Raphidophyceae (Raphidophyta)

9

Takeo Horiguchi

Abstract

The Raphidophyceae are flagellated unicellular algae that live in diverse marine, brackish, and freshwater habitats. Ten genera are currently recognized: *Gonyostomum*, *Merotricha*, *Vacuolaria*, *Chattonella*, *Chlorinimonas*, *Fibrocapsa*, *Haramonas*, *Heterosigma, Psammamonas*, and *Viridilobus* (the first three are freshwater representatives). They are wall-less heterokonts, i.e., the forward flagellum possesses tubular mastigonemes, and both flagella arise from a shallow pit at or near the apex of the cell. All known raphidophytes are photosynthetic and bear multiple plastids containing chlorophylls *a* and c_1 and/or c_2. With the exception of *Chlorinimonas sublosa*, marine species possess fucoxanthin as a major carotenoid, while freshwater representatives lack this pigment. Marine raphidophytes are widely recognized as ichthyotoxic organisms; species such as *Chattonella* spp., *Fibrocapsa japonica*, and *Heterosigma akashiwo* have been associated with finfish kills. Knowledge of the raphidophyte life cycle, cyst formation, and vertical migratory behavior is important for understanding mechanisms of bloom formation. Molecular phylogenetic analyses suggest that (1) the greenish colored freshwater species diverged from brownish colored marine raphidophytes, (2) all three species of the genus *Haramonas* and a species of *Psammamonas* are sand-dwelling and evolved from a marine planktonic ancestor by acquiring characters of benefit to benthic habitats, (3) *Chlorinimonas* is also sand-dwelling, a characteristic that must have been acquired independently from *Haramonas* and *Psammamonas*, and (4) basal lineages of the Raphidophyceae, *Fibrocapsa, Haramonas*, and *Psammamonas*, possess unique carotenoids such as fucoxanthinol (*F. japonica* and *P. australis*)

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T. Horiguchi (✉)
Department of Biological Sciences, Faculty of Science, Hokkaido University, Sapporo, Hokkaido Prefecture, Japan
e-mail: horig@mail.sci.hokudai.ac.jp

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and 19′-butanoyloxyfucoxanthin (*H. dimorpha*), but the significance of the presence of these pigments is currently unknown.

Keywords
Chattonella • *Fibrocapsa* • Flagellate • *Gonyostomum* • HAB • Heterokontophyta • *Heterosigma* • Ichthyotoxic • Raphidophyceae • Stramenopiles

Contents

Summary Classification

●**Raphidophyceae**
●●**Chattonellales**
●●●**Vacuolariaceae** (e.g., *Chattonella*, *Fibrocapsa*, *Gonyostomum*, *Heterosigma*, *Vacuolaria*, *Viridilobus*)

Introduction

General Characteristics

Members of the Raphidophyceae are flagellate unicellular algae. They are wall-less heterokonts: the forward flagellum (approximately the same length as the cell) bears tubular mastigonemes, and both flagella arise from a shallow pit at or near the apex of the cell (Heywood 1978b; Mignot 1976) (Fig. 1). They live as either motile or palmelloid individuals with a usual length of 10–80 μm. They bear multiple plastids containing chlorophylls *a* and c_1 and/or c_2. Marine species possess a xanthophyll, fucoxanthin, as a major carotenoid, although freshwater representatives lack this

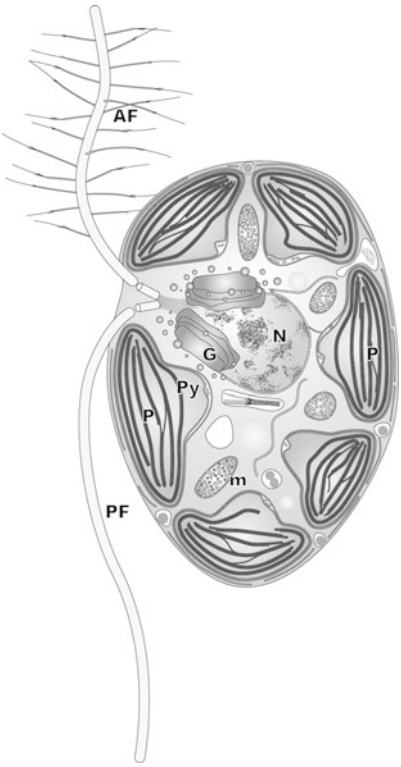

Fig. 1 Schematic illustration of a longitudinal section through a typical marine raphydophyte (*Heterosigma*). A nucleus is surrounded by Golgi body (*G*) and mitochondria (*m*). Plastids (*P*) are located in the periphery of the cell and each chloroplast possesses a projected pyrenoid (*Py*), which is traversed by several thylakoids. The cell possesses an anterior flagellum (*AF*) with tubular mastigonemes and a smooth posterior flagellum (*PF*) (Illustration by Dr. Takeshi Nakayama)

pigment. Sexual reproduction has been documented for a freshwater species (Cronberg 2005; Figueroa and Rengefors 2006). Cyst formation involving alternation of haploid and diploid phases without apparent gamete conjugation in marine raphidophytes has been reported (Yamaguchi and Imai 1994), although another type of cyst formation involving sexual fusion has also been suggested (Demura et al. 2012). The Raphidophyceae is a small group of organisms, with only ten genera (three freshwater and seven marine representatives) currently recognized, all of which are photosynthetic.

Occurrence

Freshwater raphidophyte species usually occur in acidic or neutral pH habitats where the vegetation is abundant. They occur as plankton, among aquatic plants, or adjacent to the mud. Marine species are found in coastal waters, embayments, or in the brackish waters of estuaries. Members of the marine genera *Chlorinimonas*, *Haramonas*, and *Psammamonas* are sand-dwelling (Horiguchi 1996; Yamaguchi et al. 2010; Grant et al. 2013). Although some raphidophyte species are rather rare, e.g., *Haramonas* spp., members of the genera *Gonyostomum*, *Vacuolaria*,

Chattonella, *Heterosigma*, and *Fibrocapsa* are often locally abundant and widely distributed.

Raphidophyte cultures can be obtained from the following sources: Commonwealth Scientific and Industrial Research Organization (CSIRO), The Australian National Algae Culture Collection (ANACC) (Australia), The National Institute for Environmental Studies (NIES) (Japan), The Provasoli-Guillard National Center for Marine Algae and Microbiota (NCMA) (USA), and the Culture Collection of Algae at the University of Texas at Austin (UTEX) (USA). For further information regarding algal collections of the world, see Kasai et al. (2005).

History of Knowledge

Ten genera are currently recognized in the class Raphidophyceae. *Gonyostomum* (Diesing 1865), *Vacuolaria* (Cienkowsky 1870), and *Merotricha* (Mereschkowsky 1879), the three freshwater genera, were first described over a century ago. These genera were grouped into the Chloromonadida (Klebs 1892). Later, Biecheler (1936) recognized that the marine alga *Chattonella* also belongs to this group. Subsequently, the genus *Heterosigma* was established in 1968 by Hada (invalid, no designation of type species) and was later validated by Hara and Chihara (1987). The genus *Fibrocapsa* was established based on material from Japan analyzed by Toriumi and Takano (1973) and the genus *Haramonas* was proposed later (Horiguchi 1996). More recently, the genera *Chlorinimonas* (Yamaguchi et al. 2010), *Viridilobus* (Demir-Hilton et al. 2012), and *Psammamonas* (Grant et al. 2013) have been established.

