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Taxonomic re-examination of snow-inhabiting species of *Chloromonas* (Volvocales, Chlorophyceae)
（氷雪性クロロモナス属（緑藻綱，ボルボックス目）の種レベルの分類学的再検討）

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学位論文

Taxonomic re-examination of snow-inhabiting species of *Chloromonas* (Volvocales, Chlorophyceae)

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Contents

Abstract

The microalgae which are only active in melting snow or ice are called “snow algae.” The bloom of snow algae stains snow green, yellow, brown, red or other colours, and a large part of snow algae found within green snow and red snow is generally classified as the genus *Chloromonas* (Volvocales, Chlorophyceae). Based on the 1970’s life cycle studies of four snow species of *Chloromonas* with light microscopy of North American field-collected materials, aplanozygote morphology is treated as an important criterion in the taxonomic system of snow-inhabiting species of *Chloromonas*. Among the four species, *Chr. brevispina* and *Chr. nivalis* are thought to be cosmopolitan since the aplanozygotes assignable to these two species are distributed worldwide. Currently, the two species are identified based solely on aplanozygote morphology, because of the difficulty of induction of vegetative cells from the field-collected aplanozygotes. Recent phylogenetic analyses of the field-collected aplanozygotes indicated possible polyphyly of *Chr. nivalis*, but the phylogenetic positions of the aplanozygotes are not robustly resolved, possibly due to the single genes analysed. Although many culture strains of snow *Chloromonas* species have been recently available from public culture collections, their species identifications are lacking or unreliable because aplanozygote formation cannot be easily induced in culture. Therefore, an alternative methodology is required for taxonomy of snow *Chloromonas* species in culture. On the other hand, recent taxonomic studies of green microalgae demonstrated that combined analyses of comparative light and electron microscopy and molecular analyses using cultured materials (i.e. polyphasic methods) are useful for delineating species. However, no snow-inhabiting species of *Chloromonas* have been examined using such polyphasic methods. Thus, both multigene phylogeny of field-collected materials and polyphasic analysis of culture strains are needed to reveal correct species identification and actual diversity of snow species in *Chloromonas*.

In order to evaluate the validity of species identification of field-collected aplanozygotes using robust phylogenetic results, I established a new method to obtain long sequences of multiple regions

of DNA from field-collected aplanozygotes of a single species. Phylogenetic positions of field-collected aplanozygotes identified as *Chr. brevispina* or *Chr. nivalis* were determined with high statistical support values using multigene phylogeny. Additional phylogenetic analyses using highly evolving DNA region showed that cultured material and field-collected aplanozygotes belong to the same species in two lineages. The results demonstrated that both *Chr. brevispina* and *Chr. nivalis* include possible multiple cryptic species.

In order to delineate snow species of *Chloromonas* by using only cultured material, I performed a polyphasic analysis of culture strains of snow-inhabiting *Chloromonas* species with elongate or ellipsoidal vegetative cells. My comparative light and electron microscopy demonstrated that the strains were clearly distinguished into seven species based on differences in vegetative cell shape and chloroplast morphology, the number of zoospores within the parental cell and the presence or absence of cell aggregates in old cultures. The seven species are: *Chr. chenangoensis*, *Chr. fukushimae* sp. nov., *Chr. hohamii*, *Chr. krienitzii* sp. nov., *Chr. pichinchae*, *Chr. tenuis* sp. nov. and *Chr. tughillensis*. Although four of the seven species formed a small clade based on phylogenetic analysis of multiple genes, their separation was supported by comparison of secondary structures of the internal transcribed spacer 2 of nuclear ribosomal DNA and genetic differences of nuclear- and plastid-encoded genes.

The present multigene phylogeny of both field-collected aplanozygotes and culture strains and polyphasic analysis of culture strains demonstrated that both *Chr. brevispina* and *Chr. nivalis*, which are considered cosmopolitan based only on aplanozygote morphology, actually contain multiple cryptic species; one of the present aplanozygote specimens of *Chr. brevispina* was assigned to *Chr. krienitzii*. These results indicate that the species diversity of snow-inhabiting species of *Chloromonas* might have been underestimated for a long time. Therefore, further taxonomic re-examination of snow *Chloromonas* species should be carried out based on light and electron microscopic examinations of culture strains as well as on multigene phylogeny of both aplanozygotes and strains

originating from various regions of the world.

