentrations (70, 90, 95 and 100 %). It was subsequently critical-point dried (Balzers CPD 030, Bal-Tec, Vaduz, Liechtenstein), gold-coated (Balzers SCD 050), and examined in on a Zeiss Sigma VP. For transmission electron microscopy (TEM), cells were dehydrated in acetone series (50, 70, 90, 95 and 100%),

embedded in Spurr resin, thin sectioned, stained, and examined on a Philips CM120 TEM.

DNA extraction, PCR and sequencing

Material for molecular analyses was obtained as specified in the pigment extraction section. Genomic DNA was obtained using *NucleoSpin® Plant* II kit in accordance with the manufacturer instructions. PCRs of nuclear SSU (nSSU), nucleomorph SSU (nmSSU), and ITS2 rDNA were performed with Platinum® *Taq* DNA polymerase kit (Invitrogen™, Carlsbad, USA). The primers used for PCR and sequencing reaction as well as cycling conditions are available in Supplementary Table 1.

The PCRs products producing a single band of the expected size on an agarose gel (1%) were purified using GFX Illustra kit (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK) in accordance with the manufacturer instructions. Sequencing reactions were done with Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems™, Hammonton, NJ, USA), and then samples were sequenced on a 3730 Applied Biosystems. We sequenced the genes nSSU and nmSSU rDNA of BMAK265, RCC659, RCC2614, RCC3575, RCC4102, and RCC4216. The ITS2 rDNA was also sequenced for the strains BMAK265, RCC659, RCC1504, RCC2614, RCC3436, RCC3575, RCC4102, and RCC4116. All generated contigs were searched by BLAST against NCBI sequences to ensure that each contig corresponded to the organisms we were investigating. We discarded contigs with high levels of ambiguity and noise.The consensus sequences of nSSU, nmSSU, and ITS2 rDNA were assembled using *Geneious* 9.7 (Biomatters, Auckland, New Zealand), by comparison to a reference sequence obtained from BLAST. All sequences were deposited to Genbank under accession numbers MF179473, MF179479, MF574711, MF179476, MF179483, MF574713, KY711345, KY711347, KY711346, MF179475, MF179481, MF179474, MF179480, MF574712, MF179477, MF179484, MF574715, MF589232, and MF574714 (Supplementary Table 2).

Phylogenetic analyses

A dataset for phylogenetic analysis was built using the sequences we determined to search by BLAST the NCBI database (https://blast.ncbi.nlm.nih.gov/). Alignments were performed in AliView (Larsson 2014) with the Muscle algorithm (Edgar 2004). Alignments were refined by visual inspection. The appropriate evolution model was chosen using JModelTest 2.1.7 analysis (Darriba et al. 2012).

We performed likelihood mapping tests with the alignment with varying degrees of indel regions in Tree-Puzzle 5.3 (Schmidt et al. 2003), aiming to inspect whether the phylogenetic signal where enhanced. We applied the specific molecular evolution models for each gene in the Tree-Puzzle tests. We used the alignments with best-solved quartets for the phylogenetic inference. We decided to include the nuclear SSU rDNA in phylogenetic inferences of *Hemiselmis amylosa*, although it lacks a sequence in the database for nucleomorph SSU rDNA. Highly incomplete taxa can be accurately placed in phylogenies and improve results in cases misleading long branches. Moreover, Bayesian methods seems to be more appropriate to deal with missing data (Wiens 2006).

To inspect if the topologies were congruent between the nSSU and nmSSU rDNA genes, we performed some test analysis by Bayesian analysis (BA) as described below (Supplementary figure 1). As the topologies were congruent, we decided to concatenate both genes and to apply separately the model of nucleotide molecular evolution. The concatenated dataset genes of SSU rDNA was built using SeaView (Gouy et al. 2010). It had 2841 base pairs (bp), 1346 pb of nucleomorph and 1494 bp of nuclear SSU rDNA. Fifty sequences were included from the genera *Hemiselmis Chroomonas, Komma, Cryptomonas, Rhodomonas, Rhinomonas, Storeatula, Guillardia, Hanusia, Geminigera, Teleaulax,* and *Plagioselmis* genera. The sequences of *Rhodomonas, Rhinomonas,* and *Storeatula* were used to root the tree due to their distant phylogenetic relationship to the *Hemiselmis* clade (Deane et al. 2002, Hoef-Emden 2008).

Sequences of partial 5.8S, complete ITS 2, and partial 28S rDNA were used to build an alignment of 433 bp with five sequences of *Hemiselmis* and one sequence of *Chromoonas,* the last was used to root the tree due to the close phylogenetic relationship

of these genera (Hoef-Emden 2008, Lane and Archibald 2008). Both alignment files are available online (Supplementary Data 3 and 4).

Phylogenetic inferences were performed with MrBayes 3.2 (Ronquist et al. 2012) with two consecutive runs of $2x10^8$ generations, four Markov chains, with a sampling frequency of 100 generations. Run convergence and likelihood were checked in Tracer V1.7 (Rambaut et al.). The split frequency of the runs was above the guidance recommendation. A relative burn-in of 25% was applied.

TAXONOMIC SECTION

Hemiselmis aquamarina K. Magalhães & M. C. Oliveira, sp. nov.

Description: free-swimming cells, reniform in lateral view, with rounded ends, ranging in size from 4.5 to 7.5µm (length) by 2.5-4.5 µm (width). Cells ovate in ventral/dorsal view, and circular apical/antapical view. One dorsal parietal plastid, blue-green in color, with a ventral globular structure. One pyrenoid, with a starch coat, is found at the cell apex. The plastid also harbor a starch grain in surrounded by thylakoids. Single thylakoids penetrating into pyrenoid core. Major accessory pigment Cr-PC564. A Golgi body behind flagellar apparatus, in the middle of the cell. Refractive body above flagellar region. The nucleomorph is situated above pyrenoid, and the nucleus in the antapical part of the cell. Tubular unbranched mitochondria disposed along to the flagellar region, between plastid and periplast, and near the nucleus. Two sub-equal flagella emerge from vestibulum. Gullet ventrally located, almost middle of the cell. Ejectosomes disposed close to the gullet and along the periplast of the cell. Superficial periplast component with hexagonal plates.

Holotype (here designated)*:* Frozen pellet of strain BMAK265 (in a metabolically inactive state) has been deposited as type at the Roscoff Culture Collection under Cryopreservation record.

Molecular diagnosis: nSSU (KY711345), ITS2 (KY711346), and nmSSU (KY711347) rDNA.

Type strain: BMAK265 (RCC5634)

Other strain: RCC4102

Type locality: 23.59745 S, 45.02833W, a coastal area of Ubatuba, São Paulo, Brazil.

Etymology: the epithet refers to the color of the cells and culture, light blue-green also called aquamarine.

RESULTS

The cells of *Hemiselmis aquamarina* are asymmetrical and reniform in lateral view, sizing from 4.5 to 7.5 µm. The size and form of the cells varies in the culture. Some cells are smaller, while other are bigger, some are more elliptical and others more rounded (Figure 1A-B). One parietal plastid, light blue-green, occupies the dorsal part of the cell and extending around most of lateral sides (Figure 1). The plastid harbor enclosed by four membranes a conspicuous apical pyrenoid, which has a starch coat (Figure 1A-B, and Figure 2A, D-F). Moreover, a starch grain close the antapical pole of the cell (Figure 2A). Thylakoids fill the plastid and sometimes the parallel disposition could be observed (Figure 2A). The pattern of paired thylakoids seems to not occur in *Hemiselmis* species, which thylakoids seems to be more distended and anastomosed than other genera of cryptophytes (Santore, 1982). Single thylakoids penetrate in the apical pyrenoid core (data not shown). A globular structure at the ventral part of the plastid is visible when the cell was excited under 543 nm in confocal microscopy (Figure 1E-F).

The nucleomorph is situated at the ventral view of the plastid, in the periplastidial space. Disposed below the apical pyrenoid almost the middle of the cell, it have a double-membrane envelope and a granular matrix with a nucleus like-structure (Figure 2A-C). The main nucleus of the cell lies in the antapical pole (Figure 2A,C-E), which

the last membrane is continuous with plastid-complex. One conspicuous refractive body, also called Corps-de-Maupas (Lucas 1970, Santore 1984), is located near the nucleus and the plastid (Figure 1A-D). This structure is attributed to function as a lysosome and lipid accumulation body, which is clearly seen in old cultures (Santore 1984). A small Golgi body, with many vesicles, is located near the flagellar region of the cell, almost at the middle part of the cell (Figure 2B-E). A tubular mitochondrion extends mainly through longitudinal direction of the cell, surrounding the flagellar apparatus, nucleus and between the periplast and the plastid (Figure 2A and D). Cryptophytes mitochondrion are described as unitary and with flattened finger-like morphology of cristae (Santore and Greenwood 1977). The mitochondrion of *Hemiselmis rufescens* and *H. virescens* were described as single unbranched botuliform organelle, which contrasts the branched complex pattern of *Cryptomonas* and *Chroomonas* (Santore and Greenwood 1977, Santore 1982, 1984).

Two unequal flagella are inserted ventrally, almost in the middle of the cell (Figures 1, 2 and 3). The plasma membrane is continuous with flagellar membrane (Figure 2F). One flagella slightly larger than the other (Figure 1F), and mastigonemes were found in both (Figure 3 B,D-E). The longer flagellum carries two rows of mastigonemes while the smaller a single row (Hibberd et al 1971, Pennick 1981, Santore 1982, 1983, 1984). Close to the flagellar insertion, a small gullet extends to antapical pole of the cell, which is surrounded by large ejectosomes (Figures 1C-D and 2B-D). Cells are free swimming and very active. Frequently, when resting, cells quickly rotate on their own axis and suddenly go away (see Supplementary Data 1 and 2).

The cell coverage of cryptophytes, the periplast, consists of a plasma membrane with internal and external proteinaceous plates (Hibberd et al 1971). The surface periplast

component (SPC) is composed of large hexagonal plates on the dorsal part of the cell, and the plates become slight smaller in the ventral part (Figure 3). Variances in SPC form of the plates and size of *Hemiselmis rufescens* (named as *H. bunnescens*) was already described, particularly at the apical and antapical ends of the cell (Wetherbee et al 1986). The periplast of the cells is more delicate than other cryptophytes and cells can collapse after critical point drying (Figure 3B), also reported in literature (Santore and Greenwood 1977, Santore 1982). The periplast is intermittent solely at the vestibulum/ gullet and flagellar region, where the plasma membrane is not associated with periplast plates (Hibberd et al 1971, Wetherbee et al. 1986). Few gaps in the periplast may also occur, which are occupied by ejectosomes (Figure 2A, 3D-E), suggesting that the plates are large. A mid-ventral line at the antapical pole of the cell was observed (Figure 3F). The EM stub of the species was deposited in the Herbarium of the Botanical Institute of São Paulo (**SP**), voucher number SP469.780

The phycobiliprotein of the strains BMAK265 and RCC4102 have very close spectral characteristics. Both pigment extracts are purple. BMAK265 strain has maximum absorption at 564 nm and a second peak at 616-620 nm. RCC4102 has the highest peak of absorption at 557- 566 nm and another one at 616-619 nm (Figure 4). The Cr-PCs closer spectral characteristics to Cr-PC564 are Cr-PC569 and Cr-PC615/612, which are from *Falcomonas daucoides* (Conrad and Kufferath) Hill (1991), and from *Hemiselmis virescens, H. tepida* and *H. amylosa*, respectively (Clay and Kugrens 1999, Lane and Archibald 2008, Hoef-Emden 2008). The Cr-PC569 of *Falcomonas daucoides* is the type of phycobiliprotein with the maximum absorption peak closest to Cr-PC564 of *H. aquamarina*. However, the second highest peak of Cr-PC569 is at 630 nm while in Cr-PC564 is at 616-620 nm. Cr-PC612 of *Hemiselmis virescens, H. tepida* and *H. amylosa*

is close to Cr-PC564 too. In this case, the secondary peak of Cr-PC564 is close to the highest peak of Cr-PC612.

In order to attempt to clarify the phylogenetic relationship of *Hemiselmis* among other genera of cryptophytes, we sequenced several strains of cryptophytes and performed a phylogenetic analysis using our data and from Genbank in a concatenated nSSU and nmSSU genes. The genus *Hemiselmis* forms a distinct and monophyletic clade, highly supported by posterior probability (PP), which is a sister group of the *Chroomonas* (Figures 5 and Supplementary Table 3). This is congruent with previous molecular phylogeny studies, which place *Chroomonas* as paraphyletic group leading into *Hemiselmis* (Deane et al. 2002, Clay and Kugrens 1999, Hoef-Emden et al. 2002, Hoef-Emden 2008, Lane and Archibald 2008, Marin et al. 1998).

Regarding Hemiselmidaceae evolution, previous works also reconstructed the basal position of *H. cryptochromatica*, and *H. amylosa* in the family as in our results. Additionally, the close phylogenetic relationship of *H. pacifica* and *H. virescens* was reconstructed (Hoef-Emden 2008, Lane and Archibald 2008). The sequence of UTEX2000 has also been found to be a sister of *H. tepida* and closely related to *H andersenii* and *H. rufescens* (Hoef-Emden 2008).

Within the genus *Hemiselmis*, *H. cryptochromatica* is a sister taxon of all other species (Figure 5). The identity of nSSU and nmSSU rDNA sequences between *H. cryptochromatica* and the other species is varies in the ranges 98.1- 98.7%, and 97.3 to 97.9%, respectively (Supplementary Table 3). Additionally, *H. cryptochromatica* is the only species of the genus that has Cr-PC630, a pigment also present in the *Chroomonas*

clade. *Hemiselmis amylosa*, the unique freshwater species described for the genus, occupies a basal position within the genus with full PP support (Figure 5).

The close relationship of species *H. pacifica* and *H. virescens* was observed in all phylogenies and presented full PP support (Figures 5). For these species, the sequence identity was 99.3 % for nSSU, and 99.6- 99.7% for nmSSU. The sister relationship of the species *H. andersenii* and *H. rufescens* is not well supported (Figure 5), although these species share the unique pigment Cr-PE555 and high SSU sequence identity. For the ITS2 sequences, the identity of these two species is 81% (Supplementary Table 3).

Based on the concatenated nuclear and nucleomorph SSU, as well as ITS2 sequences, strains BMAK265 and RCC4102 form a well-supported monophyletic clade corresponding to a new species, *H. aquamarina* (see Figures 5 and 6). Sequences from two strains present 99.8%, 99.8% and 100% identity for the nSSU, nmSSU and ITS2 markers, respectively (Supplementary Table 3). Strain UTEX2000, had a high sequence identity of nSSU and nmSSU rDNA to BMAK265 and RCC4102, and formed a fully supported clade with highest (PP) with BMAK265 and RCC4102, indicating that it probably belongs to the species *H. aquamarina*.

The phylogenetic relationship of *H. aquamarina* with other *Hemiselmis* species was not well resolved in our results. It seems to be a sister taxon of *H. tepida*, but the PP was not high (Figure 5). Indeed, *H. tepida* had the highest value of inter-specific similarity with *H. aquamarina*. Our results indicate that *H. aquamarina* and *H. tepida* are closely related to *H. andersenii* and *H. rufescens* in the SSU phylogenies. Additionally, the identity of the sequences among these species is relatively high. In the ITS2 data, the sequences of *H. aquamarina* and *H. andersenii* is share 81.2% identity, the highest value among sequences of different species.

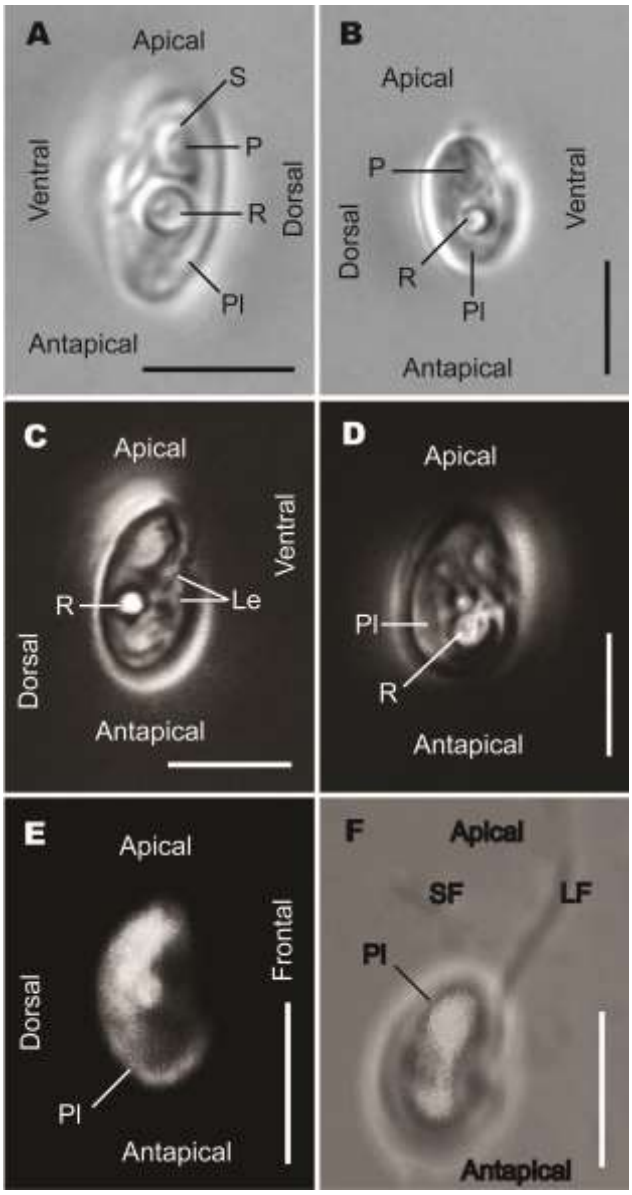

Figure 1: Morphology of Hemiselmis aquamarina, strain BMAK 265. Scale bars 5µm. (A) Lateral view of the cell in bright field in polarized light. Cell bean-shaped, slightly acute in apical/ basal ends and a . (B) Dorso-lateral view in bright field with polarized light. Cell more rounded at the apical/ basal end. (C) Lateral view in phase contrast image evidencing rows of ejectosomes and the shining refractive body. (D) Dorsal view in phase contrast showing the ovate shape of the cell and two refractive bodies. (E) Lateral view obtained with confocal microscopy showing the plastid fluorescence due to its photosynthetic pigments (phycocyanin and chlorophyll) and its parietal boat-shape. (F) Dorso-lateral view of the cell by confocal microscopy showing natural plastid fluorescence and the cell delimitation. Plastid (Pl), large ejectosomes (Le), large flagellum (Lf), pyrenoid (P), refractive body (R), (S) starch, and small flagellum (Sf).