In the recent phycological literature, these protists are frequently treated as a class of algae, the Raphidophyceae (Heywood 1983; Silva 1980). They have been termed "Chloromonadophyceae" by phycologists and "Chloromonadida" by protozoologists, but these terms are inappropriate since the genus *Chloromonas* does not belong to the Raphidophyceae. Loeblich and Loeblich (1978) include within the family Vacuolariaceae the following genera regarded as valid by most phycologists: *Chattonella*, *Gonyostomum*, *Merotricha*, and *Vacuolaria.* Also included are *Trentonia* and *Swirenkoimonas.* Too little is known to include *Swirenkoimonas* with the raphidophytes. *Trentonia* is probably synonymous with *Vacuolaria* (Fott 1968; Heywood 1983). However, the most controversial aspect of this scheme is treating *Fibrocapsa*, *Heterosigma*, *Olisthodiscus*, and *Hornellia* as synonymous with *Chattonella.* The genus *Hornellia* is probably synonymous with *Chattonella*, since the description of *Hornellia marina* (Subrahmanyan 1954) resembles that of *Chattonella subsalsa* (Biecheler 1936). As noted by Heywood (1990) in the original volume of this handbook, Loeblich and Fine (1977) argued that *Fibrocapsa japonica* (Toriumi and Takano 1973) should be named *Chattonella japonica*, that *Heterosigma inlandica* (Hada 1968) should be named *Chattonella inlandica*, and that *Olisthodiscus luteus* (Carter 1937) should be named *Chattonella luteus.* Heywood (1990) discussed the taxonomic confusion concerning marine raphidophytes at both generic and species ranks. Although Loeblich and Fine

(1977) argued that *Chattonella*, *Heterosigma*, *Fibrocapsa*, and *Olisthodiscus* are congeneric, most researchers now consider these genera to be autonomous (*Olisthodiscus* may in fact not be a raphidophyte at all; see below). In the recent literature, based on molecular phylogenetic study, Yamaguchi et al. (2010) proposed that the class Raphidophyceae should consist of a single order, Chattonellales, which contains a single family, Vacuolariaceae.

Hara and coworkers (Hara and Chihara 1982; Hara et al. 1994) recognized seven species of *Chattonella*, i.e., *C. subsalsa*, *C. antiqua*, *C. marina*, *C. ovata*, *C. minima*, *C. globosa*, and *C. verruculosa*. One original member of the genus *Chattonella*, *C. verruculosa*, is now regarded to be a member of the class Dictyochophyceae and has been transferred to the new genus *Pseudochattonella* (Hosoi-Tanabe et al. 2007). Another new genus, *Verrucophora* was established for a species, formally referred to as *Chattonela* cf. *verruculosa* from the North Sea and the Skagerrak (Edvardsen et al. 2007). The type of species of *Verrucophora*, *V. farcimen*, is closely related to *P. verruculosa*, but not identical. Although Edvardsen et al. (2007) transferred *C. verruculsa* to a new genus, a new nomenclatural combination proposed by Hosoi-Tanabe et al. (2007) seems to have priority. Similarly, another member of the genus *Chattonella*, *C. globosa*, was found to be a member of the Dictyochophyceae and was transferred to a new genus, *Vicicitus* (Chang et al. 2012).

Among true *Chattonella* species, there have also been taxonomic problems. *Chattonella antiqua*, *C. marina*, and *C. ovata* have been distinguished from each other solely based on their morphological characters. Recent genetic analyses, however, revealed that these three species are almost identical (e.g., Bowers et al. 2006; Kamikawa et al. 2007). After careful examination of both morphology and genetic diversity, Demura et al. (2009) concluded that these three species should not be treated as independent species. However, they also found that there were distinct tendencies toward specific differentiation with regard to genetic divergence, morphology, and ecophysiological differences. Therefore, they concluded that these three taxa occupy an intermediate stage between a single, unified species and three distinct and independent species; they proposed to treat them as varieties within a species, i.e., *C. marina* var. *marina*, *C. marina* var. *antiqua*, and *C. marina* var. *obata*. Klöpper et al. (2013) demonstrated that the strains identified as *C. subsalsa* in fact consist of two different species, and the strains from the western Adriatic coast (Mediterranean Sea) more closely match the original species description. Using microsatellite markers, Demura et al. (2014) attempted to reveal putative sources of populations of *C. marina* var. *antiqua* and *C. marina* var. *marina* along Japanese coasts.

A toxic marine species, *Heterosigma akashiwo*, has been the focus of extensive ecological, biochemical, physiological, and molecular studies. Readers are advised to note that in the 1970s and 1980s, this alga was erroneously identified as *Olisthodiscus luteus*, until Hara and Chihara (1987) sorted out the taxonomic confusion. Not like *Heterosigma*, true *Olisthodiscus luteus* (Carter 1937) is benthic and swims without rotating movement. Although often assigned to the class Raphidophyceae, true *Olisthodiscus luteus* is different from members of the class

in many ultrastructural features (Hara et al. 1985; Inouye et al. 1992). Furthermore, preliminary molecular phylogenetic study indicates that *O. luteus* is not a member of the Raphidophyceae (unpublished data by H. Yamaguchi, Yamaguchi et al. 2008). In addition to confusion regarding *O. luteus*/*H. akashiwo*, there has been debate as to which specific epithet should be used. It is now generally accepted that there is only one species in the genus *Heterosigma* and the species name *H. akashiwo* is appropriate and valid (for details, see Hara and Chihara 1987).

Practical Importance

Freshwater raphidophytes are generally rare and, when present, often occur in low densities. However, *Gonyostomum semen* forms dense blooms and affects lakes used for recreation. The alga discharges mucilaginous strands upon contact, thereby covering bathers with a slimy layer causing itching and other allergic reactions (Cronberg et al. 1988; Figueroa and Rengefors 2006). Members of the marine genera *Chattonella*, *Fibrocapsa*, and *Heterosigma* are often locally abundant (Hollande and Enjumet 1956; Subrahmanyan 1954; Hallegraeff and Hara 1995) and are regarded as nuisance algae worldwide.

Marine raphidophytes often cause extensive negative impact on fisheries all over the world. One of the worst cases reported was the killing of >14 million yellowtail fish (*Seriola quinqueradiata*) by *Chattonella antiqua* in Harmina-nada, Seto Inland Sea, Japan, in 1972. This resulted in the loss of 71 billion yen and a loss of 6.3 billion yen was recorded in subsequent years (1977–1979) in the same area (Okaichi 1997). *C. marina* killed 1700 t of bluefin tuna (*Tunnus maccoyii*) (US $40 million loss) in South Australia (Hallegraeff et al. 1998). In New Zealand, significant mortality of Chinook salmon (NZ $17 million loss) caused by *Heterosigma* was documented (Chang et al. 1990).