Abbreviations

*atp*B	ATP synthase beta subunit
BI	Bayesian inference
bp	base pairs
BV	bootstrap value
CBC	compensatory base change
CCCryo	Culture Collection of Cryophilic Algae at the Fraunhofer Institute for Cell Therapy and Immunology
Chr.	*Chloromonas*
DIC	Nomarski differential interference optics
hLRT	hierarchical likelihood ratio test
ICN	International Code of Nomenclature for algae, fungi, and plants
ITS2	internal transcribed spacer 2
I + G	the proportion of invariable sites and Γ-distribution
LM	light microscopy
MCMC	Markov chain Monte Carlo
ML	maximum likelihood
MP	maximum parsimony
NIES	National Institute for Environmental Studies
NJ	neighbour-joining
OTU	operational taxonomic unit
PCR	polymerase chain reaction
PP	posterior probability
*psa*B	P700 chlorophyll *a* apoprotein A2

*rbc*L	the large subunit of RuBisCO
rDNA	ribosomal DNA
TEM	transmission electron microscopy
TrNef	equal-frequency Tamura-Nei model
UTEX	the University of Texas at Austin

Chapter 1. General introduction

The remaining snow in mountainous areas and melting snow fields in polar regions are often stained green, yellow, brown, red or other colours. These phenomena are called “coloured snow,” recorded from all continents except for Africa (Kol 1968; Marchant 1982) (Figs 1.1, 1.2). Red snow is especially attractive on the snow surface, sometimes called “watermelon snow” or “blood snow.” In mountainous areas, green snow and yellow snow are generally found in shady areas such as broad-leaved, evergreen or mixed forest and narrow valleys (Fukushima 1963; Kol 1968). Green snow and yellow snow often develop on the spot just under leaf litter, debris and/or dust. On the contrary, brown snow and red snow are frequently observed on the surface of snow in open places which are exposed to direct sunlight for hours (Fukushima 1963; Kol 1968). In polar regions, coloured snow is mainly seen in the vicinity of seabird rookeries (Fritsch 1912; Gain 1912; Broady 1996).

Coloured snow has long been an interesting phenomenon to human beings. Aristotle (BC 384–BC 322), the first scientist, described red snow in his book, *Historia Animalium* (Thompson 1910). In *Shoku Nihongi*, a historiography of Japan compiled in late sixth century, red snow in the north-eastern part of Japan was described (Aoki *et al.* 1990). Charles Darwin (1809–1882), the great scientist who proposed the theory of evolution, observed red snow in Andean Cordillera during the voyage of the Beagle (Darwin 1913).

Based on light microscopic observation by Bauer (1819), coloured snow was revealed to be caused by the bloom of microalgae which are only active in melting snow or ice. Such microalgae are called “snow algae.” Kol (1968) recognised more than 300 species belonging to approximately 140 genera of microalgae in snow and ice, based on light microscopy (LM) of field-collected materials. It is generally considered that colour of the snow is determined by species and life cycle stages of snow algae, and light intensity (Fukushima 1963; Kol 1968). Within green snow, vegetative cells, gametes and/or planozygotes of species of the genus *Chloromonas* (*Chr.*) Gobi (Volvocales, Chlorophyceae) are generally dominant (Kol 1968; Hoham 1980). On the other hand, aplanozygotes

or resting spores of snow *Chloromonas* species accumulate carotenoid pigments within the protoplast and usually dominate in red or reddish snow (Kol 1968; Hoham 1980). Life cycles of snow-inhabiting *Chloromonas* species were elucidated within melting snow or ice (Hoham 1980; Gamache 1990; Jones 1991; Hoham & Duval 2001) (Fig. 1.3). Aplanozygotes or resting spores of snow *Chloromonas* species are produced to tolerate high temperature during summer, since vegetative cells of large part of snow *Chloromonas* cannot survive in mesophilic temperature (Hoham & Duval 2001).