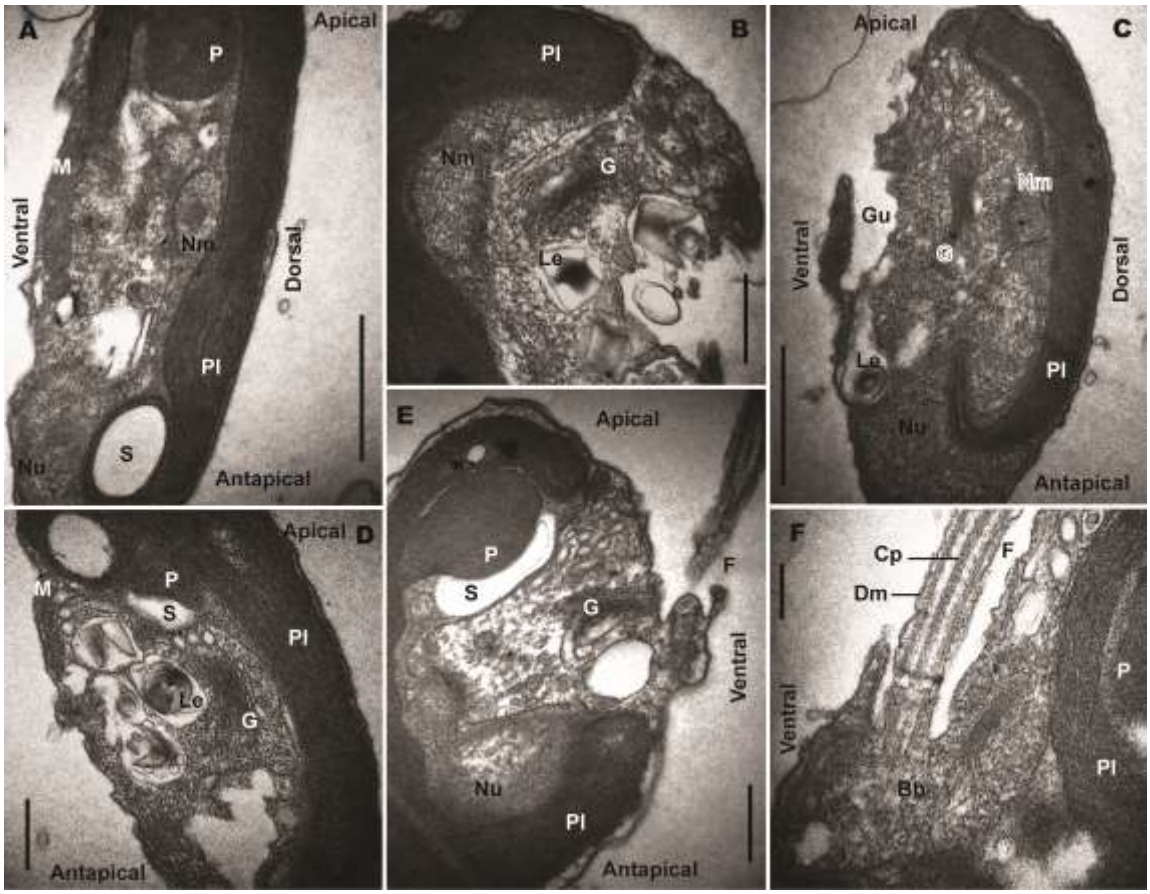

Figure 2: Ultrastructure of *Hemiselmis aquamarina* in transmission electron microscopy. (A) Longitudinal section of the cell showing the parietal single plastid, the nucleomorph position above the apical pyrenoid, the ventral tubular mitochondrion, and the antapical location of a starch grain and nucleus. (B) Middle-apical transversal section showing dorsal position of the plastid, the nucleomorph, the Golgi body and ventral large ejectosome. (C) Lateral longitudinal section of the cell showing the ventral gullet and antapical large ejectosome and nucleus, the dorsal plastid ,and middle position of nucleomorph and Golgi-body. (D) Longitudinal oblique section of the showing the ventral position of large ejectosome and mitoncodria, and dorsal parietal plastid, the apical pyrenoid and its coat of starch. (E) Logitudinal section of a cell showing the ventral flagellar insertion and close Golgi-body, the apical positon of the pyrenoid, the dorsal parietal plastid, and the antapical nucleus. (F) Longitudinal section of the flagellar region, showing the microtubular organization and its insertion into the cell. Scale bars of panels (A) and (C) represents 1µm, (B), (D) and (E) represents 500nm, and (F) is 200nm. Basal body (Bb), plastid (Pl), central pair of microtubules (Cp), double microtubules (Dm), flagellum (F), Golgi body (G), gullet (Gu), large ejectosome (LE), mitochondrion (M), nucleus (Nu), nucleomorph (Nm), starch (S).

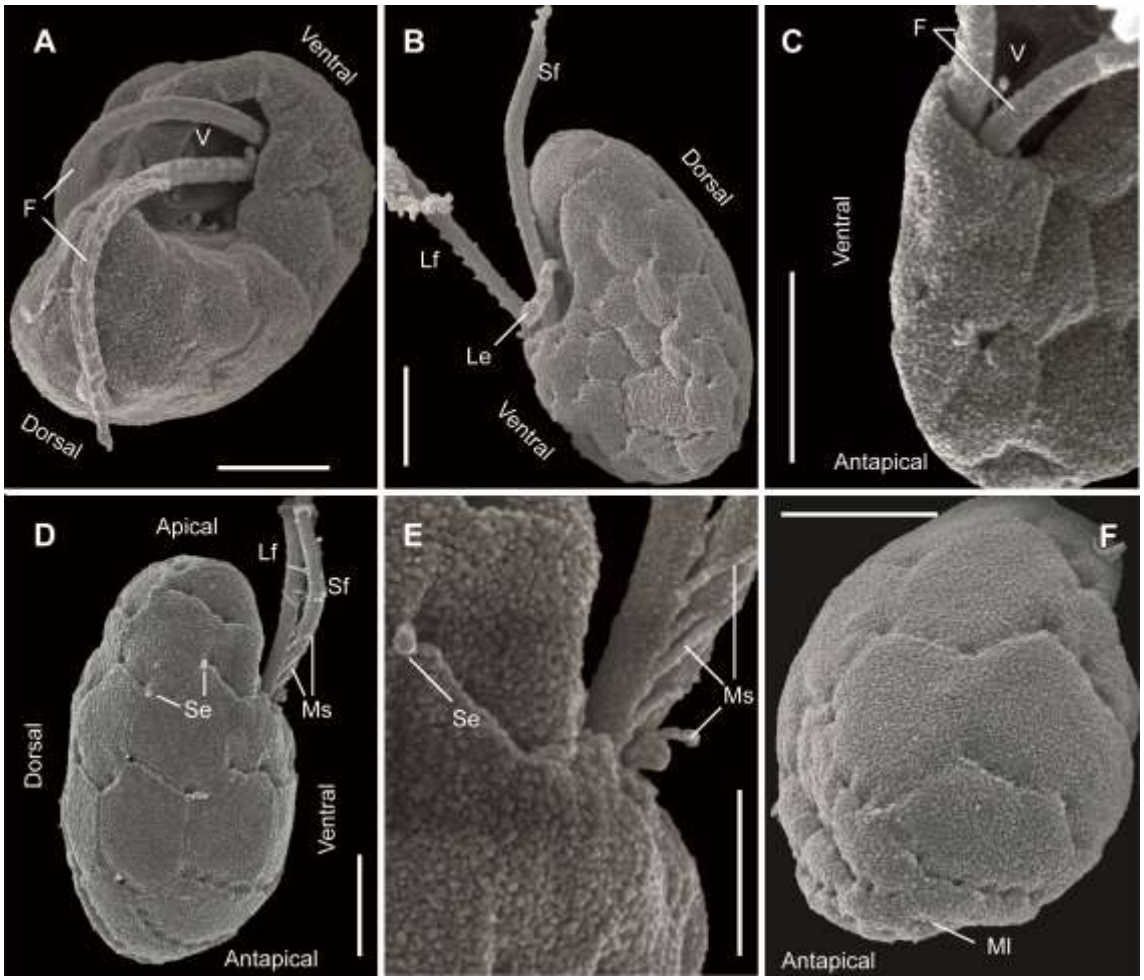

Figure 3: Morphology of Hemiselmis aquamarina strain BMAK 265 by scanning electron microscopy. (A) Apical view showing the vestibular region and the flagella. (B) Lateral view showing the SPC hexagonal plates, the flagella, and large ejectosome discharge. Cell collapsed after critical point drying. (C) Ventro-lateral view of the vestibular region showing differences in SPC plates. (D) Dorso-lateral view of the cell displaying the hexagonal SPC plates, flagellar insertion, and small ejectosomes discharged. (E) Dorso-lateral view close to the flagellar region. (F) Antapical view of the cell showing the SPC mid-ventral line. All scale bars 1µm, except in panel (E) where it is 500nm. Middle line band (Ml), flagellum (F), large ejectosomes (Le), large flagellum (LF), mastigonemes (Ms), small ejectosome (Se), small flagellum (Sf), and vestibulum (V).

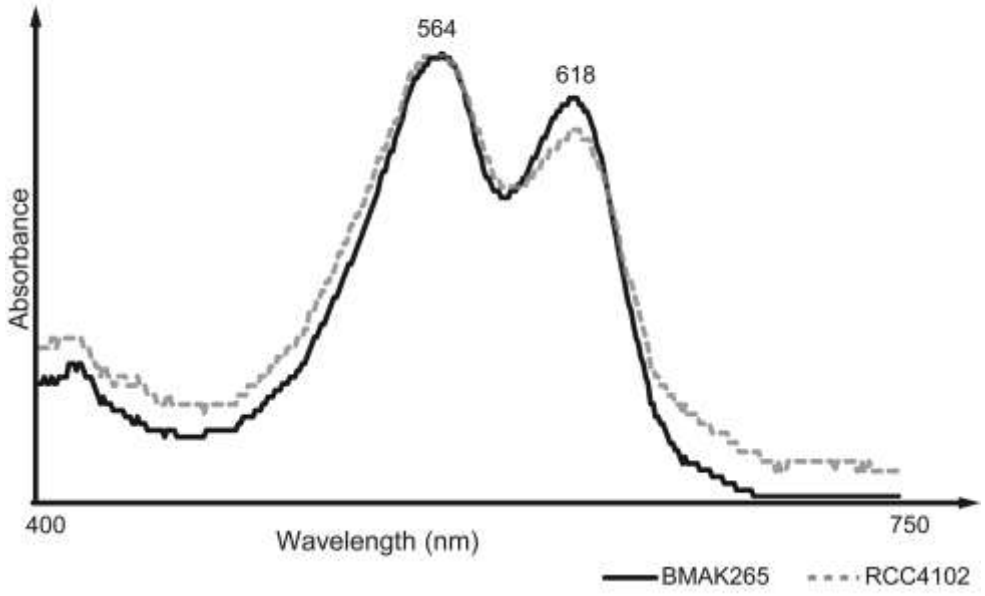

Figure 4: Absorption spectra of *Hemiselmis aquamarina* extract. Strain BMAK 265 had a major peak at 564 nm and a secondary at 616-619 nm. Strain RCC4102 had a major peak at 557-566 nm and a secondary peak at 616-620 nm.

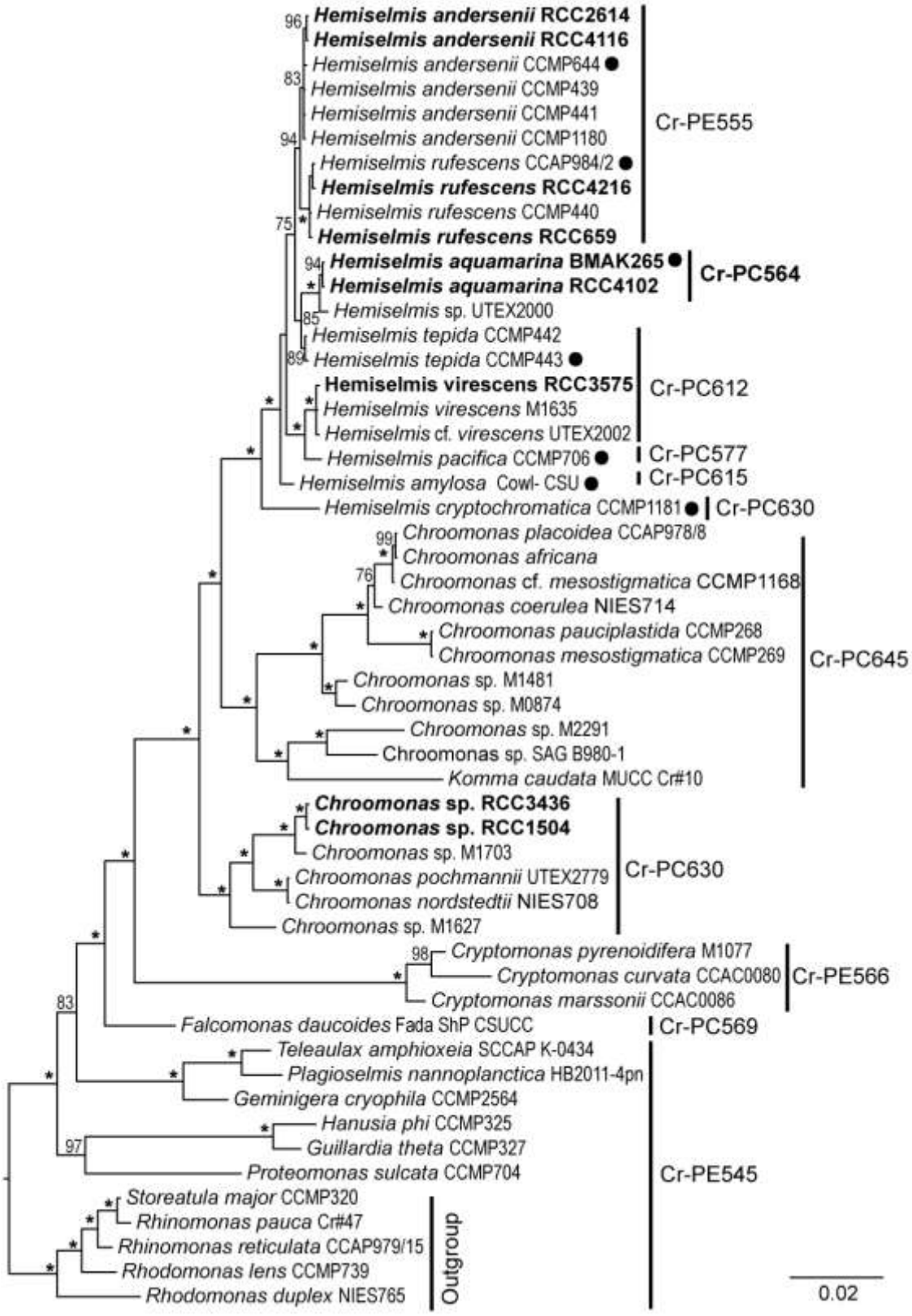

Figure 5: Phylogenetic tree of cryptophytes based on the concatenated nucleus (n) and nucleomorph (nm) SSU rDNA. Phycobiliprotein type is indicated for each clade. Bayesian inference was performed applying separate nucleotide

substitution models of evolution for the nmSSU and nSSU (GTR+G+I and TN+G+I, respectively). The nodes with * have 100% PP. Scale bar indicates the rate of nucleotide substitution per site. The taxa in bold were sequenced in this study. Bold circles represent type cultures of the species according to literature.

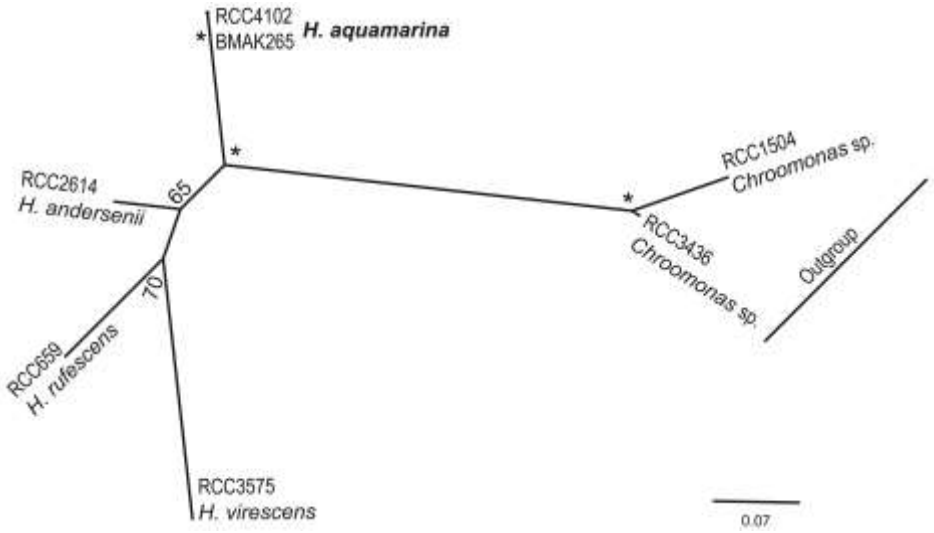

Figure 6: Hemiselmis Bayesian tree based on ITS2 sequences. Phylogenetic inference was performed with, applying K80+I as the model of nucleotide substitution. Nodes supports correspond to PP. Scale bare represents the rate of nucleotide substitution per site. The nodes with * have 100% PP.

DISCUSSION

Many features raised by electron microscopy surveys indeed unveiled important traits (sinapomorphies) for genera and other natural grouping taxa. The family Hemiselmidaceae Butcher (1967), which the diagnosis is the ventral insertion of the flagella almost in the middle of the cell with a gullet across or along the short axis of the cell, was confirmed as a natural grouping by electron microscopy observations (Santore 1982). Prior to this work, only three species of *Hemiselmis* (*H. amylosa, H. rufescens/ brunnescens, and H. virescens*) had been investigated both under TEM and SEM (Clay and Kugrens 1999, Lucas 1970, Santore 1982, Wetherbee et al. 1986). It seems that the apical starch coated pyrenoid, the position of the nucleomorph (between the pyrenoid

and nucleus), the antapical starch grain, and de SPC with hexagonal plates are synapomorphy of *Hemiselmis* (Clay and Kugrens 1999, Lucas 1970, Santore 1982, Wetherbee et al. 1986). Slight differences were detected with these species.

Although morphological characterization is important for species description, it solely is usually not enough for accurate species identification. Some traits selected can mislead identification, as happened on the description of *Hemiselmis brunnescens* Butcher (1967), who based this species diagnosis on the color and size of the strain by light microscopy observations. Posteriorly, some authors investigated the ultrastructure of *Hemiselmis* species and described more morphological variations between *Hemiselmis rufescens* and *H. brunnescens*. The first has a rudimentary gullet and no ejectosomes associated to this region (Lucas 1970), while the second has a small vestibulum (Santore 1982, Wetherbee et al 1986). Posteriorly, the authentic strains of both species (PCC563 and PCC14) were sequenced and sequences of both are identical and have almost identical nucleomorph chromosome structure, suggesting that both strains are *Hemiselmis rufescens* (Lane and Archibald 2008). For propose of future comparison, we summarized the description of the *Hemiselmis* species (Table 1).

The morphology of *H. aquamarina* investigated using different microscopy techniques (light, SEM, and TEM) shows significant similarities to that of other species of the genus, in particular the cell shape, the SPC hexagonal plates, and the distribution of the organelles (Clay and Kugrens 1999, Lane and Archibald 2008, Lucas 1970, Santore 1982, Wetherbee et al. 1986). We found an undescribed feature in the plastid of *H. aquamarina* in confocal microscopy. However, we cannot state that this is a diagnosis feature for this species due to the lack of information for other species.

Accounts of variation of the cells shape and some cellular structures morphology in a clonal culture and even among cultures of the same species are common in literature (Hoef-Emden 2007, Hoef-Emden and Melkonian 2003, Lane and Archibald 2008). Allied to this fact, in some groups of cryptophytes the alternation of morphology in haploid/ diploid stages may exist (Hill and Wetherbee 1986, Hoef-Emden 2007), and was pointed out as the . In this class, examples of cryptic species are not uncommon (Lane and Archibald, 2008, Hoef-Emden.2003, 2007).

Hemiselmis species have phycobiliproteins that are exclusive to the genus, such as Cr-PE555, while others are species-specific, such as Cr-PC612 and Cr-PC577. Cr-PC564 found in the new species *H. aquamarina* is another example of species-specific phycobiliprotein in the genus. The variability of Cr-PC654 absorption spectrum among other cryptophytes phycobiliproteins let us to define it as a new Cr-PC. These small differences can occur due to differences in the bilins composition and/or the linkage site of the bilins chromophores in the phycobiliprotein heterodimer (Glazer and Wedemayer 1995, Overkamp et al. 2014, Wedemayer et al. 1996). As reported between the Cr-PC612 and Cr-PC577, the differences of the absorption spectrum are related to differences of the bilins chromophore composition (Overkamp et al. 2014). Supposedly, Cr-PC564 could to be close to Cr-PC612 due to the close phylogenetic relationship of the species that possess these pigments.

In recent cryptophyte taxonomic studies, the sequences of nSSU, nmSSU, and ITS have been used as molecular markers to help to describe new species (Clay and Kugrens 1999, Hoef-Emden and Melkonian 2003, Hoef-Emden 2007, Lane and Archibald 2008, Majaneva et al. 2014). The ITS rRNA region has been largely used for algal species delimitation (Boenigk et al. 2012, Caisová et al. 2011, Moniz and Kaczmarska 2010,

Stern et al. 2012) and some authors have advocated the use of ITS2 rDNA for biological species delimitation in cryptophytes (Hoef-Emden and Melkonian 2003, Hoef-Emden 2007, 2012). However, prior to our study, no molecular phylogeny of Hemiselmidaceae had been inferred from studies of the ITS region. Our results, using SSU genes and ITS2 rDNA, reconstruct *H. aquamarina* strains (BMAK265 and RCC4102) as a well-supported monophyletic clade.

Our phylogeny and phycobiliprotein analyses suggest the existence of a new species for the strains from Brazil (BMAK265) and Japan (RCC4102) that we named *H. aquamarina* (see Taxonomic Section). Although the sequences of nSSU and nmSSU of these strains were not 100% identical, the ITS2 were, strongly suggesting that they belong to the same species (Hoef-Emden and Melkonian 2003, Hoef-Emden 2007, 2012). Differences in the SSU rRNA sequences of the UTEX2000 strain and the two other strains suggest that it probably does belong to *H. aquamarina* although this would have to be confirmed by ITS2 rRNA sequencing and phycobiliprotein spectrum.