Habitats and Ecology

Freshwater raphidophytes have been reported from North America (Drouet and Cohen 1935), South America (Skvortzov et al. 1969; Menezes and Bicudo 2010), Australia (Ling and Tyler 2000), Asia (Jao 1978), and Europe (Fott 1968; Kusber 2003; Cronberg 2005). Marine raphidophytes are known from the coasts of all continents except for the Antarctic.

Species of *Gonyostomum* have frequently been reported from the planktonic fraction or from the vicinity of aquatic plants in water of pH 3.2–7.0. *Gonyostomum latum* was found in water of pH 6.7–7.0 (Fott 1968). The most frequently occurring *Gonyostomum*, *G. semen*, has been reported in water of pH 4.4–6.2 (Drouet and Cohen 1935; Heywood 1980); most reports of its occurrence were from the warmer months of the year (e.g., April to October in the northern hemisphere). Since *G. semen* frequently lives in the immediate vicinity of *Sphagnum*, water squeezed from *Sphagnum* moss may provide a good source of this raphidophyte. *G. semen*

seems to have expanded its habitats to more nutrient-rich waters. Blooms of this species appear every summer in many lakes in southern Sweden, in large parts of Finland, Norway, France, and Czech Republic (Cronberg 2005). Recent studies, including genetic analyses, also show expansion of *G. semen* in Northern Europe (Lebret et al. 2013; Hagman et al. 2015). *Vacuolaria* species also occur with aquatic plants in fresh waters of acidic or neutral pH (Heywood 1983). *V. virescens*, the most frequently occurring species, has been reported from water of pH 4.0–8.3 (Graffius 1966), but it is usually found in neutral or slightly acidic conditions. *V. virescens*, reported from bogs, ponds, lakes, and mountain streams (Cienkowsky 1870; Graffius 1966; Poisson and Hollande 1943; Spencer 1971), occurs in the plankton near aquatic plants or in the layer of water adjacent to the bottom mud. *V. virescens*, tolerant of low temperatures, was found to be present in large numbers in a pond with patches of surface ice (Spencer 1971). *V. viridis* has been collected on only a few occasions from swamps and small ponds containing rich aquatic vegetation (Fott 1968). *Merotricha* (only a single species described, *M. bacillata*) has also been found in the plankton or in the vicinity of aquatic plants from bogs, reservoirs, ponds, and the mouth of a river (Graffius 1966; Mereschkowsky 1879; Palmer 1942; Skvortzov et al. 1969).

Chattonella subsalsa, first collected in southern France in organic-rich brackish water (Biecheler 1936), was also present in the port of Algiers, France (Hollande and Enjumet 1956) and in Delaware's Inland Bays, USA (Portune et al. 2009). *C. subsalsa* occurs during the late summer or early autumn in water rich in organic material, frequently at high densities (Biecheler 1936; Hollande and Enjumet 1956; Mignot 1976). *C. antiqua* has been observed from various parts of Japan and has also been found along the Dutch coast (Vrieling et al. 1995). *C. antiqua* was found to grow well at 25 °C, at salinities between 25 ‰ and 41 ‰ under light intensity above 0.04 ly min^{-1}. The pH (7.6–8.3) did not affect growth rate (Nakamura and Watanabe 1983). *C. antiqua* is known to exhibit characteristic diurnal vertical migration, i.e., the cells are concentrated near the surface during day time and near the bottom at night. The species can form red tides during summer, when the thermal stratification is striking and this vertical migration is thought to be advantageous over diatoms. The migratory ascent at daytime keeps them in the euphotic zone and the descent at night provides access to the nutrient-rich bottom waters (Watanabe et al. 1983; Imai and Yamaguchi 2012). Shikata et al. (2013) demonstrated that the blue light regulates diurnal vertical migration behavior in *C. antiqua*. *C. marina* has a wide distribution and has been found in India (Subrahmanyan 1954), Japan (Imai 1989), Hong Kong (Kai et al. 2006), Russia (Morozova and Orlova 2005), a Swedish fjord (Waite and Lindahl 2006), North America (Bowers et al. 2006), Mexico (Band-Schmidt et al. 2004), Australia (Hallegraeff et al. 1998), and New Zealand (Rhodes et al. 2001). *C. ovata* has been reported in Japan (Hara et al. 1994) and Hong Kong (Kai et al. 2006). *C. minima* was originally reported from Seto Inland Sea, Japan, and seems to have very limited distribution. Because of its ability to produce dormant resting cysts, *Chattonella* species seem to adapt well to the temperature regime in temperate seas such as the Seto Inland Sea of Japan where extensive blooms occur (Imai and Itoh 1987). For various aspects of the biology of *Chattonella* spp.,

including biological control of their blooms, see the comprehensive review by Imai and Yamaguchi (2012).

Heterosigma akashiwo also has a global distribution and occurs in subtropical or temperate, marine or brackish waters. Species occurrence includes Canada, Japan, New Zealand, North America, England, Norway, Peru, Portugal, Chile, Singapore, Korea, Ireland, Denmark, China, Spain, Thailand, Namibia, Australia, and Mexico (Ki and Han 2007 and references therein). The optimum pH for growth of *H. akashiwo* was described as 8.5–9.0 (Iwasaki and Sasada 1969). *H. akashiwo* grows well at a salinity range from 20 ‰ to 30 ‰ with maximum growth at 25 ‰ (Haque and Onoue 2002), but the organism can also tolerate low salinity such as <6 (Strom et al. 2013). During the summer, *H. akashiwo* is the dominant species in the phytoplankton of Narragansett Bay, Rhode Island. It grows to maximum densities from May to August when nitrogen concentration is low and phosphate concentration is close to its yearly maximum (Tomas 1979). Laboratory experiments indicate that at saturating and subsaturating nitrogen (N) concentrations, N uptake preference is as follows: $NH_4^+ > NO_3^- >$ urea (Herndon and Cochlan 2007). The organism is known to exhibit characteristic diurnal vertical migration as described for *C. antiqua* (Watanabe et al. 1983; Yamochi and Abe 1984). The occurrence of cysts has been reported (Imai et al. 1993; Kim et al. 2015).

Fibrocapsa japonica, the only species in the genus, was originally isolated in seawater of pH 8.4 and at a temperature of 18.6 °C (Toriumi and Takano 1973). *F. japonica* has a worldwide distribution mainly in coastal warm and cold temperate regions and has been found in North America (Pacific and Atlantic sides), South America (Brazil), Europe (Atlantic and Mediterranean Sea), East Asia, Australia, and New Zealand (De Boer et al. 2005). A culture study using three *F. japonica* strains from different climate regions revealed the species is viable between 4 °C and 32 °C, thus indicating that the species is eurythermal. The species living in cold temperate regions, e.g., the German Wadden Sea, must experience temperatures below 4 °C, and the presence of a resting stage is expected to survive in this region (De Boer et al. 2005).