Chloromonas is a unicellular green biflagellate genus that was traditionally distinguished from the genus *Chlamydomonas* Ehrenberg based solely on its pyrenoid-lacking chloroplast (Ettl 1970, 1983). In addition to approximately 130 mesophilic species, more than 10 snow-inhabiting species are recognised (Ettl 1983; Ling & Seppelt 1993, 1998; Hoham *et al.* 2006; Muramoto *et al.* 2010). Because molecular phylogeny revealed that the traditionally defined genus *Chloromonas* is polyphyletic (Buchheim *et al.* 1997), a monophyletic group composed of both pyrenoid-lacking and pyrenoid-containing species (*Chloromonas*-clade; Pröschold *et al.* 2001) within the Volvocales was assigned to *Chloromonas* by Pröschold *et al.* (2001).

Taxonomic studies of snow-inhabiting species of *Chloromonas* were mainly carried out using North American samples. On the basis of a combination of field and laboratory observations of field materials collected in North America, part of the life cycles (from vegetative cell to aplanozygote) of four snow-inhabiting species of *Chloromonas* (*Chr. brevispina* (F.E. Fritsch) Hoham, S.C. Roemer & Mullet, *Chr. hohamii* H.U. Ling & Seppelt, *Chr. nivalis* (Chodat) Hoham & Mullet and *Chr. pichinchae* Wille) were elucidated (Hoham 1975; Hoham & Mullet 1977, 1978; Hoham *et al.* 1979, 1983). The four snow *Chloromonas* species produce aplanozygotes which resemble some snow-inhabiting species of immobile chlorococcalean snow algae, such as *Chodatella* Lemmermann spp. or *Scotiella* Fritsch spp. (Hoham 1975; Hoham & Mullet 1977, 1978; Hoham *et al.* 1979, 1983). It was thus concluded that several previously described snow species of the immobile

chlorococcalean genera are conspecific with aplanozygotes of two snow-inhabiting species of *Chloromonas* and assigned to two species of the genus *Chloromonas*: *Chr. brevispina* and *Chr. nivalis* (Hoham & Mullet 1978; Hoham *et al.* 1979) (Figs 1.4, 1.5) (Table 1.1). Since the field-collected immobile cells morphologically identified as aplanozygotes of *Chr. brevispina* or *Chr. nivalis* are distributed in mountainous areas of Europe, Japan and North and South America as well as in polar regions (e.g. Fritsch 1912; Fukushima 1963; Kol 1968; Hoham & Mullet 1977, 1978; Hoham *et al.* 1979), these two *Chloromonas* species are considered cosmopolitan (Hoham & Mullet 1977; Hoham *et al.* 1979). However, vegetative cells of *Chr. brevispina* and *Chr. nivalis* have been studied using samples collected in only North America (Hoham & Mullet 1977; Hoham *et al.* 1979).

The mesophilic species of *Chloromonas* were traditionally distinguished from each other on the basis of vegetative morphologies such as vegetative cell shape, chloroplast morphology and position of nucleus within the protoplast under LM (Ettl 1970, 1983). In contrast, aplanozygote morphology is treated as an important criterion in the taxonomic identification of snow-inhabiting *Chloromonas* (Remias *et al.* 2010, 2013), based on a series of taxonomic studies using North American material (Hoham 1975; Hoham & Mullet 1977, 1978; Hoham *et al.* 1979, 1983, 2006). For instance, vegetative cells of *Chr. hohamii*, *Chr. pichinchae* and *Chr. tughillensis* Hoham, Berman, H.S. Rogers, Felio, Ryba & P.R. Miller are similar to each other under LM in having ellipsoidal cell shape with rounded posterior end, a cup-shaped chloroplast and a centrally-located nucleus, but aplanozygotes are species-specific among the three species (Hoham 1975; Hoham *et al.* 1983, 2006). According to Hoham *et al.* (1983, 2006), it is difficult to distinguish these three species from each other without information of aplanozygote morphology. On the other hand, *Chr. brevispina* and *Chr. nivalis* are generally identified based only on aplanozygote morphology (e.g. Marchant 1982; Ling 1996; Müller *et al.* 1998; Lukavský & Cepák 2010; Cepák & Lukavský 2013), according to the species concepts by Hoham & Mullet (1978) and Hoham *et al.* (1979). However, vegetative motile cells of these field-collected aplanozygotes have not been reported.