Strain BMAK265 was isolated in Brazilian coastal waters of the State of São Paulo while RCC4102 was isolated off the coast of Japan, and UTEX2000 originated from a river estuary of the US East Coast (Gloucester Point, York River, Virginia). Strains RCC4102, and BMAK265 are being cultured at 20°C suggesting that this species prefers relatively warm water conditions corresponding to those prevailing at the location where the strains were collected, the dominant currents, coming from tropical regions (Brazil and Kuroshio currents). Up to now, most species of *Hemiselmis* were described from North Atlantic Ocean, in particular in the subtropical zone. Only *H. pacifica* was described for North Pacific Ocean. *H. aquamarina* is the first species reported to occur in both the Atlantic and Pacific Oceans.

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ANNEXES

ANNEX 1: PCRs cycling conditions and primers used for PCRs and sequencing reactions

PCRs cycles for nmSSU of initial denaturation at 94°C during 5min. Followed by 35 cycles of: (i) 94°C for 30sec; (ii) 60°C for 1min and (iii) 72°C for 2min. Final extension step at 72°C for 7 minutes. PCRs for nSSU were performed as indicated in (MAJANEVA et al., 2014) ITS2 were performed as described in Hoef-Emden & Melkonian 2003.

Table S.1: Primers used for PCRs and sequencing reactions

Primers for nmSSU PCRs		
	Sequences of oligonucleotides (5'- 3')	
CrNM1F	CAG TAG TCA TAT GCT TGT CTT AAG	(HOEF-EMDEN; MELKONIAN, 2003)
SSUBR	TTG ATC CTT CTG CAG GTT CAC CTA C	(HOEF-EMDEN; MELKONIAN, 2003)
18S 5'	CCA CCT GGT TGA TCC TGC CAG T	(SOGIN, 1990)
18S 3'	GAT CCT TCT GCA GGT TCA CCT ACG GAA	(SOGIN, 1990)
Primers for nSSU PCRs		
18SNF2	TGATGGTCCCTTACTACA	(MAJANEVA et al., 2014)
SSUR	CTTGTTACGACTTCTCCT	(MAJANEVA et al., 2012)
Primers for nSSU and nmSSU sequencing reactions		
18S 5'	CCA CCT GGT TGA TCC TGC CAG T	(SOGIN, 1990)
CrNM1F	CAG TAG TCA TAT GCT TGT CTT AAG	(HOEF-EMDEN; MELKONIAN, 2003)
528F	CGG TAA TTC CAG CTC C	(SOGIN, 1990)
1055F	GGT GGT GCA TGG CCG	(BELLORIN; OLIVEIRA; OLIVEIRA, 2002)
18S 3'	GAT CCT TCT GCA GGT TCA CCT ACG GAA	(SOGIN, 1990)
536R	GAA TTA CCG CGG CTG CTG	(BIRD et al., 1992)
1055R	CGG CCA TGC ACC ACC	(BIRD et al., 1992)
18SNF2	TGATGGTCCCTTACTACA	(MAJANEVA et al., 2014)
SSUBR	TTG ATC CTT CTG CAG GTT CAC CTA C	(HOEF-EMDEN; MELKONIAN, 2003)
SSUR	CTTGTTACGACTTCTCCT	(MAJANEVA et al., 2012)
Primers for ITS PCR and sequencing reaction		
ITS03F-800	CGA TGA AGA ACG YAG CGA	(HOEF-EMDEN; MELKONIAN, 2003)
ITS05R-700	TAC TTG TTC GCT ATC GGT CTC T	(HOEF-EMDEN; MELKONIAN, 2003)

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Supplementary table 2: List of strains, associated sequences and metadata used in this study

Strain	nSSU Accession #	nmSSU Accession #	ITS 2 Acesscion #	Genbank name	Biliprotein	Maintenance temperature	Country	Habitat	Locality
CCMP1180	AM901353	AM901022		*Hemiselmis andersenii**	PE 545/555[(2)]	20°C	MEX	marine	Gulf of Mexico
CCMP441	AM901350	AM901019		*Hemiselmis andersenii**	PE 555[(2)]	20°C	MEX	marine	Gulf of Mexico, Gulf Stream
CCMP644	AM901351	DQ519365		*Hemiselmis andersenii**	PE 555[(2)]	20°C	USA	marine	Gulf of Mexico, Gulf Stream
CCMP439	AJ007283	AJ420690		*Hemiselmis andersenii**	PE 555[(2)]	24°C	USA	marine	Gulf of Mexico, Cape San Blas
RCC2614	**MF179473**	**MF179479**	**MF574711**	***Hemiselmis andersenii***		**15°C**	**GBR**	**marine**	**North Atlantic Ocean, North Sea**
UTEX2000[(5)]	AM901367	AM901034		*Hemiselmis* sp.			USA	marine	North Atlantic Ocean, Virginia, York River
RCC4099		**MF179482**		***Hemiselmis* sp.**	**PC 551-553**	**20°C**	**JPN**	**Marine**	**North Pacific Ocean**
RCC4102	**MF179476**	**MF179483**	**MF574713**	***Hemiselmis aquamarina***	**PC 564**	**20°C**	**JPN**	**marine**	**North Pacific Ocean**
BMAK265	**KY711345**	**KY711347**	**KY711346**	***Hemiselmis aquamarina***	**PC 564**	**20°C**	**BRA**	**marine**	**South Atlantic Ocean, Ubatuba, SP**
CCMP1181	AM901354	AM901023		*Hemiselmis cryptochromatica**	PC 630[(1)]	14°C	USA	marine	North Atlantic Ocean, Boothbay Harbor, Maine
CCMP706	AM901352	AM901020		*Hemiselmis pacifica**	PC 576[(1)]	14°C	USA	marine	North Atlantic Ocean, Washington, San Juan Island
RCC659	**MF179475**	**MF179481**		***Hemiselmis rufescens***	**PE 554/555**	**15°C**	**NOR**	**Marine**	**North Atlantic Ocean, North Sea**
RCC4216	**MF179474**	**MF179480**	**MF574712**	***Hemiselmis rufescens***	**PE 554/555**	**15°C**	**FRA**	**marine**	**North Atlantic Ocean, Roscoff, English Channel**
CCAP 984/2	AJ007282	AM901016		*Hemiselmis rufescens**	PE 554[(2)]		GBR	marine	English Channel

Supplementary table 2: List of strains, associated sequences and metadata used in this study

CCMP440	AM901349	AM901018		*Hemiselmis rufescens**	PE 555[3]	20°C	USA	marine	North Atlantic Ocean, Maine, West Boothbay Harbor
CCMP443	AJ007284	AJ420691		*Hemiselmis tepida**	PC 612[3]	20°C	USA	marine	North Atlantic Ocean, Galveston Channel, Texas
CCMP442	HM126533	EF594307		*Hemiselmis tepida**	PC 612[3]	20°C	USA	marine	North Atlantic Ocean, Galveston Channel, Texas
RCC3575, M1635, CACC1635	**MF179477**	**MF179484**	**MF574715**	***Hemiselmis virescens***	**PC 612[3]**	**17°C**	**SWE**	**Marine**	
M1635	AM901362	AM901030		*Hemiselmis virescens**	PC 612[3]		SWE	marine	Kristineborg, Baltic Sea
UTEX2002	AM901368	AM901035		*Hemiselmis virescens**	PC 614[3]		USA	marine	North Atlantic Ocean, Virginia, York River
	HG328376	HG328384		*Chroomonas africana*	PC645[3]		ZAF	marine	South Atlantic Ocean, Cape Province, Yzerfontein region
NIES-714	HG328381	HG328389		*Chroomonas coerulea*			JPN	freshwater	Asia, Honshu, Nagano, Sugadaira
CCMP1168	AF508268	AM901021		*Chroomonas mesostigmatica*	PC 645[3]		Unknown	marine	
CCMP269	AM901347	AM901017		*Chroomonas mesostigmatica*	PC 645[3]		USA	Marine	North Atlantic Ocean, Maryland, Assateague Island
NIES-708	HG328378	HG328386		*Chroomonas nordstedtii*			JPN	freshwater	Asia, Hokkaido, Sapporo, Hokkaido University
CCMP268	AM901346	DQ519363		*Chroomonas pauciplastida*	PC645[3]		USA	marine	North Atlantic, Nantucket Sound

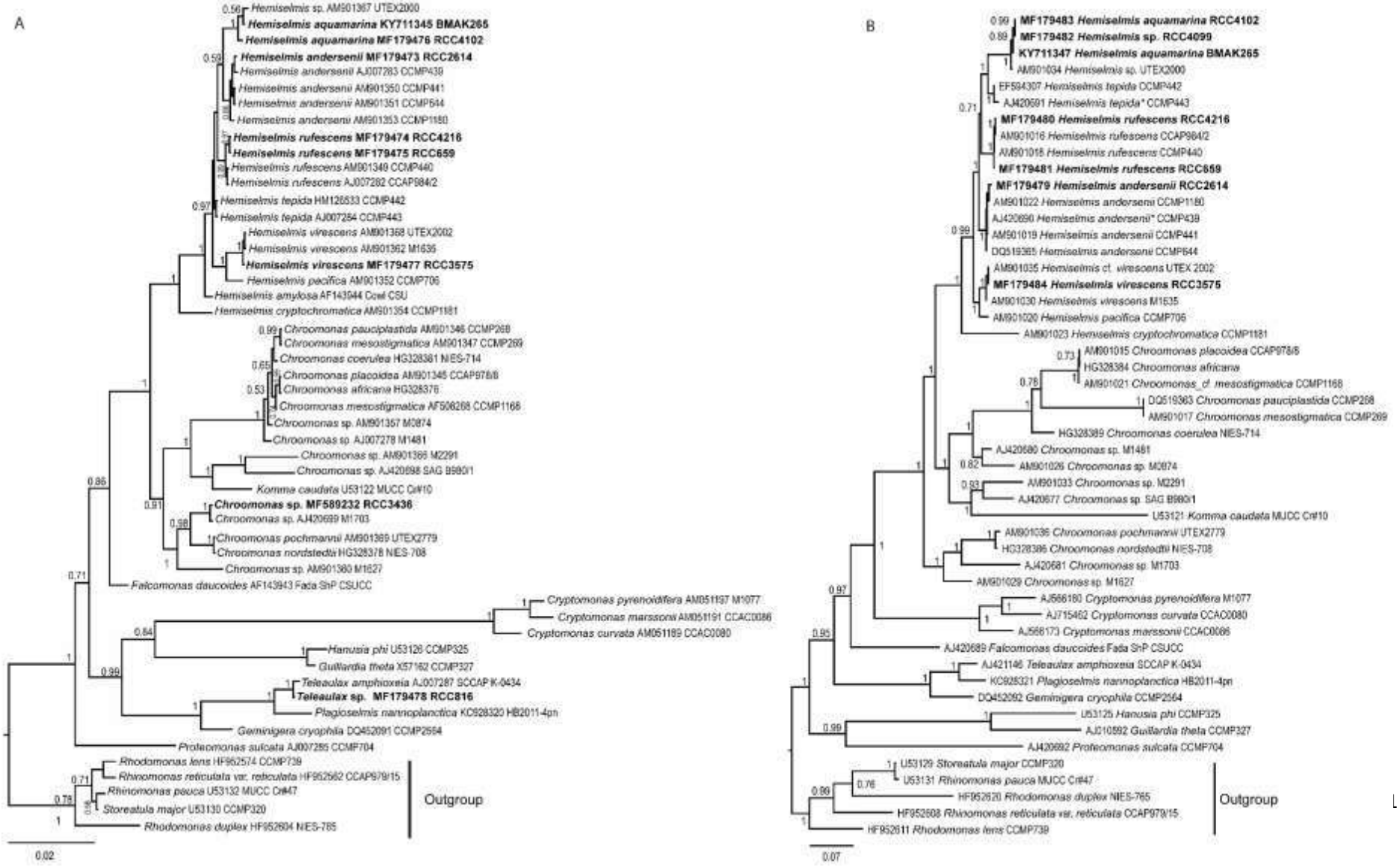
Supplementary table 2: List of strains, associated sequences and metadata used in this study

CCAP978/8	AM901345	AM901015		*Chroomonas placoidea*	PC645[3]		GBR	marine	North Atlantic, Irish Sea, Yorkshire
UTEX2779	AM901369	AM901036		*Chroomonas pochmannii*	PC 630[3]		USA	freshwater	North America, Colorado, Wellington Reservoir
RCC3436	**MF589232**		**MF574714**	***Chroomonas* sp.**		**17°C**		**marine**	**Unknown**
M0874	AM901357	AM901026		*Chroomonas* sp.	PC 630[3]		DEU	freshwater	Europe, Griether Ort
SAG B980-1	AJ420698	AJ420677		*Chroomonas* sp.	PC 645[3]		GBR		Europe, Wales
M2291	AM901366	AM901033		*Chroomonas* sp.	PC 645[3]		DEU	freshwater	Europe, Cologne, Wahner Heide
M1481	AJ007278	AJ420680		*Chroomonas* sp.	PC 645[3]		DEU	freshwater Europe	Europe, Spessart, Biebergemuend
M1627	AM901360	AM901029		*Chroomonas* sp.	PC 630[3]		DNK	marine	Baltic Sea, Sjaelland, Bellevue Strandbad
M1703	AJ420699	AJ420681		*Chroomonas* sp.	PC 630[3]		DNK		Jutland, Hjerting
CCAC0080	AM051189	AJ715462		*Cryptomonas curvata*			DEU	freshwater	Europe, Muenster
CCAC0086	AM051191	AJ566173		*Cryptomonas marssonii*			DEU	freshwater	Muenster
M1077	AM051197	AJ566180		*Cryptomonas pyrenoidifera*			DEU	freshwater	Europe, Cologne
Fada ShP CSUCC	AF143943	AJ420689		*Falcomonas daucoides*	PC 569[3]		USA	marine	North Pacific Ocean, Shannon Point, Washington
CCMP 2564	DQ452091	DQ452092		*Geminigera cryophila*			ATA	marine	Southern Ocean, McMurdo Sound
CCMP 327	X57162	AJ010592		*Guillardia theta*	PE 545[3]		USA	marine	North Atlantic, Long Island Sound
CCMP 325	U53126	U53125		*Hanusia phi*			USA	marine	North Atlantic, Milford, Connecticut USA,

Supplementary table 2: List of strains, associated sequences and metadata used in this study

								Long Island Sound
MUCC Cr#10	U53122	U53121	*Komma caudata*	PC645[3]		AUS	freshwater	Oceania, Wimmera river
Cowl- CSU	AF143944	unavailable	*Hemiselmis amylosa*[4]	PC 615[4]		USA	freshwater	North America, Cowdry Lake/ Colorado
HB2011-4pn	KC928320	KC928321	*Plagioselmis nannoplanctica*			Unknown		Unknown
CCMP 704	AJ007285	AJ420692	*Proteomonas sulcata*			Unknown		Unknown
MUCC Cr#47	U53132	U53131	*Rhinomonas pauca*	PE546[3]		AUS	marine	Bass Strait, Hobsons Bay
CCAP 979/15	HF952562	HF952608	*Rhinomonas reticulata* var. *reticulata*			GBR	marine	English Channel, Plymouth Sound
NIES-765	HF952604	HF952620	*Rhodomonas duplex*			JPN	marine	East China Sea, Okinawa, Yaga
CCMP 739	HF952574	HF952611	*Rhodomonas lens*			none	marine	Gulf of Mexico
CCMP 320	U53130	U53129	*Storeatula major*			Unknown		Unknown
SCCAP K-0434	AJ007287	AJ421146	*Teleaulax amphioxeia*			Unknown		Unknown
RCC816	**MF179478**		***Teleaulax* sp.**		**15°C**	**FRA**	**marine**	**North Atlantic Ocean, Roscoff, English Channel**

Supplementary file 3: Molecular phylogeny trees of *Hemiselmis* estimated by Bayesian analysis. (A) Phylogeny inference of nSSU gene using HKY+G+I as nucleotide substitution model. The dataset comprised 52 taxa and 1608 bp. (B) Inference of phylogeny based on sequences of the nmSSU gene with 50 taxa, and 1986bp, using the nucleotide evolutionary model GTR+G+I.



Supplementary file 4: Identity matrix (black) and sequence different counts matrix (gray) of SSU rDNA for *Hemiselmis* and *Chroomonas* sequences. (A) Sequences of nSSU rDNA gene. (B) Sequences of nmSSU rDNA gene (C) Sequences of ITS2 and partial LSU rDNA. Bold names indicates the authentic strains.

(A)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**
1. BMAK265 *H. aquamarina*		0	2	6	7	5	5	6	8	8	10	14	13	14	10	19	32	32
2. RCC4102 *H. aquamarina*	1,000		2	6	7	5	5	6	8	8	10	14	13	14	10	19	32	32
3. UTEX2000 *Hemiselmis* sp.	0.998	0.998		6	7	7	7	6	10	10	10	14	15	14	10	20	34	34
4. CCMP442 *H. tepida*	0.995	0.995	0.995		1	3	3	2	4	4	4	10	11	10	4	18	28	28
5. CCMP443 *H virescens*	0.995	0.995	0.995	0.999		4	4	3	5	5	5	11	12	11	5	19	29	29
6. RCC2614 *H. andersenii*	0.996	0.996	0.995	0.997	0.997		0	1	3	3	5	13	12	13	7	20	29	29
7. RCC4116 *H. andersenii*	0.996	0.996	0.995	0.997	0.997	1,000		1	3	3	5	13	12	13	7	20	29	29
8. CCMP644 *H. andersenii*	0.995	0.995	0.995	0.998	0.997	0.999	0.999		4	4	4	12	13	12	6	20	30	30
9. RCC4216 *H. rufescens*	0.994	0.994	0.993	0.997	0.996	0.997	0.997	0.997		0	2	14	13	14	8	19	27	27
10. RCC659 *H. rufescens*	0.994	0.994	0.993	0.997	0.996	0.997	0.997	0.997	1,000		2	14	13	14	8	19	27	27
11. CCAP984/2 *H. rufescens*	0.993	0.993	0.993	0.997	0.996	0.996	0.996	0.997	0.998	0.998		14	15	14	8	20	29	29
12. CCMP706 *H. pacifica*	0.99	0.99	0.99	0.993	0.992	0.991	0.991	0.991	0.99	0.99	0.99		11	10	14	28	32	32
13. RCC3575 *H. virescens*	0.991	0.991	0.989	0.992	0.991	0.991	0.991	0.991	0.991	0.991	0.989	0.992		1	15	26	35	35
14. M1635 *H. virescens*	0.99	0.99	0.99	0.993	0.992	0.991	0.991	0.991	0.99	0.99	0.99	0.993	0.999		14	26	36	36
15. *H. amylosa*	0.993	0.993	0.993	0.997	0.996	0.995	0.995	0.995	0.994	0.994	0.994	0.99	0.989	0.99		18	30	30
16. CCMP1181 *H. cryptochromatica*	0.987	0.987	0.986	0.987	0.987	0.986	0.986	0.986	0.987	0.987	0.986	0.981	0.982	0.982	0.987		32	32
17. RCC1504 *Chroomonas* sp.	0.978	0.978	0.977	0.981	0.98	0.98	0.98	0.979	0.981	0.981	0.98	0.978	0.976	0.975	0.979	0.978		0
18. RCC3436 *Chroomonas* sp.	0.978	0.978	0.977	0.981	0.98	0.98	0.98	0.979	0.981	0.981	0.98	0.978	0.976	0.975	0.979	0.978	1,000	

Supplementary file 4: Identity matrix (black) and sequence different counts matrix (gray) of SSU rDNA for *Hemiselmis* and *Chroomonas* sequences. (A) Sequences of nSSU rDNA gene. (B) Sequences of nmSSU rDNA gene (C) Sequences of ITS2 and partial LSU rDNA. Bold names indicates the authentic strains.