Three species in the genus *Haramonas* have been described. *H. dimorpha* was found in the bottom sand (mud) of a tropical mangrove river in northeast Australia (Horiguchi 1996) and later it was found in sand samples from Okinawa, subtropical Japan (Horiguchi, unpublished observation). A relatively localized bloom of *H. dimorpha* on the sand surface below the low tide mark on an Australian beach was noted (Chiovitti et al. 2006). *H. viridis* is a cold temperate species described from island of Sylt (eastern North Sea), Germany (Horiguchi and Hoppenrath 2003). The third species, *H. pauciplastida* was found in the beach sand of Vancouver Island, Canada (Yamaguchi et al. 2008). This genus is thus distributed from tropical to cold temperate regions. Another characteristic of *Haramonas* is having dimorphic phases in a life cycle, i.e., spherical nonmotile cells alternate with elongated motile cells (Fig. 2a, b).

The genus *Chlorinimonas* presently contains only one species, *C. sublosa*, which was discovered in sand samples of temperate regions in Japan. In culture, *C. sublosa* stays at the bottom of the culture vessel and does not behave like “typical” plankton.

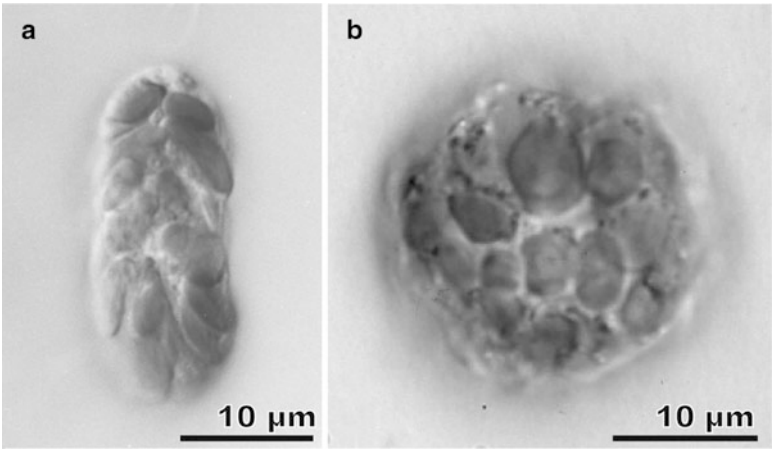

Fig. 2 LM photographs of the marine raphidophyte *Haramonas dimorpha*. (**a**) Typical motile cell. (**b**) Nonmotile spherical cell

The genus *Viridilobus* contains a single species, *V. marinus*, which can form dense blooms in Delaware's Inland Bays in the United States and can even grow when the salinity is almost zero (Demir-Hilton et al. 2012). The genus *Psasmmosa* also consists of a single species, *P. australis*, which is sand-dwelling and possesses two different morphological phases in a cell cycle. It can also produce unique "rafts," formed from 2 to 30 or more cells. The amoeboid movement of cells was also noted (Grant et al. 2013).

Although members of the Raphidophyceae are photosynthetic, mixotrophy, ingestion of bacteria in particular, was observed in *Heterosigma akashiwo* and in *Chattonella ovate*, *C. subsalsa*, and *Fibrocapsa japonica* (see Jeong 2011 and references therein).

Characterization and Recognition

Cell Structure

Raphidophyte cells vary from ovoid or pyriform to approximately spherical in shape; some species are flattened dorsiventrally and bear a furrow on the ventral surface. Biochemical, ultrastructural, and molecular information suggests that raphidophytes belong to the Heterokontophyta (photosynthetic stramenopiles) (e.g., Ali et al. 2002; Horn et al. 2007). The anterior flagellum beats rapidly and is responsible for the forward movement of the cell. The other flagellum moves infrequently and lacks tubular mastigonemes; it trails posteriorly over the ventral surface of the cell.

Plastids of freshwater species are usually bright green in color, while marine representatives are yellowish brown, although there are a few exceptions.

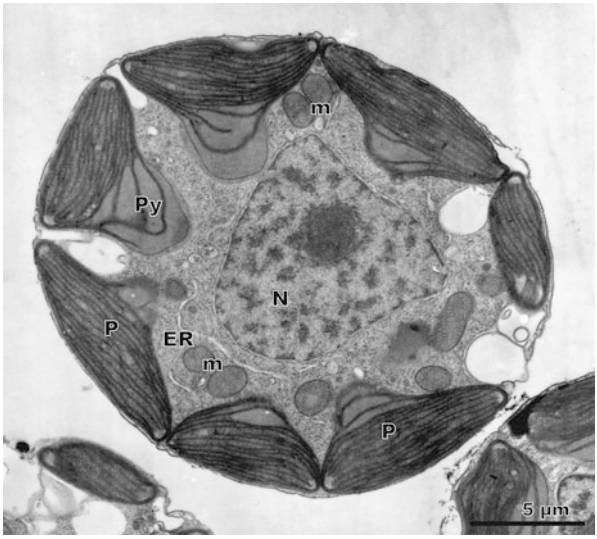

Fig. 3 TEM cross section through the cell of a marine raphidophyte (*Heterosigma akashiwo*) showing the general arrangement of organelles. *ER* endoplasmic reticulum, *N* nucleus, *m* mitochondria, *P* plastid, *Py* pyrenoid (Photograph courtesy of Dr. Yoshiaki Hara)

Chlorophylls *a* and c_1 and/or c_2 are present. The carotenoid pigments of freshwater raphidophytes are β, β-carotene, diadinooxanthin, heteroxanthin, and vaucheriaxanthin (Bjørnland and Liaaen-Jensen 1989). Fucoxanthin has been identified in all marine genera as a major carotenoid (Bjørnland and Liaaen-Jensen 1989), except for one species, *Chlorinimonas sublosa*. In the latter species, like freshwater representatives, no fucoxanthin was detected and diadinoxanthin was identified as a major xanthophyll (Yamaguchi et al. 2010). Distribution of minor carotenoids among marine raphidophytes is variable (Mostaert et al. 1998). Multiple plastids are present in the outer region of the cell between the plasmalemma and the layer of cytoplasm surrounding the nucleus (=exoplasm) (Figs. 1, 2a, b, and 3). Plastids are usually planoconvex or discoid in shape and may attain sizes up to 3 µm wide by 5 µm long. Lamellae, consisting of three thylakoids, extend approximately parallel to the longitudinal axis of the plastid (Figs. 1, 3, and 4a). A girdle band is present in *Gonyostomum*, *Vacuolaria*, and *Heterosigma* (Fig. 1) (Heywood 1980; Hara and Chihara 1987) but typical girdle lamellae appear to be absent in *Chattonella*, *Fibrocapsa*, *Haramonas*, and *Chlorinimonas* (Mignot 1967, 1976; Hara and Chihara 1985; Yamaguchi et al. 2008, 2010). Pyrenoids, present in the plastids of most marine species (Figs. 1, 3, and 4a), have not yet been reported in freshwater species (Heywood 1980; Loeblich and Fine 1977; Mignot 1967, 1976; Hara and Chihara 1982, 1985, 1987; Horiguchi 1996; Horiguchi and Hoppenrath 2003; Yamaguchi et al. 2008, 2010; Demir-Hilton et al. 2012). However, some species of freshwater representatives, e.g., *G. depressum*, may in fact possess a pyrenoid (Fig. 4a, Yoshiaki Hara and Hanae Takahira, personal communication 2013). The reserve food material is suggested to be 1, 3-β-D-glucan, which is comparable to chrysolaminarin of diatoms (Chiovitti et al. 2006). No eyespots have been reported.