Since the 1970s, cultured materials of snow-inhabiting *Chloromonas* were established and used for taxonomic and phylogenetic studies (Hoham *et al.* 2002, 2006; Muramoto *et al.* 2008, 2010; Remias *et al.* 2010, 2013) and number of culture strains increased in the public culture collections (see below). Hoham *et al.* (2002, 2006) resolved a robust clade composed entirely of snow algae species lacking pyrenoids (subclade 2) within clade A (corresponding to the genus *Chloromonas sensu* Pröschold *et al.* [2001]), using culture strains. Although aplanozygote morphology is an important criterion in the taxonomic system of snow-inhabiting species of *Chloromonas* as described above, induction of aplanozygote formation in culture was reported in only *Chr. tughillensis* (Hoham *et al.* 1998, 2000, 2006). More than 120 strains of snow-inhabiting *Chloromonas* are maintained in the Culture Collection of Algae at the University of Texas at Austin (UTEX) (Starr & Zeikus 1993) and the Culture Collection of Cryophilic Algae at the Fraunhofer Institute for Cell Therapy and Immunology (CCCryo; http://cccryo.fraunhofer.de/web/strains/); however, the cultures of which the taxonomic identification at species level is unclear account for 44.5% of the total of the strains of snow *Chloromonas*, possibly due to the lack of the information of aplanozygote morphology.

Several recent taxonomic studies of snow *Chloromonas* species were performed using both culture strains and field-collected materials as well as molecular phylogeny (Muramoto *et al.* 2008, 2010; Remias *et al.* 2010, 2013). Very recently, Matsuzaki *et al.* (2012, 2013) delineated five mesophilic species of *Chloromonas* based on comparative LM and transmission electron microscopy (TEM), multigene phylogeny and comparison of secondary structures of nuclear ribosomal DNA (rDNA) internal transcribed spacer 2 (ITS2) of cultured materials (i.e. polyphasic methods; Pröschold & Leliaert 2007). However, no snow-inhabiting species of *Chloromonas* have been classified using comparative analyses of LM, TEM and molecular data of culture strains.

Recently, Muramoto *et al.* (2008) obtained gene sequences of the large subunit of RuBisCO (*rbc*L) from the aplanozygotes of *Chr. nivalis* from Japanese mountainous areas, using the single-cell sequencing method (Sebastián & O'Ryan 2001), and demonstrated that the aplanozygotes contained

two haplotypes of *rbc*L gene. The two haplotypes are separated from each other in their phylogenetic analysis, suggesting that the aplanozygotes morphologically identified as *Chr. nivalis* actually include multiple species. However, statistical support values for the phylogenetic positions of haplotypes are quite low, possibly due to their short sequences analysed (340–400 base pairs [bp]). Remias *et al.* (2010) determined 1,746 bp of nuclear-encoded 18S rDNA of "*Chr. nivalis*" collected in Austrian mountainous areas, on the basis of the cloning of polymerase chain reaction (PCR) products from a field-collected sample containing almost exclusively *Chr. nivalis* aplanozygotes. They discussed difference in phylogenetic position between the *Chr. nivalis* aplanozygotes and the two strains labelled as "*Chr. nivalis*" (strains UTEX SNO66 and CCCryo 005-99) based on their 18S rDNA tree. However, objective data for identifying these two strains are lacking and their 18S rDNA data cannot be compared with the *rbc*L results by Muramoto *et al.* (2008). Thus, multiple gene sequence data obtained from a single field-collected aplanozygote sample and a taxonomic system at species level based on only cultured material in snow *Chloromonas* species are needed for correct species identification of the field-collected aplanozygotes. For the aplanozygotes of *Chr. brevispina*, no molecular data have been demonstrated.

The present study was undertaken to establish a taxonomic system to clarify actual species diversity of snow-inhabiting species of *Chloromonas*, on the basis of the use of both field-collected materials and culture strains. In chapter 2, I established a new method to obtain long sequences of multiple regions of DNA from field-collected aplanozygotes of a single species in order to evaluate the validity of species circumscription based on only aplanozygote morphology. In chapter 3, I used an alternative taxonomic methodology to delineate snow-inhabiting species of *Chloromonas* based on LM, TEM and molecular data using only cultured material. On the basis of my polyphasic analyses, eight North American and four Japanese strains were classified into seven species: *Chr. chenangoensis* Hoham, Berman, H.S. Rogers, Felio, Ryba & P.R. Miller; *Chr. fukushimae* Matsuzaki sp. nov.; *Chr. hohamii*; *Chr. krienitzii* Matsuzaki sp. nov.; *Chr. pichinchae*; *Chr. tenuis* Matsuzaki sp.