(B)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
1. BMAK265 *H. aquamarina*		0	3	7	8	10	12	13	12	11	12	21	19	19	32
2. RCC4102 *H. aquamarina*	1,000		3	7	8	10	12	13	12	11	12	21	19	19	32
3. UTEX2000 *Hemiselmis* sp.	0.997	0.997		10	11	13	15	16	15	14	15	24	22	22	34
4. CCMP442 *H. tepida*	0.994	0.994	0.992		1	6	8	9	8	7	8	18	16	16	29
5. CCMP443 *H virescens*	0.994	0.994	0.991	0.999		7	9	10	9	8	9	19	17	17	30
6. RCC2614 *H. andersenii*	0.992	0.992	0.99	0.995	0.994		2	3	4	3	4	15	13	13	27
7. RCC4116 *H. andersenii*	0.991	0.991	0.988	0.994	0.993	0.998		5	6	5	6	17	15	15	29
8. CCMP644 *H. andersenii*	0.99	0.99	0.988	0.993	0.992	0.997	0.996		7	6	7	18	16	16	30
9. RCC4216 *H. rufescens*	0.991	0.991	0.988	0.994	0.993	0.997	0.995	0.994		1	0	19	17	17	31
10. RCC659 *H. rufescens*	0.991	0.991	0.989	0.994	0.994	0.997	0.996	0.995	0.999		1	18	16	16	30
11. CCAP984/2 *H. rufescens*	0.991	0.991	0.988	0.994	0.993	0.997	0.995	0.994	1,000	0.999		19	17	17	31
12. CCMP706 *H. pacifica*	0.984	0.984	0.982	0.986	0.985	0.988	0.987	0.986	0.985	0.986	0.985		4	4	31
13. RCC3575 *H. virescens*	0.985	0.985	0.983	0.988	0.987	0.99	0.988	0.988	0.987	0.988	0.987	0.997		0	30
14. M1635 *H. virescens*	0.985	0.985	0.983	0.988	0.987	0.99	0.988	0.988	0.987	0.988	0.987	0.997	1,000		30
15. CCMP1181 *H. cryptochromatica*	0.976	0.976	0.974	0.978	0.977	0.979	0.978	0.977	0.976	0.977	0.976	0.976	0.977	0.977	

(C)	**1**	**2**	**3**	**4**	**5**	**6**	**7**
1. BMAK265 *H.aquamarina*		0	81	93	133	122	139
2. RCC4102 *H. aquamarina*	1		81	93	133	122	139
3. RCC2614 *H. andersenii*	0.812	0.812		82	122	123	139
4. RCC659 *H. rufescens*	0.785	0.785	0.81		122	132	148
5. RCC3575 *H. virescens*	0.692	0.692	0.717	0.717		146	167
6. RCC3436 *Chroomonas* sp.	0.718	0.718	0.715	0.695	0.662		35
7. RCC1504 *Chroomonas* sp.	0.678	0.678	0.678	0.658	0.614	0.918	

3 INVESTIGATION OF *RHODOMONAS* (PYRENOMONADALES, CRYPTOPHYTA) STRAINS FROM BRAZILIAN COAST UNVEIL THREE NEW SPECIES

Karoline Magalhães, Daniel Vaulot, Mariana Cabral de Oliveira

Abstract: *Rhodomonas baltica* Karsten was by from the Baltic Sea as swarming red flagellates. Since its description, many other "red flagellates" were assigned to the genus. However, due to a misinterpretation of the cell's pyrenoid as nucleus and the assignment of the color as a character, many authors rejected the genus, even after its reappraisal. Accordingly, the taxonomy of Pyrenomonadaceae is very controversial. Aiming to study the biodiversity of this group, 43 strains had their genes of nuclear SSU and ITS2, and nucleomorph SSU rDNA sequenced. Furthermore, six strains isolated from coastal waters of Brazil had their morphology investigated using different sources of microscopes (light, confocal, SEM and TEM). Our results point out the existence of at least five main clades in the Pyrenomonadaceae, and the strains from Brazil form three new monophyletic subclades, supported in concatenated and isolated gene phylogenies. Combining the molecular and morphological data, we propose three new species of *Rhodomonas* from Brazil. Additionally, our results indicate that the genera *Rhinomonas, Rhodomonas* and *Storeatula* are not monophyletic.

Key words: cryptomonads, molecular phylogeny, new species, phytoplankton *Rhodomonas ,Rhinomonas, Storeatula* ultrastructure.

INTRODUCTION

Cryptophytes are unicellular biflagellate organisms that inhabit aquatic ecosystems worldwide. The group has a complex chloroplast acquired by secondary endosymbiosis with a red algae, and the plastids still harbor endosymbiont nucleus, the nucleomorph (DOUGLAS; PENNY, 1999; DOUGLAS et al., 2001). Cryptomonads cells also possess unique phycobiliproteins types located inside the thylakoids, differing from red algae and cyanobacteria that harbor their phycobiliproteins in phycobilisomes associated outside the thylakoid membrane (GANTT; EDWARDS; PROVASOLI, 1971; HILL; ROWAN, 1989). Although cryptophytes are strongly supported as a monophyletic group, their phylogenetic relationship with other eukaryotes is still controversial (PATRON; INAGAKI; KEELING, 2007; OKAMOTO et al., 2009; BURKI et al., 2012, 2016; STILLER et al., 2014; KIM et al., 2017, 2018).

Cells are asymmetrical due to subapical insertion of the flagella and the furrow-gullet system. They are one of the most distinctive groups of phytoflagellates and can usually be recognized by their gyrating swimming movements (SANTORE, 1984). However, taxonomy and systematics of cryptophytes from orders to species is a challenge due to the few morphological traits that are visible by light microscopy (HOEF-EMDEN, 2007; LANE; ARCHIBALD, 2008; MAJANEVA et al., 2014).

Karsten erected *Rhodomonas* in 1898 for a species of swarming red biflagellate organisms that bloomed in an enriched sample from the Baltic Sea. The type species, *Rhodomonas baltica* Karsten was described as possessing one red chromophore, two flagella that emerge from an anterior depression, from which a curved gullet-like structure continues downward the cell, and a large nucleus situated in the middle of the cell (HILL; WETHERBEE, 1989). Pascher in 1913 was one of the first to attempt to classify cryptophytes and he elected the color as a trait to classify species and genera. Accordingly, the genera *Cryptochrysis* Pascher, *Rhodomonas* and *Chroomonas* Hansgirg were merely distinguished based on their color (PRINGSHEIM, 1944). On the other hand, Butcher (1967) stated that color is so variable in cryptophytes that it must be ignored as a taxonomic trait for the genera identification, while the nature and structure of the furrow/ gullet system is the most important feature for primary classification. The genus *Rhodomonas* was considered superfluous because according to the author it

differs from *Cryptomonas* Ehrenberg only in color (BUTCHER, 1967). Owing to the Karsten's misinterpretation of the pyrenoid as the cell's nucleus in *Rhodomonas baltica* description, Butcher proposed a new name, *Cryptomonas pseudobaltica* Butcher, for the type species of the invalid *Rhodomonas*. However, just three species of *Rhodomonas* where transferred to *Cryptomonas,* whereas other species where relocated in *Hillea* Schiller and *Chroomonas.* Moreover, Butcher emended the *Chroomonas* genus diagnosis and created two new subgenera based on color differences, *Cryptochrysis* and *Chroomonas*, if cells are blue/ green or yellow-brown/ red, respectively (BUTCHER, 1967).

Nevertheless, a study comparing the ultrastructure of two subgenera of *Chroomonas* sensu Butcher concluded that the differences between then justified a generic distinction (SANTORE, 1982b). Subsequently, the Santore erected a new genus *Pyrenomonas* Santore. The type species, *Pyrenomonas salina* (Wislouch) Santore, was newly combined from the subgenus *Cryptochrysis* sensu Butcher (SANTORE, 1984). Moreover, he suggested that all taxa of the subgenus *Cryptochrysis* sensu Butcher are indeed *Pyrenomonas.* Furthermore, Santore elected differences that distinguish *Pyrenomonas* from *Chroomonas sensu stricto*, such as the plastidial complex organization, the cell size and shape, and the morphology of the periplast plates (SANTORE, 1982b, 1984).

The descriptions of the genera *Rhinomonas* Hill &Wetherbee and *Storeatula* Hill were reexaminations of species descriptions of *Chroomonas* and *Cryptomonas* sensu Butcher. Four species of *Cryptochrysis* Butcher were remarked as diminutive cell size (less than 8µm) in the taxonomic key to species identification of *Chroomonas*. Accordingly, *Rhinomonas pauca* Hill & Wetherbee, the type species was described previously as *Chroomonas nana* Butcher (HILL; WETHERBEE, 1988). Moreover, *Storeatula major* Hill was previous reported as *Cryptomonas major* Butcher (HILL, 1991). Like *Pyrenomonas*, both genera have Cr- phycoerythrin Cr-PC545 as major accessory pigment and the nucleomorph is located inside the pyrenoid (HILL; WETHERBEE, 1988; HILL, 1991).

Due the controversy regarding the features that are diagnostic to *Rhodomonas*, Hill & Wetherbee (1989) emended the diagnosis of the genus, using a strain-isolated from the

Baltic Sea, adding ultrastructural morphology descriptions and the type of phycobiliprotein. Moreover, the genus *Pyrenomonas* was considered superfluous and synonymous of *Rhodomonas* (HILL; WETHERBEE, 1989). However, Novarino (1991) disagreed with the use of *Rhodomonas* since it is impossible to recover the truly form that corresponds to the original description. Accordingly, the name *Pyrenomonas* should be used instead *Rhodomonas* (NOVARINO, 1991). Subsequently, proposed system of cryptophytes classification, based on ultrastructural features, regarded the genera *Storeatula, Pyrenomonas/ Rhodomonas* and *Rhinomonas* together in the family Pyrenomonadaceae Novarino & Lucas. The group was based on traits such as location of the nucleomorph in the pyrenoid and the Cr- PE545 (NOVARINO; LUCAS, 1993; CLAY; KUGRENS; LEE, 1999).

Molecular phylogenies surveys using SSU rDNA genes of nucleus and nucleomorph pointed out a monophyletic relationship of *Rhodomonas/ Pyrenomonas, Rhinomonas,* and *Storeatula*, supporting the Pyrenomonadaceae family (CAVALIER-SMITH et al., 1996; DEANE et al., 2002; HOEF-EMDEN; MARIN; MELKONIAN, 2002). However, while *Rhinomonas* clade seems to be monophyletic, *Rhodomonas* is paraphyletic (HOEF-EMDEN; MARIN; MELKONIAN, 2002; MAJANEVA et al., 2014).

Molecular phylogenies studies also indicated the presence of dimorphism in Pyrenomonadaceae species (HOEF-EMDEN; MARIN; MELKONIAN, 2002; MAJANEVA et al., 2014). The mixture of *Storeatula* into the *Rhinomonas* and *Rhodomonas* clades may be explained by heteromorphic life history, as in *Proteomonas sulcata*, which the periplast plates indicates the haploid stage, whereas the sheet-like periplast represents the diploid phase (MAJANEVA et al., 2014).

Cryptophytes taxonomy as well as its biodiversity knowledge is limited and confusing. Due to the scarcity of morphological traits for the taxa diagnosis, most cryptophytes species descriptions represents complex of cryptic species (HOEF-EMDEN; MELKONIAN, 2003; LANE; ARCHIBALD, 2008). In Brazil, the study of cryptophytes biodiversity is very incipient, and all surveys were performed solely with light microscopy observations (MENEZES; NOVARINO, 2003; TUCCI et al., 2011), which is insufficient for reliable species identification. Aiming to enhance the knowledge of cryptophytes biodiversity, mainly in Brazilian marine waters, 14 strains

of red cryptophytes from distinct marine environments of Brazil were newly isolated or obtained from culture collections, and investigated under different microscopy sources and distinct molecular markers for phylogenetic inferences. Other 29 strains from a wide variety of marine environments were obtained from Roscoff Culture Collection and added to enhance the robustness of the molecular analysis.

MATERIAL AND METHODS

Sampling, isolation, and maintenance of cultures

Ten strains were isolated from the estuarine region of Cananéia, São Paulo state, Brazil (Lat 25°2' S, Long 47°55' W), in April of 2011 (TABLE 1). Superficial water sample was collected between Cardoso and Comprida Islands. Enrichment cultures were established using Guillard *f/2* medium (GUILLARD; RYTHER, 1962) without silicate solution in a proportion of 3 parts of sample for one of medium. These cultures were kept under 20°C temperature, 32 salinity, photoperiod of 12:12 L:D cycle, and 50 µmol $m^{-2}.s^{-1}$ of light intensity for 15 days. Afterwards, enrichment cultures with cryptomonads were used for isolating the cells by the micropipetting and washing technique. A drop of enrichment culture was put is a glass slide, where cells were selected manually using a micropipette, and successively washed in a drop of sterile medium until only one cell remained. Each isolated cell was put in a well with 1.5 mL of sterile medium of an acrylic 24 well plate, which was posteriorly sealed with Parafilm and kept under conditions cited above for 20 days. Colorful wells were transferred to test tubes containing new media, and then routinely transferred to new media monthly.

Other strain were isolated in April 2014 from water collected in surfing zone of Cotovelo beach, at Parnamirim, Rio Grande do Norte state, Brazil (Lat. 5°57'S, Long. 35°7'W). An amount of water sample was used for serial dilution technique, in four steps of dilution (1/10, 1/100/, 1/1000, 1/10000) in sterile *f/6* media, and each step of dilution contained five replicates. After 15 days, two tubes of the third step dilution (1/1000) contained cryptomonads cells, and so they were purified by micropipetting and washing technique, as described above. After a month, successful cells purifications

were moved to test tubes, and then monthly transferred to new media. Incubation conditions are the same as described above. All isolated strains are deposited in the Culture of Collection Aydar & Kutner (BMA&K) from Oceanographic Institute of University of São Paulo and in Roscoff Culture Collection from the Station Biologique de Roscoff, except one strain (Can12) that died before strains incorporation to the collections. Other strains were received from BMA&K (BMAK39), RCC, and the Elizabeth Aydar Collection from Fluminense Federal University (RJ02, RJ03) (TABLE 1).

Phycobiliprotein analysis

The extractions of phycobiliprotein pigments were performed in accordance to Hill & Rowan (1989). Cultures flasks of 30 mL were harvested by centrifugation after 15-20 days of incubation at the conditions described above. The cells pellets were frozen in liquid nitrogen and kept in -80°C until the analysis. Afterwards, the cells pellets were melted and frozen twice for cellular lysis, and then 1.5 ml of phosphate buffer (pH6.8) was added. Cellular debris were removed with high speed centrifugation (16,000g) for 30 min, and 400 µL of the supernatant with the pigments extracted of each strain was put in a well of 96 well plates and was read in an Epoch 2 microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA).

TABLE 1: List of strains, associated sequences and metadata in this study; seq: sequences obtained in this work (GenBank number will be added in the publication); nd: non determined. Species names in bold show strains sequenced in this study.(*) Represents authentic strain,, which the type was obtained according to information on their respective culture collections.

	Strain name	Other name	nSSU	ITS	nmSSU	Habitat	Country	Location
***Rhodomonas* sp. 1**	RCC5614	BMAK243	seq	nd		marine	BRA	Cananéia, São Paulo, South Atlantic
****Rhodomonas* sp. 1**	RCC5615	BMAK247	seq	seq		marine	BRA	Cananéia, São Paulo, South Atlantic
***Rhodomonas* sp. 1**	RCC5616	BMAK248	seq	seq		marine	BRA	Cananéia, São Paulo, South Atlantic
***Rhodomonas* sp. 1**	RCC5617	BMAK249	seq	seq		marine	BRA	Cananéia, São Paulo, South Atlantic
***Rhodomonas* sp. 1**	RCC5618	BMAK250	seq	seq		marine	BRA	Cananéia, São Paulo, South Atlantic
***Rhodomonas* sp. 1**	RCC5619	BMAK251	seq	nd		marine	BRA	Cananéia, São Paulo, South Atlantic
***Rhodomonas* sp. 1**	RCC5621	BMAK253	seq	seq		marine	BRA	Cananéia, São Paulo, South Atlantic
***Rhodomonas* sp. 2**	Can12		seq	seq	seq	marine	BRA	Cananéia, São Paulo, South Atlantic
****Rhodomonas* sp. 2**	RCC5613	BMAK254	seq	seq		marine	BRA	Cananéia, São Paulo, South Atlantic
****Rhodomonas* sp. 3**	RCC5621	BMAK276	seq	seq		marine	BRA	Cotovelo Beach, Parnamirim, Rio Grande do Norte
Rhodomonas sp.	RJ02		seq	seq	seq	marine	BRA	Unknown

Rhodomonas sp.	RJ03		seq	seq	seq	marine	BRA	Unknown
Rhodomonas marataiama	BMAK39		seq	seq	seq	marine	BRA	Cananéia, São Paulo, South Atlantic
Rhodomonas sp.	RCC1978		seq, KT861341	seq		marine	FRA	Plage de Santec, English Channel
Rhodomonas sp.	RCC439		seq	seq				
Rhodomonas sp.	RCC662		seq, KT861326	seq		marine	NOR	North Sea, North Atlantic
Rhodomonas sp.	RCC821		seq, KT860556	seq		marine	FRA	English Channel
Storeatula?	RCC906		seq	seq		marine	FRA	English Channel, Atlantic Ocean
Rhodomonas sp.	RCC2603		seq, KT861331	seq		marine	GBR	North Sea, North Atlantic
Rhodomonas sp.	RCC2626		seq, KT861330	seq		marine	GBR	North Sea, North Atlantic
Rhodomonas sp.	RCC2944		seq, KT861329	seq		marine	GBR	North Sea, North Atlantic
Rhodomonas salina	RCC20	CCMP322, Mel-044	seq	seq		marine	AUS	Point Hicks, Victoria, Tasman Sea, Pacific Ocean
Rhodomonas baltica	RCC350	Poulet	seq	seq	DQ228118	marine	Unknown	Unknown
Rhodomonas sp.	RCC4444		seq	nd	seq	marine	JPN	North Pacific Ocean
Proteomonas sp.	RCC4474		seq	seq		marine	ISR	Gulf of Eilat, Red Sea
Teleaulax sp.	RCC816		seq	seq		marine	FRA	English Channel, North Atlantic
Proteomonas sulcata	RCC1505		seq	seq	seq	marine	FRA	Lingurian Sea
Rhodomonas salina	RCC1506		seq, KJ513110	seq		marine	Unknown	Unknown
Rhodomonas salina	RCC1507	AC160	seq	seq		marine	FRA	English Channel, North

								Atlantic
Rhodomonas sp.	RCC1509		seq	seq	seq	marine	FRA	English Channel, North Atlantic
Rhodomonas sp.	RCC1510		seq	seq		marine	FRA	English Channel, North Atlantic
Proteomonas sulcata	RCC3574	C30	seq	seq	seq	marine	Unknown	Unknown
Rhodomonas sp.	RCC1998		seq	seq		marine		Beaufort Sea, Arctic Ocean
Rhodomonas sp.	RCC2045		seq	nd	seq	marine		Beaufort Sea, Arctic Ocean
Rhodomonas sp.	RCC2044		seq	seq	seq	marine		Beaufort Sea, Arctic Ocean
Rhodomonas sp.	RCC2513		seq, KT861333	seq	seq	marine		Beaufort Sea, Arctic Ocean
Rhodomonas sp.	RCC2516		seq, KT861332	seq	seq	marine		Beaufort Sea, Arctic Ocean
Rhodomonas sp.	RCC2007		seq,KT861338	seq	seq	marine		North East Pacific
Rhodomonas sp.	RCC2018		seq, KT861337	seq		marine		Beaufort Sea, Arctic Ocean
Rhodomonas sp.	RCC2019		seq, KT861336	seq	seq	marine		Beaufort Sea, Arctic Ocean
Rhodomonas sp.	RCC2020		seq, JN934672	seq	seq	marine		Beaufort Sea, Arctic Ocean
Proteomonassp.	RCC3072		seq	nd				
Rhodomonas sp.	RCC2001		KT861339					
Rhodomonas sp.	RCC4784		KX602216			marine	NOR	Greenland Sea, North Atlantic
Rhodomonas sp.	RCC4847		KY095062			marine	FRA	English Channel, North Atlantic
Rhodomonas sp.	RCC4848		KY095005	KY095063		marine	FRA	English Channel, North

								Atlantic
Rhodomonas sp.	RCC822		KT861342					
Storeatula major	CCMP320		U53130		U53129	marine	AUS	Tasman Sea, Bass Strait, South Pacific
Storeatula sp.	CCMP1868	Clone B	HF952565		HF952609	marine	AUS	Indic Ocean
Storeatula sp.	CCMP1868	clone A	HF952564			marine	AUS	Indic Ocean
Storeatula sp.	CCMP1868		AF508276	FJ973366		marine	AUS	Indic Ocean
Cryptomonas acuta	NIES697	CCAP 979/10	AB240956			marine	GBR	N.Wales
Pyrenomonas salina			X54276					
Cryptophyta sp	CR-MAL06		EF195733					
Proteomonassp.	CCMP321							
Pyrenomonas helgolandii	SAG 28.87		AB240964			marine	DEU	North Sea
Pyrenomonas salina					X55032			
Rhinomonas nottbecki	07B6		HF952561		HF952607	marine	FIN	Baltic Sea
Rhinomonas nottbecki	07B6, clone A		HF952558			marine	FIN	Baltic Sea
Rhinomonas nottbecki	07B6, clone B		HF952558			marine	FIN	Baltic Sea
Rhinomonas nottbecki	07B6, clone C		HF952560			marine	FIN	Baltic Sea
****Rhinomonas nottbecki***	07B3				HF952606	marine	FIN	Baltic Sea
****Rhinomonas nottbecki***	07B3, clone B		HF952555			marine	FIN	Baltic Sea
****Rhinomonas nottbecki***	07B3, clone C		HF952556			marine	FIN	Baltic Sea
Rhinomonas nottbecki	07B5		HF952557			marine	FIN	Baltic Sea
Rhinomonas pauca	MUCC Cr# 47		U53132		U53131	marine	AUS	Hobsons Bay
Rhinomonas reticulata var. reticulata	CCAP 979/15, clone D		HF952563		HF952608	marine	GBR	Plymouth Sound, English Channel
Rhinomonas reticulata var. reticulata	CCAP 979/15, clone B		HF952562			marine	GBR	Plymouth Sound, English Channel