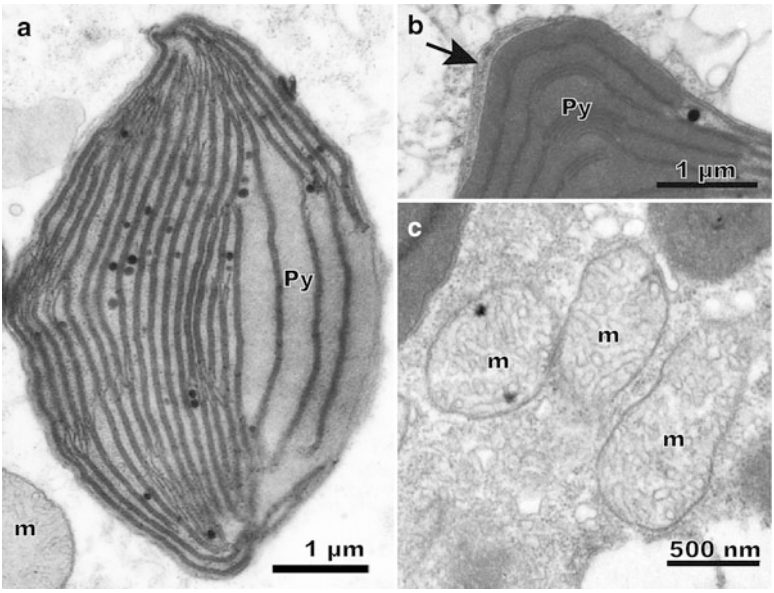

Fig. 4 TEM photographs of selected raphidophytes. (**a**) Close-up of plastid of a freshwater raphidophyte, *Gonyostomum depressum*, showing the presence of a pyrenoid (*Py*). *M* mitochondria (Photograph courtesy of Dr. Yoshiaki Hara and Ms. Hanae Takahira) (**b**) Close-up of the pyrenoid region of *Haramonas viridis*, showing the periplastidal network (*arrow*). (**c**) Close-up of raphidophyte mitochondria (*m*) (*Haramonas dimorpha*), which contains tubular cristae

The plastids of raphidophytes are of secondary endosymbiotic origin as in other heterokont algae. The plastid is surrounded by four membranes: the inner and outer envelope membranes (IEM and OEM), the periplastid membrane (PPM), and the outermost membrane, referred to as the chloroplast endoplasmic reticulum (CER) (Ishida et al. 2000). Small vesicles, termed the periplastidal network (Hibberd 1976), are present between the OEM and PPM at the surface of the projected pyrenoid (Figs. 1 and 4b). The process of plastid division in *Heterosigma akashiwo* was investigated, and it was revealed that an electron-opaque annular structure (plastid-dividing ring or PD ring) girdles the constricting isthmus of the dividing plastids. The inner membranes (IEM and OEM) constrict in advance of the outer two membranes, and the PD ring was observed at the outer surface of the inner pair (Hashimoto 1997). The membrane topology and plastid protein targeting system of *H. akashiwo* was investigated as a model system of organisms with multiple plastids of secondary origin (Ishida et al. 2000). The CER membrane is connected to the endoplasmic reticulum (ER) and in turn, the ER membrane is continuous with outer nuclear envelope. Therefore, the chloroplasts (plastids) of raphidophytes are located within the ER lumen, as in single-plastid containing heterokonts (Ishida et al. 2000). Using an in vitro system, Ishida et al. (2000) hypothesized that nuclear-encoded plastid protein precursors that have been cotranslationally transported into the ER lumen are sorted in the ER and transported to the plastid through the ER lumen

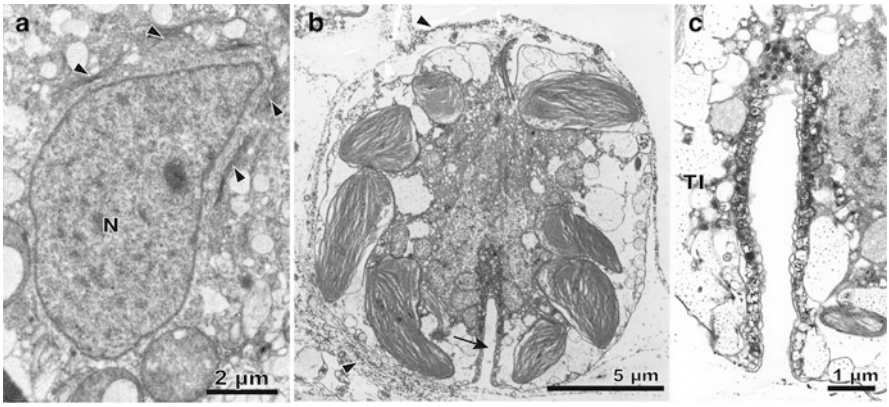

Fig. 5 TEM photographs of the marine raphidophyte *Haramonas* spp. (**a**) Close-up showing a tear-drop shaped nucleus (*N*) and surrounding Golgi bodies (*arrowhead*) in *H. viridis*. (**b**) TEM longitudinal section through a nonmotile cell of *H. dimorpha*, showing the "tubular invagination" (*arrow*). Note that the cell is surrounded by mucilaginous material (*arrowheads*). (**c**) Close-up of the tubular invagination (*TI*) of *H. dimorpha* (Images shown with permission from Phycological Research, Wiley and Sons)

(Ishida et al. 2000; Ishida 2005). *H. akashiwo* has been also used to study various aspects of plastid molecular biology (e.g., Duplessis et al. 2007).

Raphidophyte mitochondria, which possess tubular cristae, are especially numerous in the layer of cytoplasm surrounding the nucleus (Fig. 4c), although some occur in the more peripheral regions of the cell. A distinctive feature is the presence of a large Golgi network over the anterior surface of the nucleus (Figs. 1 and 5a) (Heywood 1980, 1990; Mignot 1967, 1976). A contractile vacuole, which may reach up to 10 µm in diameter, occurs between the Golgi and the kinetosomes in freshwater genera but not marine genera (Heywood 1983; Mignot 1967, 1976; Toriumi and Takano 1973; Hara and Chihara 1982, 1985, 1987; Horiguchi 1996; Horiguchi and Hoppenrath 2003; Yamaguchi et al. 2008, 2010; Demir-Hilton et al. 2012).