Rhinomonas sp.			AF508273				
Rhodomonas abbreviata	CCAP 979/16						
Rhodomonas abbreviata	CCAP 976/10		U53128	U53127			
Rhodomonas cf. abbreviata	CCMP1178		HF952573	DQ228122	marine	EUA	Gulf of Maine, North Atlantic
****Rhodomonas astrosea***	NIES-699	CCAP 978/6A	AB240957		marine	GBR	Newtown, Isle of Wight, England
****Rhodomonas atrorosea***	CCAP 978/6A		HF952566		marine	GBR	Newtown, Isle of Wight, England
****Rhodomonas baltica***	NIES-700		HF952603		marine	GBR	Channel Isls
****Rhodomonas baltica***	NIES-700		AB241128		marine	GBR	Channel Isls
****Rhodomonas chrysoidea***	NIES-701		AB240958		brackish	GBR	River Colne Essex
Rhodomonas duplex	NIES-765		AB240960			JPN	Yaga Okinawa
Rhodomonas duplex	NIES-765		HF952604	HF952620		JPN	Yaga Okinawa
****Rhodomonas falcata***	NIES-702		AB240959		marine	GBR	Aberystwyth Wales
Rhodomonas lens	CCMP739, clone C	RLENS, NEPCC588, Milford RHODO	HF952576	HF952611			Gulf of Mexico, North Atlantic Ocean
Rhodomonas lens	CCMP739, clone A	RLENS, NEPCC588, Milford RHODO	HF952574	DQ228121			Gulf of Mexico, North Atlantic Ocean
Rhodomonas lens	RlensIII		JF489951				
****Rhodomonas maculata***	CCAP 979/14		HF952567		marine	GBR	Plymouth Sound, England
Rhodomonas maculata	MUCC053		AF508274				
Rhodomonas marina	SCCAP K-0435		HF952572	HF952610	marine	DNK	Gilleleje, Kattegat
Rhodomonas marina			X81373	X81374			

Rhodomonas minuta	CPCC344		HF952602					
Rhodomonas salina	SCCAP K-0294, clone D	DH4	HF952571			marine	DNK	Amager
Rhodomonas salina	SCCAP K-0294, clone C	DH4	HF952570			marine	DNK	Amager
Rhodomonas salina	SCCAP K-0294, clone B	DH4	HF952569			marine	DNK	Amager
Rhodomonas salina	SCCAP K-0294, clone A	DH4	HF952568			marine	DNK	Amager
Rhodomonas salina	CCMP1319				DQ228119	marine	USA	Long Island Sound, North Atlantic
Rhodomonas salina								
Rhodomonas salina	SCCAP K-1487		HM126532			marine	DNK	
Rhodomonas salina	CCMP1170				DQ228120	marine	AUS	Cape Conran, Victoria, Tasman Sea, South Pacific
Rhodomonas salina	CCAP 978/24		EU926158	FJ210726				
Rhodomonas sp.	08A2		HF952599				FIN	Tvärminne
Rhodomonas sp.	08C1, clone C		HF952601				FIN	Tvärminne
Rhodomonas sp.	08C1, clone A		HF952600				FIN	Tvärminne
Rhodomonas sp.	EDK-2013		KF039722					
Rhodomonas sp.	CCAP 995/5		KU900223					
Rhodomonas sp.	CCAC 1630 B			HG328374			ITA	Castellamare, Gulf of Naples
Rhodomonas sp.	CCMP2045		HF952577		DQ228117	marine	CAN	Baffin Bay, Artic
Rhodomonas sp.	CCMP275	Mel-032	HF952605		HF952621	marine	AUS	Point Lonsdale, Victoria, Bass Strait,

						Indic Ocean
Rhodomonas sp.	CCMP324	HF952578	HF952612	marine	USA	Devil's Churn, Oregon, North Pacific
Rhodomonas sp.	CCMP740	HF952579	HF952613	marine	USA	Galveston Channel, Texas, Gulf of Mexico, North Atlantic
Rhodomonas sp.	CCMP741	HF952580		marine	USA	Galveston Channel, Texas, Gulf of Mexico, North Atlantic
Rhodomonas sp.	CCMP742	HF952581		marine	Unknown	Unknown
Rhodomonas sp.	CCMP743	HF952582	HF952614	marine	USA	South of Hopkins Marine Station, Pacific Grove, California, Monterey Bay, North Pacific
Rhodomonas sp.	CCMP744	HF952583		marine	Unknown	Unknown
Rhodomonas sp.	CCMP747	HF952584	HF952615	marine	USA	Hopkins Marine Station, California, Monterey Bay, North Pacific
Rhodomonas sp.	CCMP755	HF952598		marine	USA	Long Island Sound, near Connecticut, Long Island Sound, North Atlantic
Rhodomonas sp.	CCMP756	HF952585		marine	Unknown	Unknown
Rhodomonas sp.	CCMP758, clone D	HF952588				Falmouth Great Pond, Falmouth, Massachusetts USA
Rhodomonas sp.	CCMP758, clone B	HF952587				Falmouth Great Pond, Falmouth,

								Massachusetts USA
Rhodomonas sp.	CCMP758, clone A		HF952586					Falmouth Great Pond, Falmouth, Massachusetts USA
Rhodomonas sp.	CCMP759		HF952589			marine	USA	Woods Hole, Massachusetts, Nantucket Sound, North Atlantic
Rhodomonas sp.	CCMP760		HF952590		HF952616	marine	MEX	Acapulco Harbor, North Pacific
Rhodomonas sp.	CCMP762		HF952591		HF952617	marine	USA	Pigeon Hill Bay, Gulf of Maine, North Atlantic
Rhodomonas sp.	CCMP763		HF952592			marine	USA	Gulf of Maine, North Atlantic
Rhodomonas sp.	CCMP767	clone D	HF952595		HF952618	marine	MEX	Magdalena Bay, Baja California, North Pacific
Rhodomonas sp.	CCMP767	clone C	HF952594			marine	MEX	Magdalena Bay, Baja California, North Pacific
Rhodomonas sp.	CCMP767	clone B	HF952593			marine	MEX	Magdalena Bay, Baja California, North Pacific
Rhodomonas sp.	CCMP768		FJ973361	FJ600091				
Rhodomonas sp.	CCMP768	clone D	HF952597		HF952619	marine	NZL	North Island, South Pacific
Rhodomonas sp.	CCMP768	clone C	HF952596			marine	NZL	North Island, South Pacific
Rhodomonas sp.	CCMP768		GU594640			marine	NZL	North Island, South Pacific

Rhodomonas sp.	SCCAP K-1488	HM126535				
Rhodomonas sp.	KMMCC 574	JQ315655		marine		
Rhodomonas sp.	M1480	AJ007286	AJ420693			
Rhodomonas sp.	M1630	AJ421148	AJ420694			
Rhodomonas sp.	MBIC10225	AB183594				
Rhodomonas sp.	NIES-3921	LC129531		freshwater	JPN	Lake Kasumigaura Ibaraki

Morphological documentation

The strains BMAK254, BMAK243, BMAK247, RJ02, and BMAK276 (TABLE 1) were cultured for 15-20 days for morphological observations. Living cells as well as fixed cells (2% glutaraldehyde) were documented in light microscopy Leica DM 4000 B (Leica Microsystems, Wentzler, Germany), and in confocal microscopy Zeiss LSM 440 Axiovent 100 (Carl Zeiss, Jena, Germany). Confocal microscopy excited cells with 543 nm laser, and long pass filter of 570 nm was used for natural fluorescence documentation. For morphometric measurements, at least 35 cells were taken into account in each one of the strains species.

For both scanning and transmission electron microscopies, 30 mL of cell cultures were harvest by gentle centrifugation (100-150 g) during five minutes. Supernatant was removed and cells were fixed immediately during 90 min with glutaraldehyde (2%) buffered with sodium cacodilate trihydrate (0.1 M) and sucrose (0.8M), as described in Majaneva et al. (2014). Posteriorly, cells were washed with a buffer solution with sodium cacodilate trihydrate (0.8 M) with decreasing concentrations of sucrose until it was completely removed. The second step of fixation was performed with osmium tetroxide (1%) buffered with cacodilate (0.8 M) for 60 min. Then, samples were washed twice with cacodilate buffer. For scanning electron microscopy (SEM), samples were submitted to ethanol dehydration steps with increasing concentration (70, 90, 95, 100%) to the critical point dried (Balzers CPD 030, Bal-Tec, Vaduz, Liechtenstein) and gold coated (Balzers SCD 050). Finally, cells were observed in Zeiss Sigma VP SEM. For transmission electron microscopy (TEM), after the fixation steps, cells were dehydrated in increasing concentration of acetone (50, 70, 90, 95, and 100%), and subsequently embedded in Spurr resin. Thin sections were performed, which were stained for visualization of cellular features in the scanning mode of FEI TECNAI G^2 F20 HRTEM.

DNA extraction, PCR and sequencing

For DNA extraction, 30 mL of 1-3 weeks old cultures were harvested by centrifugation. The cells pellet were used to obtain genomic DNA with the *NucleoSpin® Plant* II

(Macherey-Nagel, Düren, Germany) kit following the manufacturer's guide. PCRs of nuclear and nucleomorph SSU rDNA, and ITS2 genes were performed with Go Taq® G2 Hot Start (Promega Corporation, Madison, USA). The complete list of primers is in Table 2. PCRs using the primers 18S5'and 18S3' were performed with initial denaturation at 94°C for 5min, followed by 35 cycles of 94°C for 30sec, 60°C for 1 min and 72°C for 2 min and final extension step for 7 min at 72°C. For PCRs using the primers NUC71F/ SSUBR, and 18SNF2/ SSUR, the cycling conditions are the same as specified in Majaneva et al. (2014). For the amplification of ITS2 region, the cycling conditions were the same as described in Hoef-Emden & Melkonian (2003). PCRs products with distinctive clear band were purified with Pure Link® PCR purification kit (Life Technologies Corporation, Carlsbad, USA) according to the instructions of the manufacturer. Sequencing reactions were performed with Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems™, Hammonton, NJ, USA). Samples were sequenced on a 3730 Applied Biosystems. We generated 48 new sequences of nuclear SSU, 38 of ITS2, and 47 of nucleomorph SSU rDNA, totalizing 133 new sequences.

Table 2: Primers for PCRs and sequencing reactions.

	Sequences of oligonucleotides (5'- 3')	References
Primers for PCRs of nuclear SSU rDNA		
*18S5'	CCA CCT GGT TGA TCC TGC CAG T	(SOGIN, 1990)
18SNF2	TGATGGTCCCTTACTACA	(MAJANEVA et al., 2014)
SSUR	CTTGTTACGACTTCTCCT	(MAJANEVA et al., 2012)
*18S3'	GAT CCT TCT GCA GGT TCA CCT ACG GAA	(SOGIN, 1990)
Primers for PCRS of nucleomorph SSU r DNA		
NUC71F	AACGATTTTGTACCGTGA	(MAJANEVA et al., 2014)
*18S5'	CCA CCT GGT TGA TCC TGC CAG T	(SOGIN, 1990)
SSUBR	TTG ATC CTT CTG CAG GTT CAC CTA C	(HOEF-EMDEN; MELKONIAN, 2003)
*18S 3'	GAT CCT TCT GCA GGT TCA CCT ACG GAA	(SOGIN, 1990)
Primers for sequencing nuclear and nucleomorph SSU rDNA		
18S 5'	CCA CCT GGT TGA TCC TGC CAG T	(SOGIN, 1990)
NUC71F	AACGATTTTGTACCGTGA	(MAJANEVA et al., 2014)
528F	CGG TAA TTC CAG CTC C	(SOGIN, 1990)
1055F	GGT GGT GCA TGG CCG	(BELLORIN; OLIVEIRA; OLIVEIRA, 2002)
18S 3'	GAT CCT TCT GCA GGT TCA CCT ACG GAA	(SOGIN, 1990)
536R	GAA TTA CCG CGG CTG CTG	(BIRD et al., 1992)

1055R	CGG CCA TGC ACC ACC	(BIRD et al., 1992)
18SNF2	TGATGGTCCCTTACTACA	(MAJANEVA et al., 2014)
SSUBR	TTG ATC CTT CTG CAG GTT CAC CTA C	(HOEF-EMDEN; MELKONIAN, 2003)
SSUR	CTTGTTACGACTTCTCCT	(MAJANEVA et al., 2012)
Primers for PCR and sequencing of ITS2 region		
ITS03F-800	CGA TGA AGA ACG YAG CGA	(HOEF-EMDEN; MELKONIAN, 2003)
ITS05R-700	TAC TTG TTC GCT ATC GGT CTC T	(HOEF-EMDEN; MELKONIAN, 2003)

* The primers 18S5' and 18S3' can amplify the nuclear or nucleomorph region using the described cycling conditions and it varies according to the strain.

Molecular phylogeny inferences analysis

Consensus sequences were assembled in Geneious 10.2.3 (BioMatters, Ann Arbor, Michigan, USA) using forward and reverse sequences. The matrix for each marker were built by search in taxonomic section in NCBI database. Additionally, sequences were blasted to certify that all close-related sequences were included. Each sequences bibliography, when available, was checked to known about authentic strains and environmental descriptions.

All sequences were aligned using AliView (LARSSON, 2014). Short sequences as well as bad quality ones were removed from the matrix. Preliminary runs were performed with each gene separately to inspect if the topologies were congruent among nuclear SSU and ITS2, and nucleomorph SSU rDNA. As the results were congruent, the database of different genes were concatenated with SeaView (GOUY; GUINDON; GASCUEL, 2010). Strains with incomplete sequence data were added in the matrix because incomplete taxa can be placed accurately in phylogeny and these data can improve results by subdividing misleading long branches (WIENS, 2006), as Bayesian analysis are the most appropriate to deal with missing data problem (DWIVEDI; GADAGKAR, 2009)

The final concatenated dataset with the three markers contained 3904 base pairs and 122 sequences. Seven strains of *Teleaulax* and *Proteomonas* were used to root the tree. The outgroup choice was based on the phylogenetic proximity of these genera with *Rhodomonas* (DEANE et al., 2002; HOEF-EMDEN; MARIN; MELKONIAN, 2002). For the Bayesian analysis, this matrix was partioned in three, according to the molecular

marker, and for each one the most appropriate evolutionary model were applied according to the analysis results of JModel Test 2.1.7 (DARRIBA et al., 2012). Phylogenetic inferences were performed using MrBayes 3.2 (RONQUIST et al., 2012). Two consecutive runs were performed with $2x10^8$ generations, four Markov Chains and sampling every 1000 generations. The runs convergence were checked in Tracer V1.7 (RAMBAUT et al.,) and relative burn-in of 25% were applied.

RESULTS

Taxonomic section

***Rhodomonas* sp. 1** Magalhães & Oliveira sp. nov.

Description: Cells elliptical in lateral view, ranging from 12.5- 7 µm of length, and 7-4 µm of width. In ventral/dorsal view cells are slightly compressed. Two flagella of similar size. One contractile vacuole at the apical portion of the cell, close to flagellar region. Small furrow and a gullet prominent gullet. Rows of ejectosomes surrounds gullet region and small ones are distribute at among the periplast plates. Hexagonal SPC plates with a slight mid basal band. One parietal lobed chloroplast with a starch sheet pyrenoid. Major accessory pigment Cr-PE545.Oil drops accumulation in chloroplast stroma. Nucleomorph inside the pyrenoid. Golgi body close to the gullet. Basal nucleus.

Type strain: BMAK247 (RCC5615)

Other strains: BMAK243 (RCC5614), BMAK248 (RCC5616), BMAK249 (RCC5617), BMAK250 (RCC5618), BMAK251 (RCC5619), BMAK253 (RCC5621).

Type locality: coastal area of Cananéia, São Paulo, Brazil (Lat. 25°2'S, Long. 47°55'W)

***Rhodomonas* sp. 2** Magalhães & Oliveira sp. nov.

Description: Cells elliptical -ovoid in lateral view, varying in length 12.5-8 µm, and 7-4 µm in with. Two flagella of similar size. Small furrow and prominent gullet. Rows of ejectisomes surrounds the gullet region and small ones distributed among the periplast plates. SPC with hexagonal plates. One parietal lobed chloroplast with starch sheet

pyrenoid. Main accessory pigment Cr-PE545. Nucleomorph inside the pyrenoid. Golgi body close to the gullet region. Tubular mitochondria close to the flagellar region. Nucleus basal.

Type strain: BMAK 254 (RCC5613)

Type locality: coastal area of Cananéia, São Paulo, Brazil (Lat. 25°2'S, Long. 47°55'W)

***Rhodomonas* sp. 3** Magalhães & Oliveira sp. nov.

Description: Cells ellipsoids to obovate in lateral view, varying 19-11 µm long and 10-6 µm of width. Laterally compressed and in ventral/dorsal view triangular-like shape, with rounded anterior and acute posterior. Two flagella of similar size. Developed furrow. Rows of ejectosomes surrounds the region of the gullet and are disposed among periplast plates. One big lobed parietal chloroplast with one starch sheet pyrenoid. Major accessory pigment Cr-PE545 (551-552nm). Rectangular-hexagonal SPC and a pronounced mid-ventral band.

Type strain: BMAK276 (RCC5621).

Type locality: Cotovelo beach, Parnamirin, Rio Grande do Norte, Brazil (Lat. 5°57'S, Long. 35°7'W).

Morphological descriptions and phycobiliprotein type

The morphology of six strains was analyzed in detail using different approaches. *Rhodomonas* .sp. 1 cells are ellipsoid with rounded anterior and posterior ends, but some cells with more or less acute ends can be found (FIGURE 1 A-C). The size and shape of the cells were different even in the same culture. Some cells are larger and thicker, while others are smaller and thin. Rows of large ejectisomes surrounds the cells furrow/gullet system, and small ones are found distributed along the cell periplast (FIGURE 2 A-D). Superficial periplast component (SPC) composed of more or less rectangular-hexagonal plates, bigger near the furrow/gullet, and smaller at the basal portion of the cell (FIGURES 2A-D). A slight middle basal band was observed (FIGURE 2 C) A small furrow was visualized in cells of BMAK243 (FIGURE 2 D).

One lobed peripheral parietal chloroplast occupies more than ¾ of the cell surface. Long well stained thylakoids are disposed in the chloroplast stroma and accumulation of oil drops in this region was detected (FIGURE 3 A-D). The chloroplast harbors a pronounced pyrenoid, located dorsally at the middle of the cell. The pyrenoid is surrounded by starch sheet. The nucleomorph is situated inside the pyrenoid (FIGURE 3 C-D). The Golgi body is disposed close to the gullet region and above it is the located the contrail vacuole (FIGURE 3 A, B and D). Two flagella emerge from the vestibular region (FIGURE 3 B). One big nucleus is placed at the basal part of the cell (FIGURE 3 A, C and D). The main accessory pigment is Cr-PE 545, with maximum absorption at 544-545 nm (FIGURE 4).

Rhodomonas sp. 2 cells are ovoid with anterior end more rounded than posterior one (FIGURE 2F-H). Two unequal flagella emerge from the vestibular region (FIGURE 1 F and I). Large ejectisomes surrounds the cells furrow/gullet system and small ones are disposed along the cell body (FIGURE 1 F-H, and 3 F). SPC with more or less hexagonal plates (FIGURES 2 E-F). A small furrow and a gullet were detected (FIGURE 3 E and I). One parietal lobed chloroplast occupies most of the cell periphery. Natural fluorescence of the chloroplast was obtained using 543 nm laser (FIGURE 1 J). Chloroplast is well filed by long thylakoids and harbors a big pyrenoid starch sheet. The nucleomorph is located inside the pyrenoid (FIGURE 3 E, G-H). Oil drops are accumulated in the chloroplast stroma (FIGURE 3 F, I). Golgi body is disposed near the gullet region where apparently vesicles are discharged (Figure 3 E). Tubular mitochondria found near the flagellar region. Cells possess Cr- PE545 as the major accessory pigment (FIGURE 4).

Rhodomonas sp.3 cells are ellipsoids, sometimes obovate in lateral view, ranging from 11 to 19µm in length, and 6 to 10µm in width. In ventral/dorsal view, cells are very compressed laterally, and it shapes sometimes are resembles a triangle. The anterior of the cell is anterior is more rounded while the basal portion is very acute. Two unequal flagella emerge from the vestibular region and several rows of ejectisomes go along with the furrow/gullet system (FIGURE 1 K-L). Ejectisomes are also found along the cell body. SPC has rectangular-hexagonal plates covering almost the cell body, less the basal portion, where a prominent antapical band emerges and goes almost until the middle of the ventral part of the cell (FIGURE 2 G-H). Dorsal part of the cell is convex

(FIGURE 3 I). A prominent furrow is present. One large parietal chloroplast occupies more than half of the cells periphery. One big pyrenoid starch sheeted is situated almost in the middle of the cell. Phycobiliprotein extract resulted in maximum absorption at 551-552nm (FIGURE 4), classified as Cr-PE 545 (HILL; ROWAN, 1989).

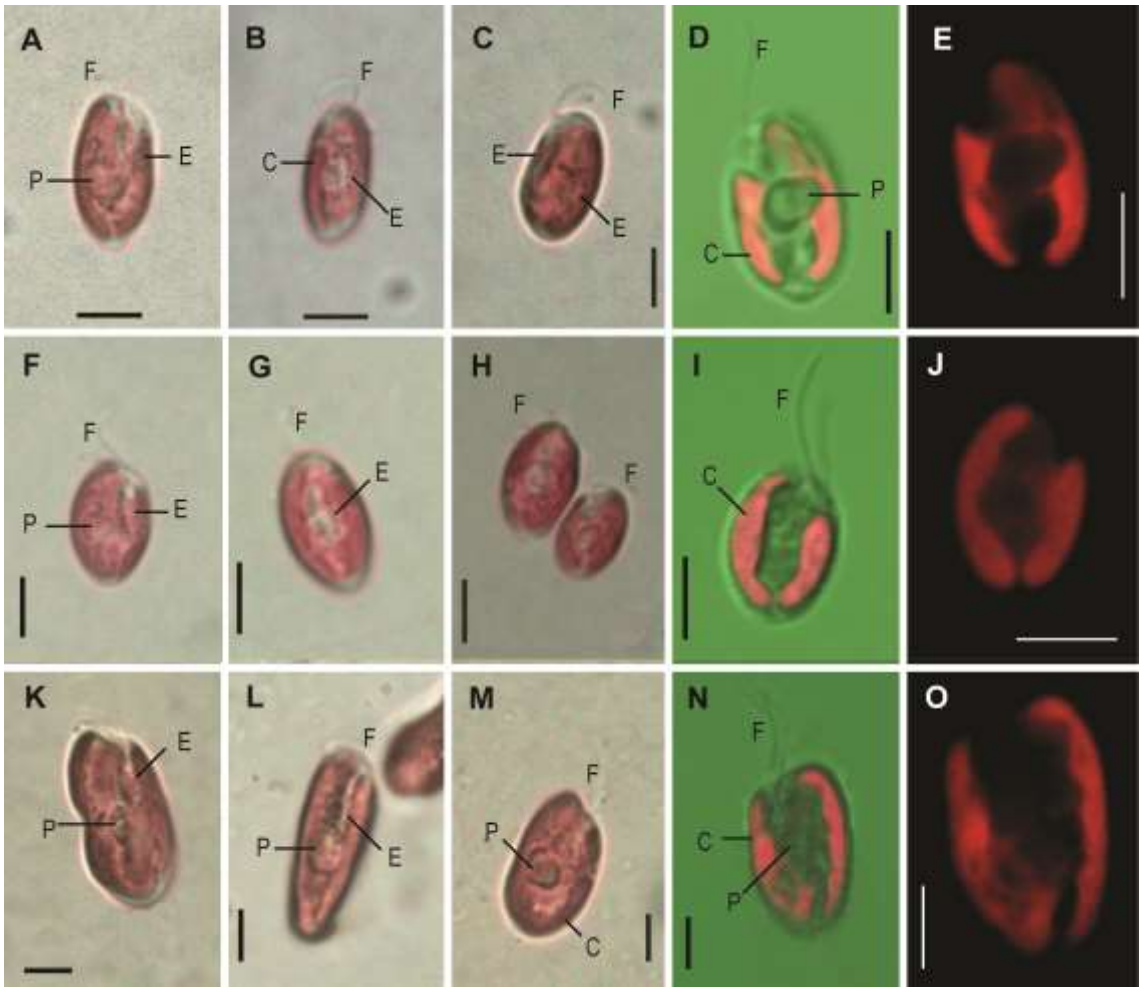


FIGURE 1: *Rhodomonas* sp. 1 (A-E), *Rhodomonas* sp. 2 (F-J) and *Rhodomonas potiguaris* (K-O) cells morphology under light (A-C, F-H and K-M) and confocal microscopy (D, E, I, J, N, O). Scale bars represent 5 µm. The pictures in D-E, I-J, and N-O shows natural fluorescence of chloroplast using a laser of 543 nm. (C) Chloroplast, (E) ejectisome, (F) flagella, and (P) pyrenoid.

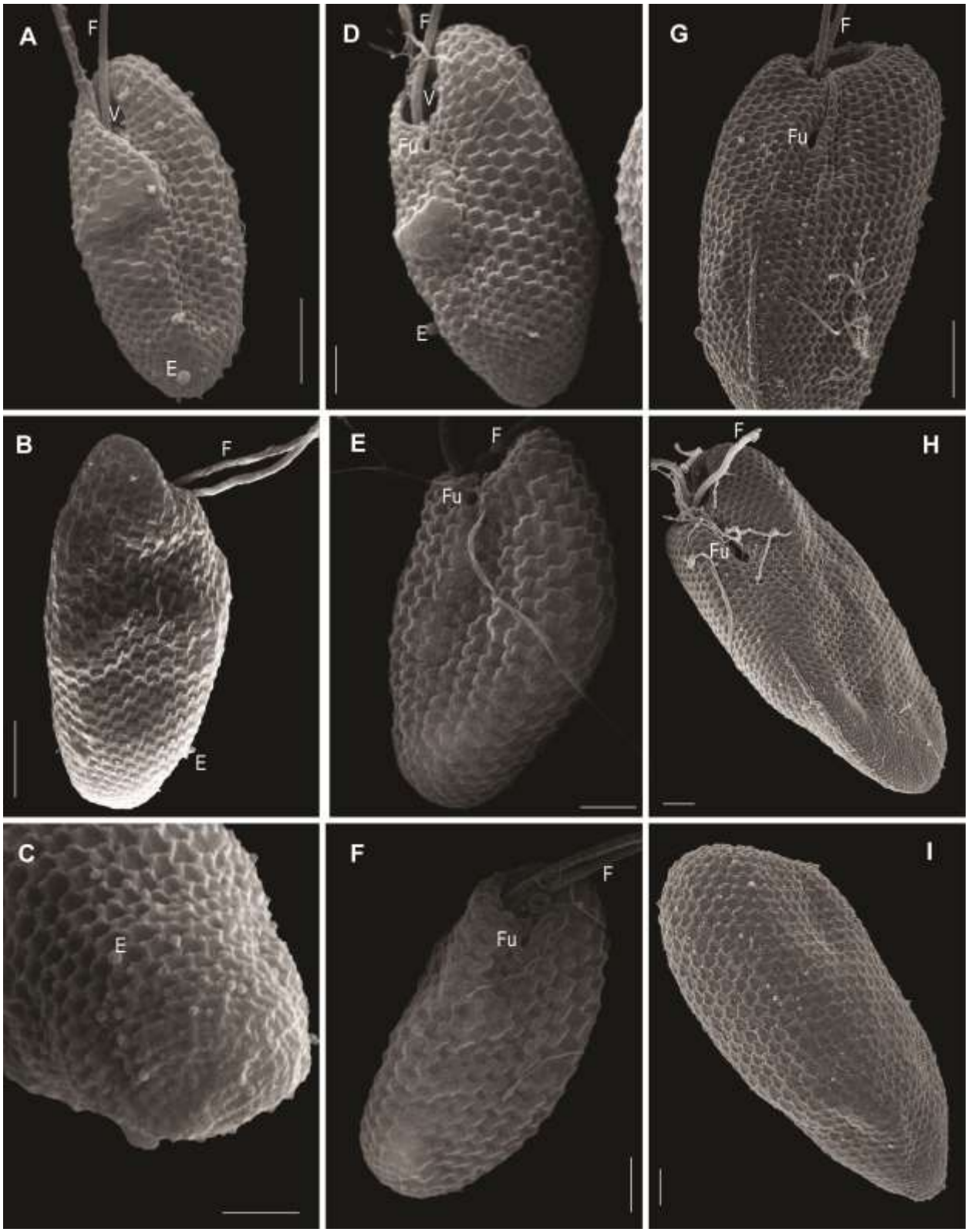


FIGURE 2: Scanning electron microscopy of *Rhodomonas* sp. 1 (A-D), Rhodomonas sp. 2 (E-F), and *Rhodomonas* sp. 3 (G-I). Scale bars represent 1 µm, except A, B, and G, where they represent 2 µm. (E) ejectisome, (F) flagella, (Fu) furrow.

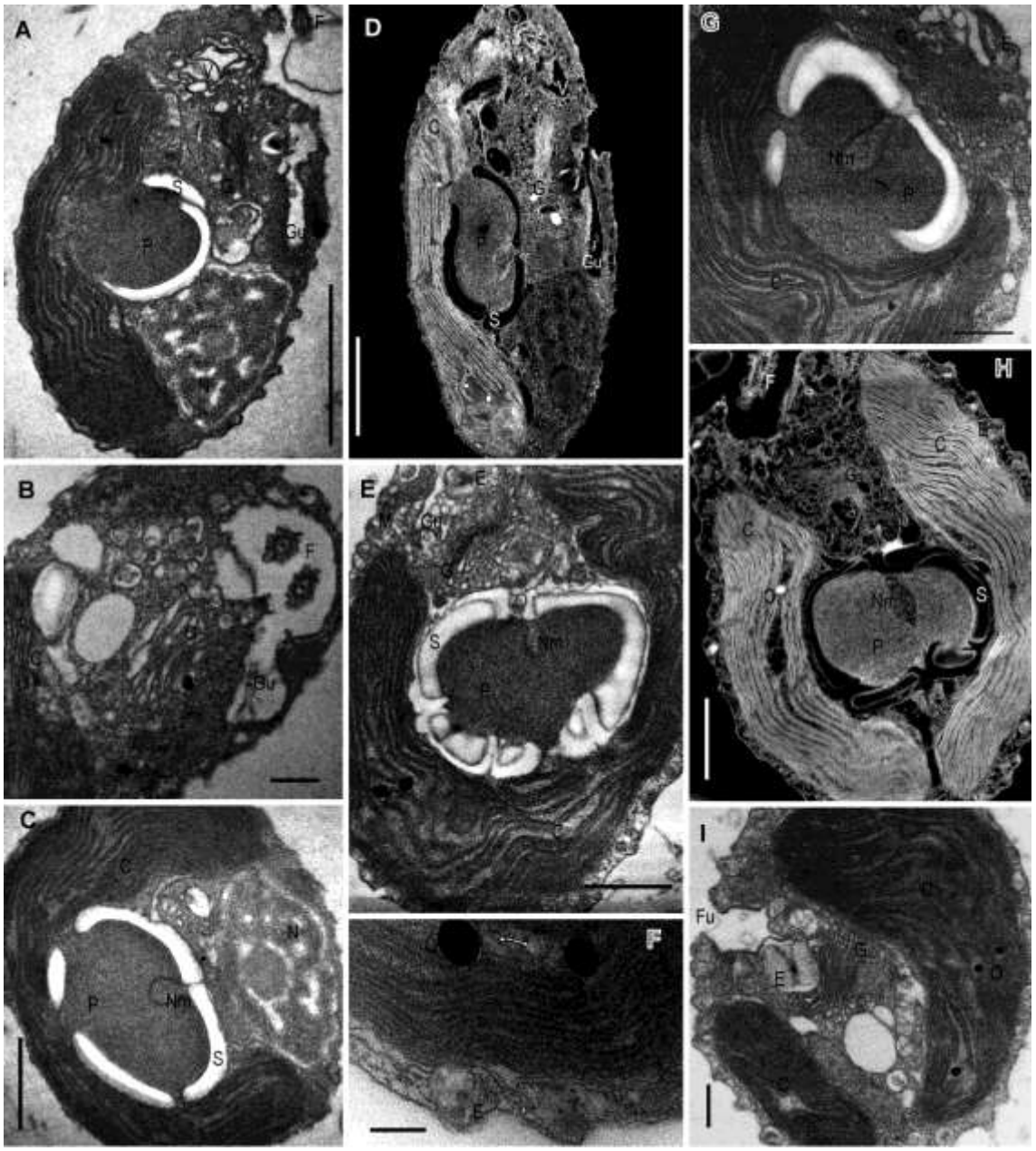


FIGURE 3: Transmission electron microscopy in bright and dark fields of *Rhodomonas* sp. 1 and *Rhodomonas* sp. 2 *(*BMAK254). (A) *Rhodomonas* sp. 1 lateral-longitudinal section in bright field. Scale bar 2 µm. (B) *Rhodomonas* sp. 1 apical-cross section in bright field. Scale bar 500 nm. (C) *Rhodomonas* sp. 1 antapical cross section in bright field. Scale bar 1 µm. (D) Dark field lateral-longitudinal section of *Rhodomonas* sp. 1*.* Scale bar 2 µm. (E) *Rhodomonas* sp. 2 lateral-cross section. Scale bar 1 µm. (F) *Rhodomonas* sp. 2 section evidencing the oil accumulation (O) in the chloroplast stroma and the small ejectisomes associated to the periplast structure. Scale bar 200 nm. (G) *Rhodomonas* sp. 2 mid cross-section. Scale bar 500 nm. (H) *Rhodomonas* sp. 2 lateral-longitudinal section. Scale bar 1 µm. (I) *Rhodomonas* sp. 2 apical cross section. Scale bar 500 nm. (C) Chloroplast, (E) ejectisome, (F) flagella, (Fu) furrow, (G) Golgi body, (Gu) gullet, (M) mitochondria, (N) nucleus, (Nm)

nucleomorph, (P) pyrenoid, (S) starch, (O) oil drops accumulation in chloroplast stroma, (V) contractile vacuole.

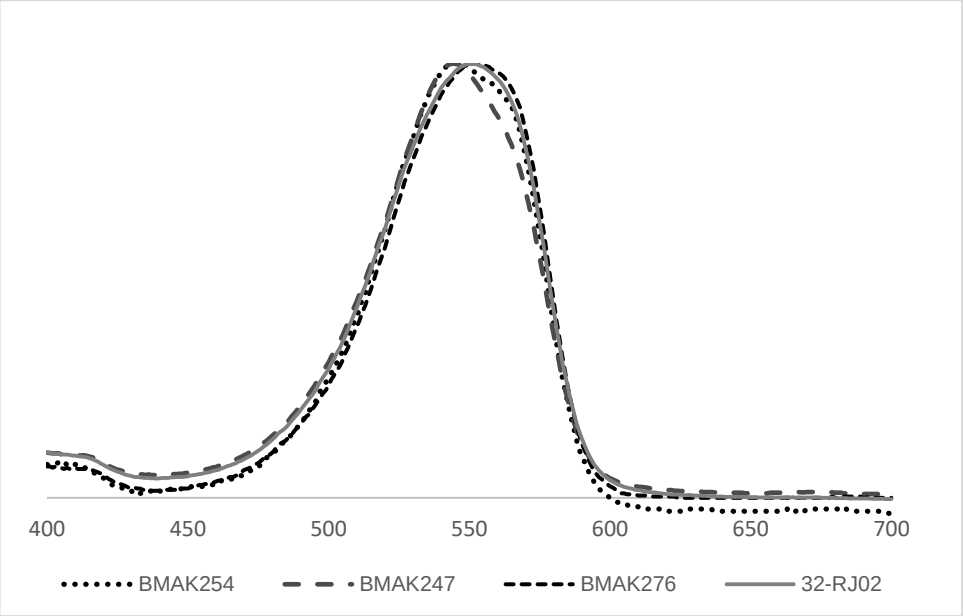


FIGURE 4: Spectral signature of phycobiliprotein extract in phosphate buffer (pH 6.8) of *Rhodomonas* sp. 1 (BMAK247), *Rhodomonas* sp.2 (BMAK254), *Rhodomonas* sp. 3 (BMAK276), and *Rhodomonas* sp. (RJ02). All strains possess Cr-PE545

Molecular phylogeny relationships

According to concatenated analysis of nuclear SSU and ITS2, and nucleomorph SSU rDNA regions, at least five main clades were observed in Pyrenomonaceae (FIGURE 5). The first to diverge in our analysis is composed of *Rhodomonas abbreviata, R. marina* (P.A.Dangeard) Lemmerman and some other unidentified strains. Another clade is composed of *Rhodomonas duplex, Rhodomonas* sp. 3 and *Storeatula* sp. The third clade is composed by the sequences of *Rhodomonas* sp. 1 strains. Following, the fourth clade includes *Rhodomonas baltica, R. salina, R. lens* and some other unidentified strains. The last clade is a "*Rhinomonas/ Rhodomonas* clade", including *Rhodomonas* sp. 2. All new species designated in this study form new monophyletic clades (FIGURE 5). Although the relationships among some taxa are not well supported, the clades of the

new proposed species here were recognized in all the separated phylogenetic analysis of each gene. *Rhodomonas* sp. 1 forms a distinct clade with poorly supported relationship with other clades. Although the support of the clade in the concatenated analysis is not strong, in ITS2 and nucleomorph SSU rDNA individual phylogenetic analysis the clade was recovered with 100% posterior probability. Contrarily, *Rhodomonas* sp. 2 is a full-supported clade in the concatenated tree, with maximum support, closely related with the authentic strain of *Rhinomonas pauca*, MUCC Cr#47 (HILL; WETHERBEE, 1988). While *Rhodomonas* sp. 3 is related to *Rhodomonas duplex* (FIGURE 5).

The other strains sequenced in this study grouped in a wide variety of clades within the Pyrenomonadaceae. The strains RCC350, RCC1507 and RJ02 are joined in a very confusing clade where many sequences of different authentic cultures are encountered. The strains RCC20, RCC906 and RCC821 seem to be *Rhinomonas pauca.* The strains RJ03 and RCC4444 are in the "*Rhodomonas baltica* clade". These strains seems to be the same species and sister group of *Rhodomonas salina* (Wislouch) D.R.A.Hill & R.Wetherbee and *Rhodomonas lens* Pascher & Ruttner strains, although these sequences are not confirmed to be from the type species. Ten strains, RCC662, RCC1998, RCC2007, RCC2018, RCC2019, RCC2020, RCC2044, RCC2045, RCC2513, and RCC2516 group with other strains specified as *Rhodomonas marina* (P.A.Dangeard) Lemmermann and four strains, RCC1510, RCC2603, and RCC2626 clustered with a strain assigned as *Rhodomonas abbreviata* Butcher ex D.R.A.Hill & R.Wetherbee. However, as in the clade of *Rhodomonas salina/ R. lens*, none of the two species strains in database were assigned as from the type. Additionally, our results pointed out that the genus *Storeatula* is invalid, due to his great spread in the phylogenetic tree, where it can be found in "*Rhinomonas/Rhodomonas"* and "*Rhodomonas duplex"* clades. Accordingly, it may represent a dimorphic form of cellular life cycle in Pyrenomonadaceae.