Neither scales nor cell walls are present in the raphidophytes, but extracellular material may be produced by extrusome organelles, mucocysts, and trichocysts, which occur in many species. Trichocysts can expel their mucilaginous contents considerable distances (Drouet and Cohen 1935; Toriumi and Takano 1973). Oboe-shaped mucocysts are a characteristic feature of *Chattonella subsalsa* (Biecheler 1936; Klöpper et al. 2013). Material produced by the mucocysts may surround a motile individual with mucilage so that it becomes palmelloid. Members of the genus *Haramonas* produce copious amounts of mucilage (Fig. 5b) (Horiguchi 1996; Horiguchi and Hoppenrath 2003; Yamaguchi et al. 2008).

An unusual structure, the tubular invagination, has been found in all three species of the genus *Haramonas*. The structure can be observed throughout the cell cycle. It opens directly to the outside of the cell (Fig. 5b, c) and appears hollow and devoid of any kind of material. The plasmalemma of the tubular invagination is supported by a

single layer of many underlying small, flattened vesicles, resembling the amphiesma of dinoflagellates (see ▶ Dinoflagellata). These vesicles are, in turn, surrounded by one or two layers of small spherical vesicles, which contain fibrous materials. The function of this structure is currently unknown (Horiguchi 1996; Horiguchi and Hoppenrath 2003; Yamaguchi et al. 2008).

The large nucleus (up to 20 μm in length) and chromosomes (1–12 μm in length at metaphase) have prompted several investigations of nuclear cytology (Heywood 1978a, 1980; Mignot 1967; Poisson and Hollande 1943). Interphase chromatin is often recognizable as fine threads. Chromosomes condense during mitosis and their chromatids become attached to opposite poles by kinetochore microtubules. Spindle microtubules, formed around the kinetosomes, enter the nucleus through gaps at the poles of the nuclear envelope at prophase. By metaphase the chromosomes have become aligned across the equator of the nucleus, and the one or more nucleoli have begun to disperse. Well-spread chromosome preparations from metaphase cells indicate that there are 97 ± 2 chromosomes in *V. virescens* and 65–75 chromosomes in *G. semen* (Heywood 1980). In V. *virescens*, a Golgi and contractile vacuole occur at each pole of the mitotic nucleus; this arrangement ensures their segregation to progeny cells. The original nuclear envelope remains intact over most of its surface until telophase; at this stage new nuclear envelope has begun to be assembled over much of the surface of the chromosome groups (Heywood 1978a). Light microscopic investigation suggests that nuclear envelope behavior is similar in other raphidophytes (Heywood 1978a). Little is known about the biochemistry of raphidophyte nuclei, but their nuclear DNA has been analyzed and found to have a guanine plus cytosine content of 35% in *G. semen* and 34% in *V. virescens* (Rae 1976). Nemoto et al. (1987) reported that light irradiation is necessary for nuclear DNA replication in *Chattonella antiqua* and that the timing of the replication is dependent upon only the timing of the onset of the last irradiation.

Flagella and Flagellar Apparatus

The raphidophytes possess two heterodynamic flagella. The anterior flagellum possesses tripartite tubular mastigonemes, while the posterior flagellum is smooth in surface (Fig. 1) (Karpov 2000). There is no transitional helix in the transition zone of the flagella (Hibberd 1979). Neither flagellar swelling nor flagellar autofluorescence has been detected in the raphidophyte algae (Kawai and Inouye 1989). Only a limited amount of information concerning flagellar apparatuses is available for the Raphidophyceae (Mignot 1967, 1976; Heywood 1980; Vesk and Moestrup 1987; Horiguchi and Hoppenrath 2003; Yamaguchi et al. 2008, 2010). The flagellar root system of *H. akashiwo* was described as comprising three roots, i.e., (1) the rhizoplast, a massive crossbanded fibrous root, which extends from near the proximal ends of both basal bodies to the anterior surface of the nucleus (Fig. 6a), (2) a compound microtubular root with a layered structure, associated with the anterior flagellum and extending the anterior surface, and (3) the rhizostyle, which passes between the two basal bodies leading anteriorly to a vesicle in the flagellar

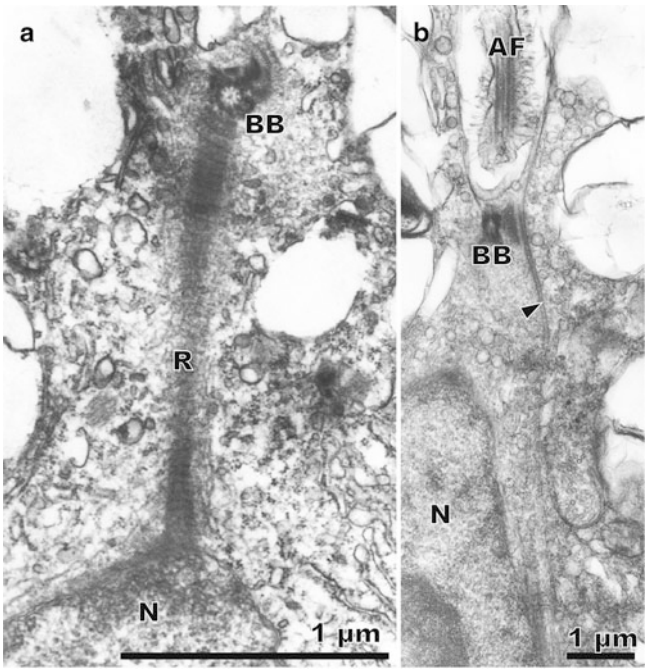

Fig. 6 TEM photographs of the marine raphidophyte *Haramonas viridis*. (**a**) Close-up showing part of the flagellar apparatus, with one of basal bodies (*BB*) and rhyzoplast (*R*) visible, the latter connecting the nucleus (*N*) and basal body. (**b**) Image shows the rhyzostyle (*arrowhead*) running toward the anterior and posterior parts of the cell. *AF* anterior flagellum, *BB* basal body (Images shown with permission from Phycological Research, Wiley and Sons)

groove region and following the nucleus posteriorly, terminating deep in the cytoplasm (Vesk and Moestrup 1987). All raphidophyte species so far examined possess a rhizoplast (Fig. 6a). The presence of a rhizostyle (Fig. 6b) also seems to be a common feature of the class (Vesk and Moestrup 1987; Horiguchi 1996; Horiguchi and Hoppenrath 2003). The presence of a layered structure associated with basal body of the anterior hairy flagellum was reported in *H. akashiwo* (superficially resembling the MLS (multilayered structure) of green plants but with a different structure) (Vesk and Moestrup 1987). This structure has been found in *Chattonella subsalsa* and *Vacuolaria virescens* and *Gonyostomum semen. Haramonas* spp. seem to have similar structures, but details have yet to be confirmed.

Toxicity

Members of marine raphidophytes are widely recognized as ichthyotoxic organisms. The following species have been associated with finfish kills: *Chattonella antiqua*, *C. marina*, *C. subsalsa*, *C. ovata*, *Fibrocapsa japonica*, and *Heterosigma akashiwo*.

The mechanism(s) of toxicity by these raphidophycean flagellates are not fully understood. Production of brevetoxin or brevetoxin-like compounds was reported for *C. antiqua*, *C. marina*, *F. japonica*, and *H. akashiwo* (Khan et al. 1997; Keppler et al. 2006). *C. antiqua*, *C. marina*, and *C. ovata* are known to produce reactive oxygen species (ROS) such as superoxide, hydroxyl radicals, and hydrogen peroxide, and the ROS generated by *Chattonella* spp. was thought to involve gill tissue injury (Ishimatsu et al. 1996; Hiroishi et al. 2005). It was demonstrated that *F. japonica* and *H. akashiwo* also generate superoxide and hydrogen peroxide (Oda et al. 1997). Marshall et al. (2002) demonstrated that *C. marina* cells contain high levels of potentially toxic polyunsaturated fatty acids such as eicosapentaenoic acid (EPA). Later they found that the presence of superoxide together with a low concentration of EPA accelerated fish mortality rates threefold and thus hypothesized that a synergistic effect between ROS and FFA accounts for the ichthyotoxicity of *C. marina* (Marshall et al. 2003). In a study using *C. marina*, *F. japonica*, and *H. akashiwo* (and a few toxic dinoflagellates), Dorantes-Aranda et al. (2015) demonstrated that ROS plays an important role only with *C. marina* and that ROS may also cause a synergistic effect with the lipids in the alga, producing other toxic compounds through lipid peroxidation. They also suggested that other unknown compounds are involved in ichthyotoxicity by *H. akashiwo*, *F. japonica*, and *C. marina*, some of which clearly have a lipid component (Dorantes-Aranda et al. 2015). *H. akashiwo* was known to have allelopathic interactions with a diatom species, *Skeletonema costatum* (Yamasaki et al. 2007).

Life Cycle and Cyst Formation

Members of the Raphidophyceae reproduce asexually by binary fission. Sexual reproduction was demonstrated in a freshwater species, *Gonyostomum semen* (Cronberg 2005; Figueroa and Rengefors 2006). The fusion of gametes was observed under stressed conditions, such as in old cultures or in medium with N or P depletion (Figueroa and Rengefors 2006). The gametes seem smaller and lighter in color than the vegetative cells (Cronberg 2005; Figueroa and Rengefors 2006). There are discrepancies between two reports concerning the sexual process. According to Cronberg (2005), meiosis, i.e., gamete formation, takes place within the cyst (resting cyst) and fused gametes become diploid vegetative motile cells, while Figueroa and Rengefors (2006) reported that the resting cyst is formed by fusion of gametes and a motile diploid vegetative motile cell is released from the resting cyst. The resting cyst is reported to be spherical, 27–39 μm in diameter, and with a few red droplets (Cronberg 2005; Figueroa and Rengefors 2006).

To understand the seasonal occurrence of noxious red tide raphidophytes such as *Chattonella* spp., *F. japonica*, and *H. akashiwo*, information on life cycles and cyst formation is extremely important. Subrahmanyan (1954) documented sexual reproduction and zygote formation in *C. marina* (as *Hornellia marina*), but the fate of the zygote was not observed. As for *Chattonella*, the cysts of this genus were first identified in the Seto Inland Sea, Japan (Imai and Itoh 1986), and it was subsequently

found that the cysts overwinter in the sediments and play an important role in initiating red tides the following summer (Imai and Itoh 1987). The cysts of *Chattonella* are hemispherical in shape with a diameter of 25–35 μm and usually attaching to a solid surface (Imai 1989). Cyst formation was induced by N depletion in the culture medium, and for germination, the cysts required a dormancy period (>4 months) at low temperature (11 °C) (Imai 1989).

By using microfluorometric analysis, Yamaguchi and Imai (1994) reported the life cycle of *Chattonella antiqua* and *C. marina*. The vegetative motile cells are thought to be diploid. The cyst was formed after meiosis, thus the cyst stage is haploid. The germinated small cell becomes a diploid vegetative motile cell, thus suggesting the occurrence of DNA diploidization without cell fusion (asexual diploidization) sometime after excystment (within 2 days) (Yamaguchi and Imai 1994; Imai and Yamaguchi 2012). On the other hand, Nakamura et al. (1990) observed fusion of "small cells (gametes)" and subsequent formation of the cyst (diploid), suggesting the presence of sexual reproduction. Using a microsatellite marker genotyping technique, Demura et al. (2012) confirmed that vegetative cells of 286 strains analyzed were heterozygous for at least some loci and thus diploid. The result suggests that most *Chattonella* strains undergo sexual reproduction. If asexual diplodization were the case, vegetative cells would be expected to be homozygous, even though diploid. The cysts of *F. japonica* were found to be similar in morphology to those of *Chattonella* but smaller (15–20 μm in diameter) and attaching to the solid substrata (Yoshimatsu 1987). Cyst formation in *H. akashiwo* was also reported (Itakura et al. 1996). The cysts, which are covered with sediment particles and can form a cyst cluster, are mostly spherical, about 10 μm in diameter, possessing a distinct wall and a diagnostic feature called the "structure underneath the lid of germination pore" or SLUG (Kim et al. 2015).

Cell Fixation and Molecular Identification of Species

Because of their delicate nature, it can be difficult to fix raphidophyte flagellates without their cell envelope collapsing by commonly used chemical fixatives. Katano et al. (2009) demonstrated that Hepes-buffered paraformaldehyde and glutaraldehyde works well for fixation of *Chattonella* species (and possibly other raphidophytes, too) and that these fixed cells are amenable to flow cytometry.

Members of the Raphidophyceae can easily change morphology, and it is sometimes difficult to identify species with certainty. For example, Imai (2000) reported that in *Chattonella antiqua* cultures, *C. marina*-like cells were occasionally produced. Precise identification of these harmful species is extremely important to fisheries management. Because some of these harmful species seem to have expanded their distribution rather recently, and toxicity can differ between strains, it is important to know the genetic relationships between strains and species located in geographically separated regions. Molecular methods for species identification

have been developed (Connell 2000, 2002; Tyrrell et al. 2001; Akase et al. 2004; Kai et al. 2006; Bowers et al. 2006; Hosoi-Tanabe et al. 2006; Ki and Han 2007; Kamikawa et al. 2007), and microsatellite markers for identification of *Chattonella* spp. (Demura et al. 2007) and *Heterosigma akashiwo* (Nagai et al. 2006) have been developed.

Maintenance and Cultivation

Enrichment of raphidophytes from mixed natural samples has been achieved by phototaxis (Chapman and Haxo 1966; Mignot 1976; Spencer 1971). Clonal cultures have been obtained for most genera (e.g., Heywood 1973; Loeblich and Fine 1977), and in some instances axenic cultures have been established (Cattolico et al. 1976; Iwasaki and Sasada 1969). Raphidophyte cells are usually sufficiently large and distinctive to be distinguished from other protists under a dissecting or inverted microscope and picked out by a micropipette to establish clonal cultures.