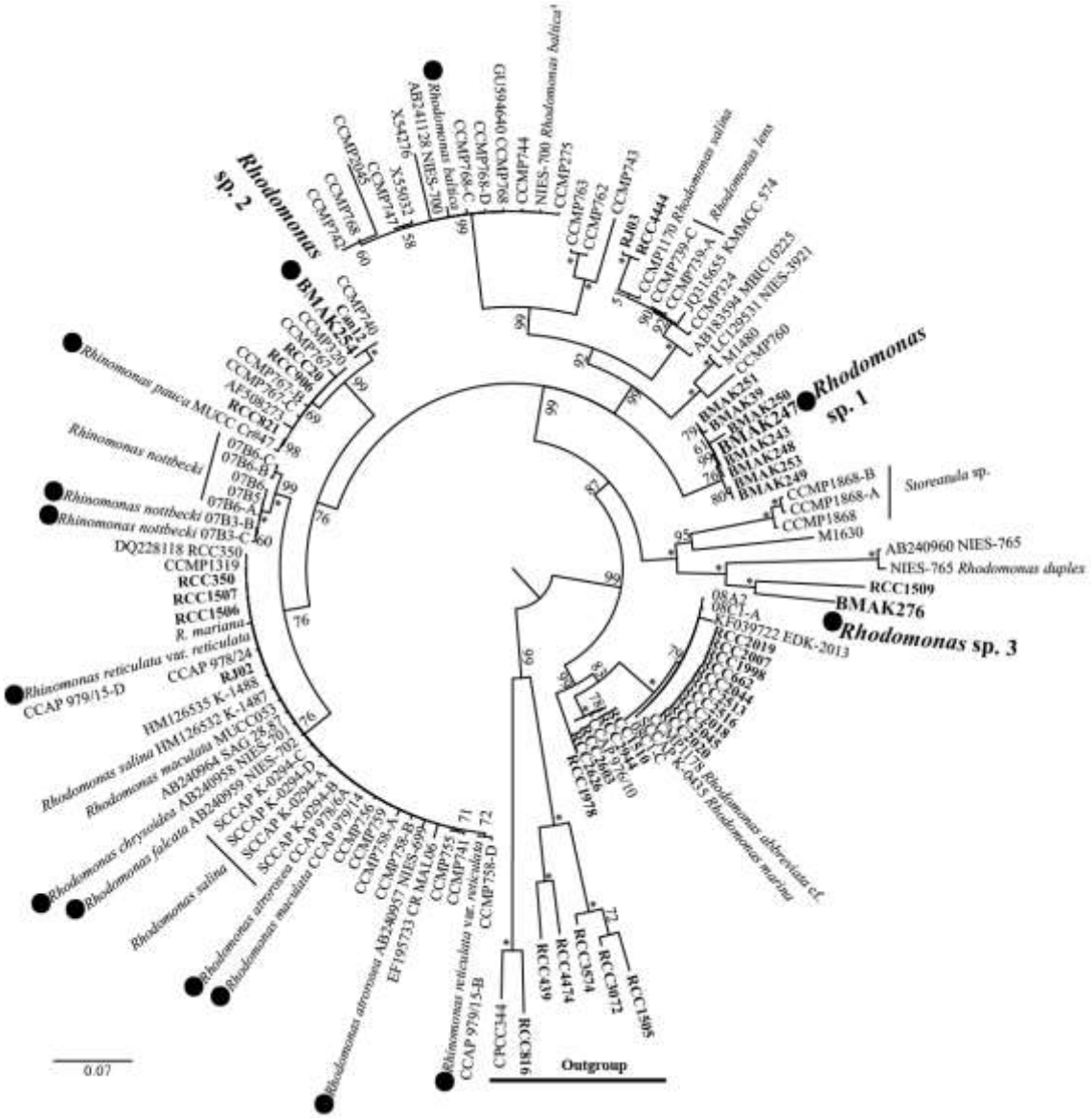


FIGURE 5: Phylogenetic inference tree based on a concatenated dataset composed by the sequences of nuclear SSU and ITS2, and nucleomorph SSU rDNA. The molecular evolution models used were Tr+I+G, SYM+G, and SYM+I+G, respectively. New sequences added in this study are represented in bold. Bold circles represents the authentic cultures, which the type was obtained according to the information of the respective culture collection. * Represents nodes supported by 100% of posterior probability. The branch lengths represent the rate of nucleotide substitution per site.

DISCUSSION

The species *Rhodomonas* sp. 1 and *Rhodomonas* sp. 2 coexist in the same environment. Strains of both species were isolated from the same collection site at the same date. The first strain from Cananéia of *Rhodomonas* sp. 1 was isolated in 1990 by serial dilution technique. This strain is deposited in BMA&K culture collection under the access number BMAK39. Both species are morphologically very similar under light microscopy observations and the cells sizes overlap. In SEM and TEM observations, both species showed a small furrow, and cellular arrangement of the organelles are the same between these species. However, *Rhodomonas* sp. 2 showed a more serrated periplast structure in TEM while *Rhodomonas* sp. 1 has a slight mid-basal band in SEM. Additionally, in light microscopy, cells of *Rhodomonas* sp. 2are slightly more rounded-ovoid than *Rhodomonas* sp. 1, but it is not possible to differentiate these species just using light microscopy data.

Rhodomonas sp. 3 is the most different species among the new proposed herein. The superficial periplast morphology resembles the morphospecies described as *Rhodomonas maculata* Butcher ex D.R.A.Hill & R.Wetherbee and *Rhodomonas duplex* in Hill and Wetherbee (1989) regarding the presence of a pronounced mid-ventral band and the larger furrow. However, cells of *R. duplex* are described as possessing two plastidial complexes, allied to the fact that the cell size is smaller than *Rhodomonas* sp. 3. *Rhodomonas maculata* has cells length very close to *Rhodomonas* sp. 3, but *R. maculata* cells width is smaller.

Rhodomonas sp.2 is a sister group of *Rhinomonas pauca* type strain sequence and it possess a small furrow, while in *Rhinomonas pauca* this structure was not found in the species description. Allied to this evidence, the sister clade of *Rhinomonas nottbecki* Rintala, Majaneva, Remonen & Blomster contains several authentic strains sequences of different morphological species, such as *Rhodomonas chrysoidea* Butcher ex Hill & Wetherbee (NIES-701), *Rhodomonas falcata* Hill & Wetherbee (NIES-702), *Rhodomonas atrorosea* Butcher ex Hill & Wetherbee (NIES-699), *Rhodomonas maculata* (CCAP979/14), *Rhinomonas reticulata* var. *reticulata* (CCAP979/15). Accordingly, all the last morphological species represents a single clade in our

concatenated dataset and in literature (MAJANEVA et al., 2014). Additionally, One of the examined strains in this study grouped in this clade, and this strain also possess a small furrow. The genus *Rhinomonas* was defined by the absence of furrow and the periplast plates (internal and external) more or less hexagonal. Furthermore, *Rhinomonas* was considered closely related to *Pyrenomonas*/ *Rhodomonas* due to the nucleomorph location, inside the pyrenoid (HILL; WETHERBEE, 1988). If *Rhinomonas* still considered a valid genus, the genus *Rhodomonas* become paraphyletic, as described in this and previous studies (HOEF-EMDEN; MARIN; MELKONIAN, 2002; MAJANEVA et al., 2014).

More studies regarding Pyrenomonadaceae family are necessary aiming to clarify the relation of morphological differences and molecular phylogeny. However, morphological investigations needs to be carefully executed aiming to evaluate if the suspicion concerning the morphological modifications on periplast forms are the result of fixation conditions and biomass collection damages (GANTT, 1971; KUGRENS; LEE, 1987, 1988), resulting in artificial morphological characters. Other variables needs to be considered too, as the sexual reproduction and alternating forms, as described for other taxa, and indicated by previous works (HOEF-EMDEN; MARIN; MELKONIAN, 2002; MAJANEVA et al., 2014).

Previous works indicate that SEM is more suitable for morphological species examination (NOVARINO, 2003, 2005). Additionally, although very important for cellular characterization, TEM was shown to be insufficient for the diagnosis of some cryptophytes species (SANTORE, 1982a, 1982b, 1984; HILL; WETHERBEE, 1989). Therefore, a combined analysis using different microscopy techniques and molecular data is essential for the precise identification of cryptophytes species and detection of different forms, related to life cycle in the same species.

Much more data is needed to be able to uncover the phylogeny and morphology relations in Pyrenomonadaceae, once the samples are very limited and there is only little sampling from tropical environments.

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4 PERSPECTIVES AND FINAL CONSIDERATIONS

Although cryptophytes are easily detectable at class level, species identification is a great challenge and laborious work. Most of cryptophytes described are in the nanoplankton fraction (less than 20µm), and some cells are very diminutive. Allied to it, cells are nude and some kinds of chemical fixation can damage them, therefore they became unrecognizable morphologically. Morphological identification of cryptophytes species is possible only by using different sources of microscopy images to obtain the necessary features. Additionally, the use of molecular tools is also a need, due to presence of dimorphic cell life cycles, in which cells can change its morphology. Authors disagree which traits are valuable for species identification, and many gaps of morphological descriptions are found in the literature. Furthermore, little information is available for cryptophytes from the South hemisphere oceans. Consequently, the diversity of cryptomonads is poorly known worldwide and restricted to limited sampling. In this study, even with limited collections and samples, we gave an initial contribution to a better understanding of the morphological traits, diversity and molecular phylogeny for the group. The contributions of the present study can be summarized as:

- Initial detection of cryptophytes biodiversity in Brazilian coastal waters and understanding of their distribution;
- Discovery of four new species collected on the coast of Brazil, *Hemiselmis aquamarina, Rhodomonas s*p. 1, *Rhodomonas* sp. 2, and *Rhodomonas* sp. 3. Probably co-occurrence of *Rhodomonas* sp. 2 and *Hemiselmis aquamarina* in North Atlantic and North Pacific coastal waters, respectively.
- Description of one new type of phycobiliprotein in *Hemiselmis* species;
- New records of species for marine waters of Brazil, such as *Nephroselmis viridis*, a genus of Chlorophyta which was initially described in Cryptophyceae;
- Contribution to the taxonomy of Pyrenomonadaceae, showing evidence that the genera proposed based on morphology are non monophyletic and their circumscription need to be reviewed;

- Discovery of strains that phylogenetically represents new undescribed clades in Pyrenomonadaceae (*Rhodomonas*) and Geminigeraceae (*Proteomonas*) that must be morphologically investigated for the new species description.

Cryptophytes are important as food source for planktonic heterotrophic organisms and are often used as food source in aquaculture. Consequently, is common to have reports of cryptophytes blooms that were followed by blooms of their predators. Additionally, many organisms are described to harbor kleptoplastids from cryptophytes. Some of the cryptophytes plastids hosts are toxic and capable to form toxic blooms. Accordingly, understanding and describing cryptophytes biodiversity can help to predict the occurrence of co-evolved organisms, which can have negative impact for human populations.

Annex 1: *NEPHROSELMIS VIRIDIS* (NEPHROSELMIDOPHYCEAE, CHLOROPHYTA), A NEW RECORD FOR THE ATLANTIC OCEAN BASED ON MOLECULAR PHYLOGENY AND ULTRASTRUCTURE

Lubiana, K. M. F., Gianesella, S. M. F., Saldanha-Corrêa, F. M. P., Oliveira, M. C. Nephroselmis viridis (Nephroselmidophyceae, Chlorophyta), a new record for the Atlantic Ocean based on molecular phylogeny and ultrastructure. **Marine Biodiversity Records**, v. 10, p. 5, 2017.

ABSTRACT

Nephroselmis is composed by unicellular nanoplanktonic organisms, occurring predominantly in marine environments. Currently, 14 species are taxonomically accepted. *Nephroselmis viridis* was described in 2011 and strains were isolated from Indic and Pacific Oceans. Since then, it was not recorded in other places. A strain was isolated from coastal waters of Brazil by micropipetting and washing, and cultivated in f/2 medium for morphological observations (light, confocal, SEM and TEM) and molecular phylogeny inferences (maximum likelihood and Bayesian). The cells are asymmetrical, have two unequal flagella, one cup-shaped chloroplast with an eyespot, and a large starch covered pyrenoid. Chloroplast thylakoids intrude into the pyrenoid and organic scales cover all cell body and flagella. Molecular phylogeny (18S rRNA) clustered the isolated strain with other *Nephroselmis viridis* sequences, and the species is the sister of the *N. olivacea*, the type species of the genus. Morphology and molecular phylogeny corroborate the strain identification, and it is the first time this species is recorded in Brazil and in the Atlantic Ocean.

Key words: Brazilian coast; 18S rRNA; strain isolation; morphology; biodiversity

BACKGROUND

Nephroselmis was described in 1879 by the typification of *Nephroselmis olivacea* Stein, and initially was allocated into Cryptophyceae (PARKE; RAYNS, 1964). Further studies moved it to Chlorophyta, and Bourelly, in 1970, classified it as Prasinophyceae (NORRIS, 1980). In the last decades, many studies taking into account molecular phylogeny have shown that Prasinophyceae is not monophyletic (MARIN; MELKONIAN, 1994, 2010; STEINKOTTER et al., 1994; NAKAYAMA et al., 1998). Hence, the class Nephroselmidophyceae (Nephrophyceae) was proposed to accommodate the genus (CAVALIER-SMITH, 1993; NAKAYAMA et al., 2007). This class seems to be an early derived clade of the core Chlorophyta (STEINKOTTER et al., 1994; DAUGBJERG; MOESTRUP; ARCTANDER, 1995; NAKAYAMA et al., 1998; TURMEL; OTIS; LEMIEUX, 1999; TURMEL et al., 2009), keeping a high number of ancestral characters.

Currently, 14 species of *Nephroselmis* are taxonomically accepted (GUIRY; GUIRY, 2017), and except for *N. olivacea* which is freshwater, all other species are brackish or marine. The nuclear gene coding for the ribosomal small subunit RNA (18S rDNA) is the most widely used molecular marker for this group (NAKAYAMA et al., 1998, 2007; BELL, 2008; FARIA et al., 2011; YAMAGUCHI et al., 2011; FARIA; KATO; SUDA, 2012). However, sequences for just nine species of this molecular marker are available in Genbank, representing less than 65% of the genus biodiversity.

Nephroselmis viridis Inouye, Pienaar, Suda & Chihara was described in 2011 and strains were isolated from marine waters of Fiji, Japan and South Africa, in the Pacific and Indic Oceans (YAMAGUCHI et al., 2011). In the Atlantic Ocean, just five *Nephroselmis* species were recorded previously, vis., *N. discoidea* Skuja (MENEZES; BICUDO, 2008), *N. fissa* (LACKEY, 1940), *N. minuta* (N.Carter) Butcher (BUTCHER, 1959; DOMINGOS; MENEZES, 1998), *N. pyriformis* (N.Carter) Ettl (MOESTRUP, 1983; STEINKOTTER et al., 1994; BERGESCH; ODEBRECHT; MOESTRUP, 2008), and *N. rotunda* (N.Carter) Fott (BUTCHER, 1959; BELL, 2008). Therefore, here we report for the first time the occurrence of *N. viridis* in Atlantic Ocean, isolated from the coast of Brazil, identified by molecular and microscopy tools.

METHODS

Strain isolation and culturing conditions

The strain was isolated from a water sample collected in coastal area of Ubatuba, São Paulo, Brazil, close to Anchieta Island (23º 35.847'S, 45º 01.70' W), at a depth of 40 meters. In the laboratory, a drop of the water sample was used to select the cell, which was transferred successively to sterile sea water drops until just the desired cell was present. Then, the cell was placed in 3ml of medium, and after one month transferred to higher volume. The isolated strain is being maintained in f/2 medium (without Si stock solution) (Guillard and Ryther 1962), salinity 32-35, temperature 20°C (±1), photoperiod of 12h light /12h dark, and 80 µE $m^{-2}.s^{-1}$ radiation. The strain is deposited in the Microorganisms Collection Aidar & Kutner from Oceanographic Institute, University of São Paulo (strain number BMAK193).

Morphological characterization

Cultures of 1-3 weeks old were used for morphological observations. Living and fixed cells (2% glutaraldehyde) were observed under light microcopy Leica DM 4000 B (Leica Microsystems, Wentzler, Germany), and confocal microscopy Zeiss LSM 440 Axiovent 100 (lp870/543nm) (Carl Zeiss, Jena, Germany). For SEM and TEM, cultures were harvested by centrifugation (3min, 100-150g), and transferred for 90 min to a fixative solution (2% glutaraldehyde plus sodium cacodylate trihydrate 0.1M, and sucrose 0.8M buffer). For the SEM preparation, cells were washed in cacodylate plus-sucrose buffer, and then post-fixed in osmium tetroxide (1%) for 60 min. After that, the cells were washed again in buffer, and dehydrated in an ethanol series (70, 90, 95 and 100%). Finally, the sample was dried to critical point (Balzers CPD 030, Bal-Tec, Vaduz, Liechtenstein) and gold-coated (Balzers SCD 050) for visualization in Zeiss Sigma VP. For TEM, cells were dehydrated in an acetone series (50, 70, 95 and 100%), and after embed in Spurr resin. Lastly, thin sectioned, stained, and observed in Zeiss EM 900.

DNA extraction, amplification, sequencing and molecular phylogeny

Genomic DNA was extracted using *NucleoSpin® Plant* II kit (Macherey-Nagel, Düren, Germany), according to the manufactures instructions. PCRs of 18S (small ribosomal subunit) , ITS 1 and 2 (internal transcribed spacers), 5.8S and partial 28S (large

ribosomal subunit) rRNA were amplified with Platinum® *Taq* DNA polymerase kit (Invitrogen™, Carlsbad, USA) and purified with the GFX Illustra kit (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK), both done in accordance with the manufactures instructions. PCRs programs and primers are available as annex 1. Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems™, Hammonton, NJ, USA) was used for sequencing reactions, and samples were sequenced using a 3730 DNA Analyzer (Applied Biosystems™, Hammonton, NJ, USA)

Sequences were assembled with *Sequencher* 4.7 software (Gene Codes Corporation, Ann Arbor, Michigan, USA), and were used to seek for other sequences in GenBank database. Thirty-four sequences were used in the matrix data (see ANNEX 2). Four sequences of phylogenetically close species were used to root the tree (*Pyramimonas aurea*, *Pseudoscourfieldia marina*, and *Pycnococcus provasolii*). These sequences were chosen based on previous studies of *Nephroselmis* phylogeny (NAKAYAMA et al., 2007; FARIA et al., 2011; YAMAGUCHI et al., 2011; FARIA; KATO; SUDA, 2012). Introns were removed from the data. Dataset alignment was performed in AliView (LARSSON, 2014), using the Muscle algorithm (EDGAR, 2004).. The appropriate evolution method was selected according to JModelTest 2.1.7 analysis (DARRIBA et al., 2012). Maximum likelihood (ML) phylogeny inference was performed in Garli (BAZINET; ZWICKL; CUMMINGS, 2014) using 1000 bootstrap replicates (FELSENSTEIN, 1985), and two searches per run. MrBayes (RONQUIST et al., 2012) was used to perform Bayesian analysis, with nodes confidence supported by posterior probability. Two runs were done consecutively, each one with $4x10^6$ generations, four chains, and sampling at 100 generations. MrBayes generated $8x10^4$ trees, whereas $6x10^4$ were used to build the consensus tree (burn -in $2x10^4$).

RESULTS

SYSTEMATICS

Order NEPHROSELMIDALES

Family NEPHROSELMIDACEAE

Genus *Nephroselmis* Stein 1878

Nephroselmis viridis Inouye, Pienaar, Suda & Chihara, 2011 (Figures 1 and 2)

Description

The cells decant on the flasks bottom and the color of the culture is green in exponential phase and become olive in stationary and senescent phases. (YAMAGUCHI et al., 2011)(Yamaguchi et al. 2011). Cells are flattened when observed in ventral view and almost symmetrical in lateral view, bean-shaped, ranging 5 to 7.5 µm in length and 5.5 to 9µm width (Figures 1 and 2). During the cellular cycle, cells enlarge becoming more rounded, and the first noticeable feature is the expansion of the pyrenoid. The cells reproduce by bisection in the longitudinal axis (Figure 1 b), and sexual reproduction was not observed. Two unequal heterodynamic flagella emerge from a frontal groove, ventrally located (Figure. 1 a, c and 2 a). The bigger flagellum (F1), ranged from 20-27 µm (3-4X), and the smaller flagellum (F2), ranged between 8.5 to 11.5 µm (1-1.5X) (Figure 1 d). The cells commonly coil both flagella around the body when resting (Figure 1 a). An unique green parietal cup-shaped chloroplast was located at cells dorsal face (Figure 1 a, c, d, e and 2 c) which has an eyespot in the anterior/ventral face (not show in figures). The chloroplast has a large sinus in the ventral portion (Figure 1 d), and a big cup-shaped pyrenoid starch sheath is at the dorsal region (Figure. 1 b, c and 2 c).Thylakoids sheets penetrate the pyrenoid (Figure. 2 c). A disc-like structure is located at the dorsal part of the cell (Figure. 1 e and 2 c). The nucleus is located in the right position, near the ventral face (Figure 2 b and c). A single reticulate mitochondrion (Figure 2 b) is situated in the inner part of the chloroplast cavity, and a high number of Golgi vesicles are visible (Figure 2 c and d).

Molecular phylogeny

The 18S rDNA of BMAK193 does not have introns. The sequences of ITS 1 and 2, 5.8S, and partial 28S rRNA obtained in this work were not used to infer phylogeny, due to few sequences of these markers in the Genbank for the genus and the absence for the species. In the alignment matrix of 18S rDNA, *Nephroselmis viridis* strains sequences are 100% identical (DNA matrix is available upon request). Maximum likelihood and Bayesian phylogenetic analysis clustered BMAK193 into *Nephroselmis viridis* strains (Figure 3). It also pointed out that *Nephroselmis* is a monophyletic genus, and *N. viridis* is the sister group of the freshwater species *N. olivacea*.

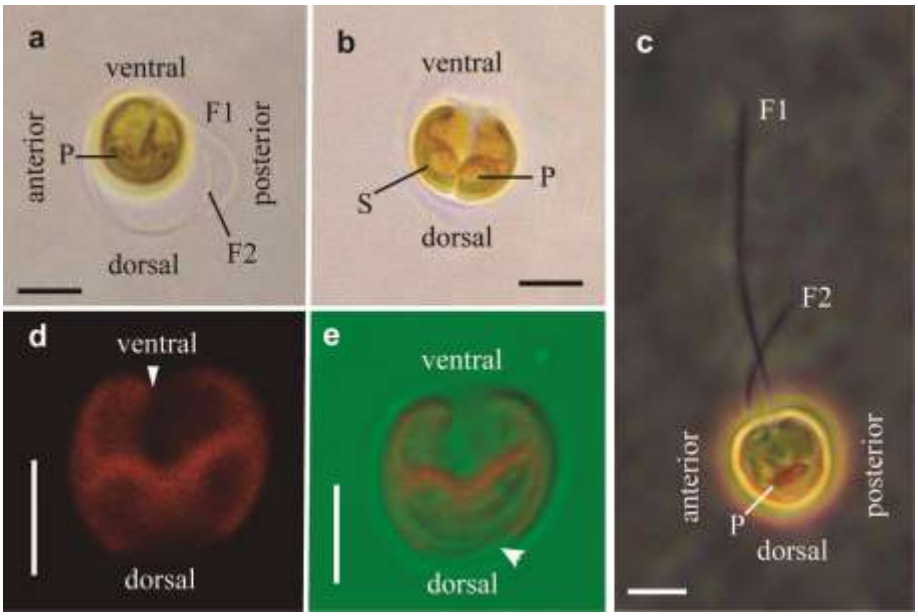

FIGURE 1: *Nephroselmis viridis* morphology by light and confocal microscopy. Scales bars represents 5µm (a) Living cell in bright field coiling the flagella around the body; (b) Living cell in duplication observed in bright field . (c) Fixed cell in phase contrast evidencing the flagella length and pyrenoid . (d) Chloroplast natural fluorescence evidencing the chloroplast sinus (arrow) and pyrenoid . (e) Chloroplast fluorescence and cell morphology showing disk-like structure (arrow). (*F1*) longer flagellum, (*F2*) shorter flagellum, *(P)* pyrenoid. *(S)* starch sheath.

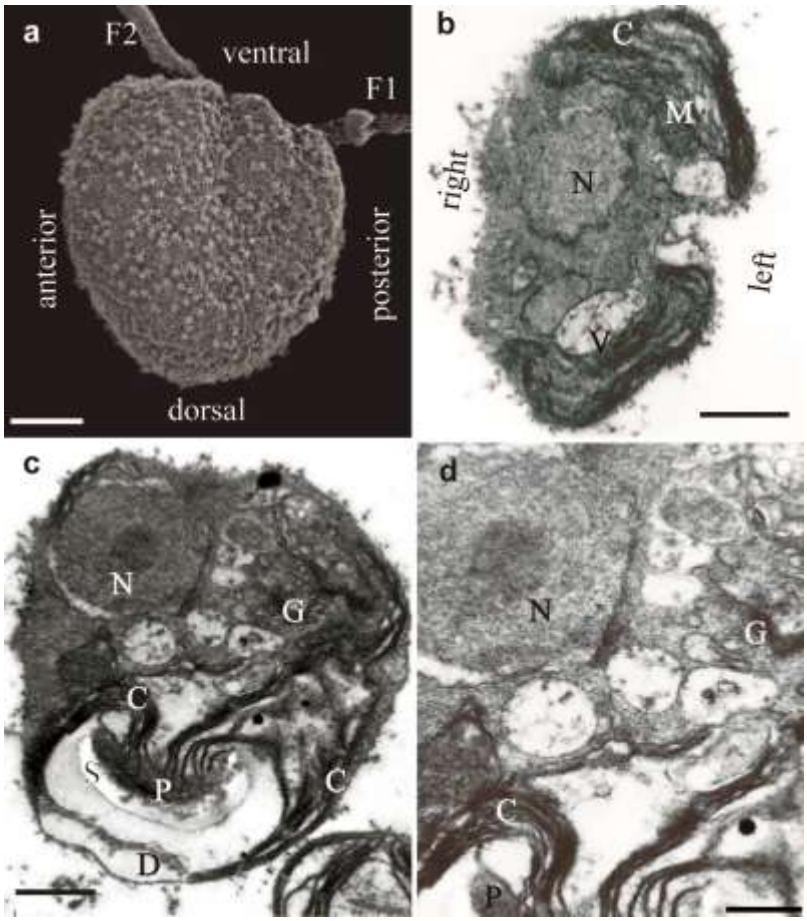

FIGURE 2: *Nephroselmis viridis* morphology by electron microscopy. (a) SEM image showing cell surface and organic scales (Scale bar 1µm). (b) TEM image of the ventral view a cell showing the right nucleus (Scale bar 1 µm). (c) TEM image form the right- anterior view evidencing the organellar placement (Scale bar 1µm). (d) More detailed view of organellar arrangement (Scale bar 0.5µm). *(C)* chloroplast, (*D*) disk-like structure, (*F1*) longer flagellum, (*F2*) shorter flagellum, (*G*) Golgi body, *(M)* mitochondria, *(N)* nucleus, *(P)* pyrenoid. *(S)* starch sheath, and *(V)* vacuoles.

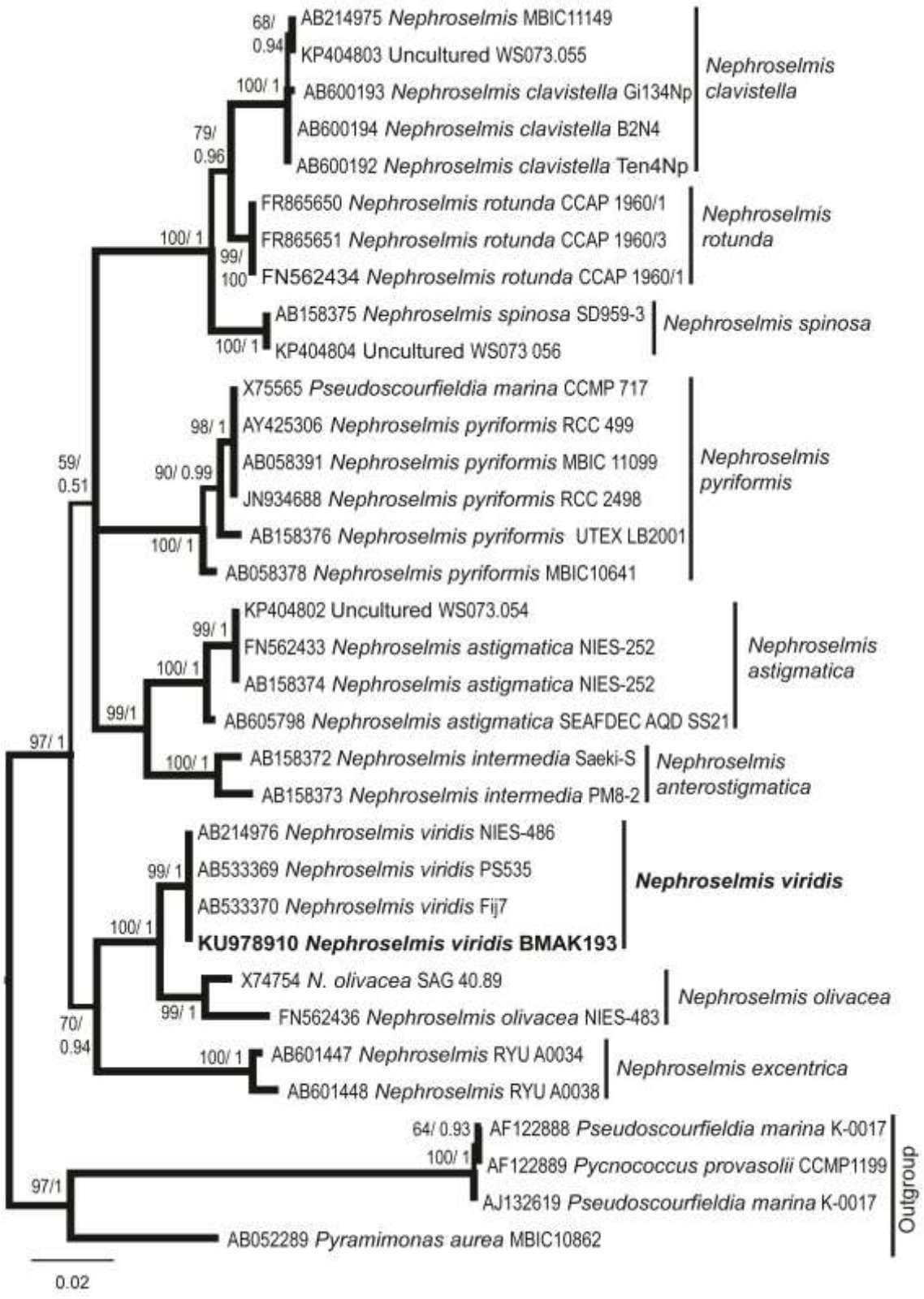

FIGURE 3: *Nephroselmis* maximum likelihood phylogeny tree inferred by 18S rRNA performed with 2000 bootstraps replicates. General time reversible with invariant sites (I= 0.6902) and gamma distribution rate (α= 0.6101) was the evolutionary substitution nucleotide model used (GTR+G+I, InL -5465.48501). Nodes supports are bootstrap/ posterior probability. Branch width represents the node bootstrap support. Scale bar is the rate of nucleotide substitution per

site.

DISCUSSION

The cell measures of *Nephroselmis viridis* from the Atlantic Ocean, such as width and length of cell body and flagella, are exactly the same of the type described in Yamaguchi et al. (2011). Ultrastructural features observed also endorse the identification, such as the chloroplast form and location, the pyrenoid cup shaped and its starch sheath, the thylakoid sheets penetrating into the pyrenoid, the disk like structure, and the positions of the reticulate mitochondrion, nucleus, and Golgi apparatus. The shape and location of these organelles are the same as observed by Yamaguchi et al. (2011). However, the color of the cells and culture are different from the species description. The isolated strain is olive when in stationary and senescent physiological culture stage, different from the green color of the type.

The most common cell shape in *Nephroselmis* species is bean-shaped or semicircular, and symmetrical in anterior/posterior and right/left axis, as in *N viridis* (FARIA et al., 2011; YAMAGUCHI et al., 2011; FARIA; KATO; SUDA, 2012). The cell and flagella size are overlapping in some *Nephroselmis* species. Another common feature widespread in this genus is coiling the flagella around the cell body when cells are resting (SUDA, 2003; FARIA et al., 2011; YAMAGUCHI et al., 2011; FARIA; KATO; SUDA, 2012). For these reasons, *N. viridis* could be easily mistaken with *N. rotunda* in light microscopy investigation. Therefore, for reliable morphological identification ultrastructural information is need.

Molecular markers are more suitable for identification of species, once are less affected by erroneous or incomplete observations and morphological plasticity. The clustering of *Nephroselmis viridis* isolated from coastal waters of Brazil with other *N. virids* strains from Japan, Fiji and South Africa give a clear evidence that they are the same species. The 18S rDNA pointed out that *Nephroselmis* is a monophyletic genus, and *N. viridis* is the sister group of the freshwater species *N. olivacea*, as observed in previous studies (YOSHII et al., 2005; MARIN; MELKONIAN, 2010; FARIA et al., 2011; YAMAGUCHI et al., 2011; FARIA; KATO; SUDA, 2012). The 18S rDNA of BMAK193 does not have introns, such as the strain isolated in Fiji (Fiji7). But, introns

in the 18S rDNA are present in other two strains of *N. viridis*, NIES-486 and PS537, isolated from Japan (YAMAGUCHI et al., 2011).

Other species of *Nephroselmis* were detected in Brazilian coastal waters, such as *N discoidea* (MENEZES; BICUDO, 2008), *N. minuta*,(DOMINGOS; MENEZES, 1998), and *N. pyriformis* (BERGESCH; ODEBRECHT; MOESTRUP, 2008). However, most of the studies performed in South Atlantic Ocean investigate the composition of diatoms and dinoflagellates (Garcia and Odebrecht 2012; Jardim and Cardoso 2013; Lubiana and Dias Júnior 2016, and others). Consequently, the biodiversity status is rarely updated for other groups, as the chlorophytes.

The small cell size, the challenge of morphological identification, and the species tendency to reside in sediments makes *Nephroselmis viridis* detection difficult. However, the species geographic distribution ought to be worldwide, especially in tropical and temperate marine regions (YAMAGUCHI et al., 2011), such as coastal waters of Brazil. Therefore, using an integrate methodology with culturing, morphological description, and molecular phylogeny we contribute to the knowledge of the biodiversity of the Atlantic Ocean, presenting the first record of *Nephroselmis viridis* in coastal waters of Brazil.

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List of Abbreviations:

SEM- scanning electron microscopy

TEM- transmission electron microscopy

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and materials: The DNA sequence generated in this study are available in GENBANK repository, https://www.ncbi.nlm.nih.gov/genbank/. Reference number KU978910. The strain isolated in this study is available in Marine Microorganisms Collection Aydar & Kutner, http://www.io.usp.br/index.php/infraestrutura/banco-de-microorganismos. Reference number BMAK193. The sequence data matrix are available under request to corresponding author. Other data used in this publication are available in annex.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions

KL isolated the strain, obtained morphological and molecular data, and wrote the manuscript. SG assisted article composing. FSC did strain culturing for experiments. MCO draw the experiment, did the phylogenetic analysis, and assisted article composing.

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SUPPLEMENTARY MATERIAL

ANNEX 1: PCRs programs and primers used for amplification and sequencing

PCRs cycles for SSU of initial denaturation at 94°C during 5min. Followed by 35 cycles of: (*i*) 94°C for 30sec; (*ii*) 60°C for 1min and (*iii*) 72°C for 2min. Final extension step at 72°C for 7 minutes. PCRs for ITS, 5.8S and partial LSU were performed as described in (Hoef-Emden & Melkonian 2003).

Table 1: Primers used for PCR and sequencing reactions

Primers for SSU PCR		
	Sequences of oligonucleotides (5'- 3')	
18S5'	CCA CCT GGT TGA TCC TGC CAG T	(SOGIN, 1990)
18S 3'	GAT CCT TCT GCA GGT TCA CCT ACG GAA	(SOGIN, 1990)
Primers for SSU sequencing		
18S5'	CCA CCT GGT TGA TCC TGC CAG T	(SOGIN, 1990)
528F	CGG TAA TTC CAG CTC C	(SOGIN, 1990)
1055F	GGT GGT GCA TGG CCG	(BELLORIN; OLIVEIRA; OLIVEIRA, 2002)
18S 3'	GAT CCT TCT GCA GGT TCA CCT ACG GAA	(SOGIN, 1990)
536R	GAA TTA CCG CGG CTG CTG	(BIRD et al., 1992)
1055R	CGG CCA TGC ACC ACC	(BIRD et al., 1992)
Primers for ITS1 and 2, 5.8S and partial LSU PCR		
SSU1400F	CTG CCC TTT GTA CAC ACC GCC CGT C	(HOEF-EMDEN; MELKONIAN, 2003)
ITS05R-700	TAC TTG TTC GCT ATC GGT CTC T	(HOEF-EMDEN; MELKONIAN, 2003)
Primers for ITS1 and 2, 5.8S and partial LSU sequencing		
SSU1400F	CTG CCC TTT GTA CAC ACC GCC CGT C	(HOEF-EMDEN; MELKONIAN, 2003)
ITS03F-700	CGA TGA AGA ACG YAG CGA	(HOEF-EMDEN; MELKONIAN, 2003)
28S5'R	ATA TGC TTA ART TCA GCG GGT	(BELLORIN; OLIVEIRA; OLIVEIRA, 2002)
ITS05R-800	TAC TTG TTC GCT ATC GGT CTC T	(HOEF-EMDEN; MELKONIAN, 2003)

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ANNEX 2: Sequences used from Genbank and their information

Access #	Genbank Name	Accepted name	Strain	collection site
AB158373	*Nephroselmis intermedia*	*Nephroselmis anterostigmatica*	PM8-2	Unknown
AB158372	*Nephroselmis intermedia*	*Nephroselmis anterostigmatica*	Saeki-S	Japan, Saeki port, Oita Prefecture
KP404802	Uncultured	*Nephroselmis astigmatica*	WS073.054	China, Southwestern south china sea
AB605798	*Nephroselmis astigmatica*	*Nephroselmis astigmatica*	SEAFDEC AQD SS21	Philippines
FN562433	*Nephroselmis astigmatica*	*Nephroselmis astigmatica*	NIES 252	Japan, Chiba port
AB158374	*Nephroselmis astigmatica*	*Nephroselmis astigmatica*	NIES 252	Japan, Chiba port
KP404803	Uncultured	*Nephroselmis clavistella*	WS073_055	China, Southwestern south china sea
AB600193	*Nephroselmis clavistella*	*Nephroselmis clavistella*	Gi134Np	Japan, Ginanzaki, Higashi, Okinawa
AB600192	*Nephroselmis clavistella*	*Nephroselmis clavistella*	Ten4Np	Japan, Teniyazaki, Nago, Okinaea
AB214975	*Nephroselmis* sp.	*Nephroselmis clavistella*	MBIC11149	Republic of Palau, Koror
AB600194	*Nephroselmis clavistella*	*Nephroselmis clavistella*	B2N4	Japan, Bisezaki, Motobu, Okinawa
AB601447	*Nephroselmis* sp.	*Nephroselmis excentrica*	RYU: A0034	Japan, Okinawa-Jima
AB601448	*Nephroselmis* sp.	*Nephroselmis excentrica*	RYU: A0038	Japan, Okinawa-Jima
X74754	*Nephroselmis olivacea*	*Nephroselmis olivacea*	SAG 40.89	Switzerland, Zurich, pond near Thalwiler Waldweiher
FN562436	*Nephroselmis olivacea*	*Nephroselmis olivacea*	NIES-483	Unknown
AB058378	*Nephroselmis pyriformis*	*Nephroselmis pyriformis*	MBIC10641	Pacific
AY425306	*Nephroselmis pyriformis*	*Nephroselmis pyriformis*	RCC 499 (BL_78-7)	Spain, Medirerranean Sea
AB058391	*Nephroselmis pyriformis*	*Nephroselmis pyriformis*	MBIC11099 and CCMP717	Unknown
AB158376	*Nephroselmis pyriformis*	*Nephroselmis pyriformis*	UTEX LB2001	USA, York River, Gloucester Point VA
JN934688	*Nephroselmis pyriformis*	*Nephroselmis pyriformis*	RCC2498	Bering Strait
X75565	*Pseudoscourfieldia*	*Nephroselmis pyriformis*	CCMP 717	USA, TEXAS, Galveston Channel
FR865651	*Nephroselmis rotunda*	*Nephroselmis rotunda*	CCAP 1960 3	England, UK, Tamar Estuary, Cornwall
FN562434	*Nephroselmis rotunda*	*Nephroselmis rotunda*	CCAP 1960 1	Unknown
FR865650	*Nephroselmis rotunda*	*Nephroselmis rotunda*	CCAP 1960 1	Unknown
AB158375	*Nephroselmis spinosa*	*Nephroselmis spinosa*	SD959-3	Australia, Port Hedland, WA
KP404804	Uncultured	*Nephroselmis spinosa*	WS073 056	China, Southwestern south china sea
AB533370	*Nephroselmis viridis*	*Nephroselmis viridis*	Fij7	Fiji
AB533369	*Nephroselmis viridis*	*Nephroselmis viridis*	PS535	Japan: Hyogo, Ieshima Islands
AB214976	*Nephroselmis viridis*	*Nephroselmis viridis*	NIES 486	Japan: Harima-Nada, Seto Inland Sea
KU978910	*Nephroselmis viridis*	*Nephroselmis viridis*	BMAK193	Brazil, Ubatuba, São Paulo
AJ132619	*Pseudoscourfieldia marina*	*Pseudoscourfieldia marina*	K-0017	Norway
AF122888	*Pseudoscourfieldia marina*	*Pseudoscourfieldia marina*	K-0017	Norway
AF122889	*Pycnococcus provasolii*	*Pycnococcus provasolii*	CCMP1199	Gulf of Mexico, USA
AB052289	*Pyramimonas aurea*	*Pyramimonas aurea*	MBIC10862	Republic of Palau