Media and conditions for culturing the freshwater species *Gonyostomum semen* and *Vacuolaria virescens* have been described (Chapman and Haxo 1966; Guillard and Lorenzen 1972; Heywood 1973; Spencer 1971). A series of culturing experiments on raphidophytes was reported by Heywood (1973). The medium used in these studies (Table 1) gave satisfactory growth at 22 ± 1 °C when the cultures were aerated with 4% CO_2 in air and were illuminated by Ecko brand 30 W daylight fluorescent tubes at a light intensity of 210 fc. Cultures were maintained in alternating light and dark regimes or in continuous light; under a continuous light regime, a doubling time of 70.5 h was recorded (Heywood 1973). Subsequently, a completely synthetic medium that promoted more rapid growth was developed (Table 2) which allowed a doubling time of 46.0 h at 24 ± 1 °C under continuous light.

For the culturing of marine species, various types of media have been utilized; most such species are easy to maintain in culture. The widely used media include Provasoli's enriched seawater (Provasoli 1968), f/2 culture medium (Guillard 1975), and modified SWM3 medium (Chen et al. 1969; Yamasaki et al. 2007).

Table 1 Composition of GSP medium containing soil and peat extract

KNO_3	100 mg
K_2HPO_4	10 mg
$MgSO_4$-$7H_2O$	10 mg
Ferric citrate	1 mg
Citric acid	1 mg
Soil extract	100 ml
Peat extract	100 ml
Distilled water	800 ml

From Heywood (1990)
pH adjusted between 5.2 and 6.5

Table 2 Composition of raphidophyte medium (in milligrams per liter)

KNO_3	90
K_2HPO_4	29
$MgSO_4{\cdot}7H_2O$	89
NH_4CL	20
$ZnSO_4{\cdot}7H_2O$	20
$CaCO_3$	8
H_3BO_3	1
$MnSO_4{\cdot}4H_2O$	6
$FeSO_4{\cdot}7H_2O$	4
$Na_2MO_42H_2O$	2
$CoSO_45H_2O$	2
$CuSO_45H_2O$	0.1
EDTA	50
Biotin	1
Thiamine	1
Vitamin B_{12}	0.01

From Heywood (1990)
For *Vacuolaria virescens* the pH was adjusted between 6.3 and 6.5
For *Gonyostomum semen* the pH was adjusted between 5.5 and 5.8

Evolutionary History

There is presently no raphidophyte fossil record. Molecular phylogenetic analyses clearly indicate that members of the Raphidophyceae belong to the division Heterokontophyta (autotrophic stramenopiles) (Potter et al. 1997; Ali et al. 2002; Horn et al. 2007). This phylogenetic placement is justified particularly well by the ultrastructure of their flagella, i.e., an anterior flagellum with tubular mastigonemes. However, the exact phylogenetic affinities of the Raphidophyceae to other members of the Heterokontophyta have not been elucidated.

Yamaguchi et al. (2010), Demir-Hilton et al. (2012), and Grant et al. (2013) published phylogenetic trees of the Raphidophyceae based on the SSU rRNA gene, which has been sequenced from representatives of most raphidophyte genera. Figure 7 summarizes the phylogenetic relationships between genera within the class. The genus *Fibrocapsa* appears to have diverged first within the lineage. The three species of *Haramonas* together with *Psammamonas australis* formed a robust clade as the next deepest diverging lineage, followed by a clade containing the three freshwater genera. Sister to the freshwater raphidophyte clade, a clade containing the marine genera *Chlorinimonas*, *Heterosigma*, and *Chattonella* is resolved. The tree allows some insights into the evolution of raphidophycean algae to be inferred. First, the greenish colored freshwater species diverged from brownish colored marine raphidophytes (Figueroa and Rengefors 2006; Yamaguchi et al. 2010). It is highly likely that the freshwater species are derived from a marine raphidophyte, and loss of fucoxanthin and gain of diadinooxanthin, heteroxanthin, and vaucheriaxanthin

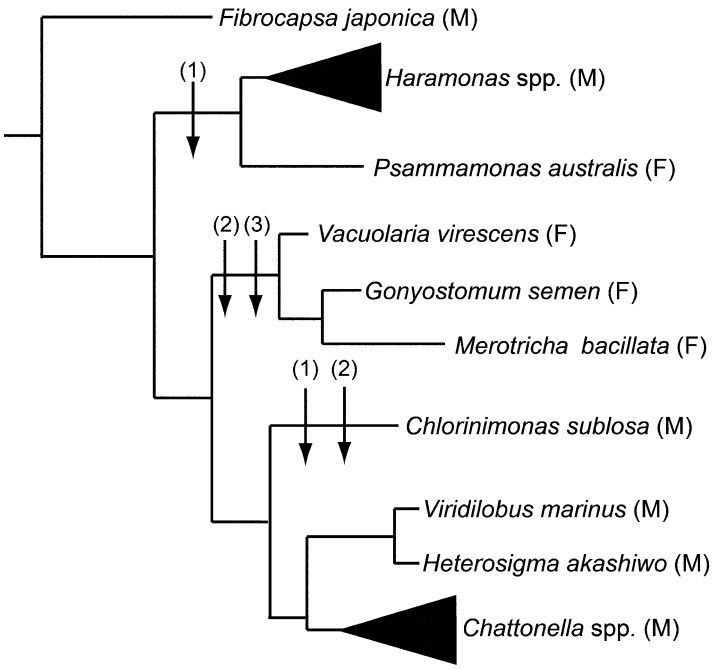

Fig. 7 Schematic diagram depicting the evolutionary relationships between raphidophyte genera based on SSU rDNA phylogenies (see text). (*M*) marine species, (*F*) freshwater species. (*1*) Indicates gain of sand-dwelling habit. (*2*) Indicates loss of fucoxanthin and gain of diadinoxanthin. (*3*) Indicates gain of freshwater-dwelling habit

(Bjørnland and Liaaen-Jensen 1989) took place only once in the lineage leading to freshwater raphidophytes. Second, although being a marine species, *Chlorinimonas sublosa* lacks fucoxanthin and possesses diadinoxanthin like in freshwater representatives. If this SSU-based tree topology is correct, replacement of photosynthetic pigments must have occurred independently in this lineage. Third, all three species of the genus *Haramonas* and a species of *Psammamonas* are sand-dwelling in habit. Since all other marine raphidophytes are planktonic, these three species appear to be derived from a single marine planktonic ancestor, and to have acquired characters that helped them adapted to a benthic habitat. Fourth, *Chlorinimonas* is also sand-dwelling a characteristic it presumably acquired independently from the *Haramonas*/*Psammamonas* lineage. Finally, basal lineages of the Raphidophyceae, viz., *Fibrocapsa*, *Haramonas*, and *Psammamonas*, possess unique carotenoids such as Fucoxanthinol (*F. japonica* and *P. australis*) and 19′-butanoyloxyfucoxanthin (*H. dimorpha*); the significance of the presence of these pigments is currently unknown (Mostaert et al. 1998; Grant et al. 2013). Molecular data from more raphidophyte taxa and additional genes will hopefully provide a more complete framework for understanding the evolutionary history of this fascinating and important algal group.

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