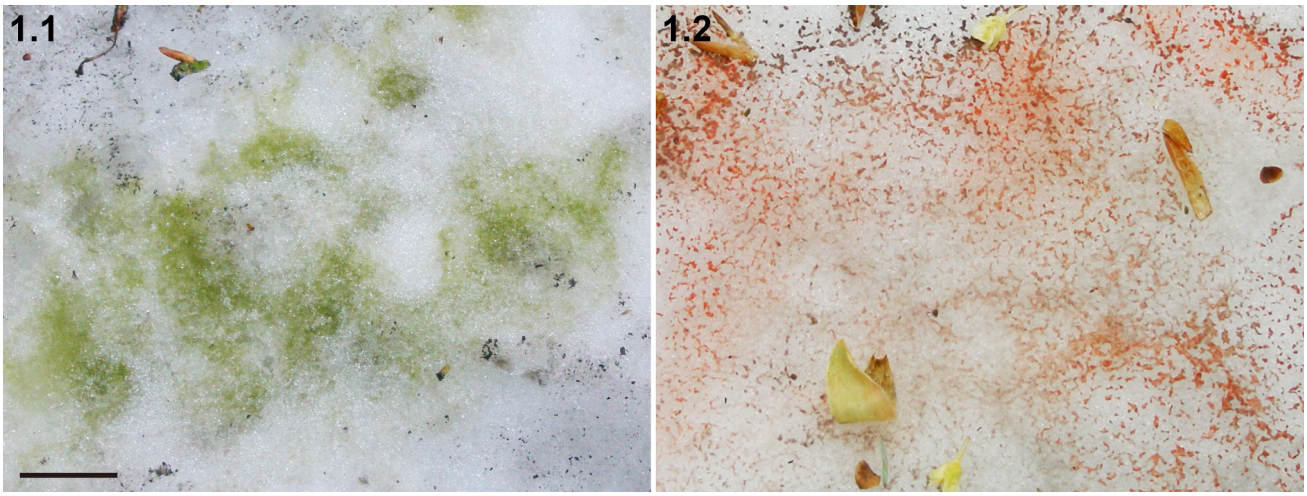
nov.; and *Chr. tughillensis*.

TABLE AND FIGURES

Table 1.1. Synonyms of *Chloromonas brevispina* and *Chr. nivalis*, recognised by Hoham & Mullet (1978) and Hoham *et al.* (1979).

Taxon	Type locality
***Chloromonas brevispina* (F.E. Fritsch) Hoham, S.C. Roemer & Mullet 1979**	
≡ *Chodatella brevispina* F.E. Fritsch 1912 [basionym]	South Orkney, Antarctica
≡ *Cryocystis brevispina* (F.E. Fritsch) Kol 1968 (nom. inval.[1])	
= *Trochiscia nivalis* Lagerheim 1892	Mt. Pichincha, Ecuador
= *Oocystis lacustris* Chodat f. *nivalis* F.E. Fritsch 1912	South Orkney, Antarctica
≡ *Cryodactylon antarctica* Kol 1968 (nom. inval.[1])	
= *Cryodactylon glaciale* Chodat 1921	Great St. Bernard Pass, Switzerland
= *Trochiscia cryophila* Chodat 1921	Great St. Bernard Pass, Switzerland
= *Trochiscia cryophila* f. *longispina* Kol 1942	Alaska, USA
= *Trochiscia cryophila* f. *brevispina* Kol 1942	Alaska, USA
= *Chodatella brevispina* f. *groenlandica* Kol 1959	Cape York, Greenland
≡ *Cryocystis brevispina* f. *groenlandica* Kol 1968 (nom. inval.[1])	
= *Trochiscia cryophila* var. *rubra* Kol 1963	Finse, Norway
≡ *Trochiscia rubra* (Kol) Kol 1968 (nom. inval.[1])	
= *Cryocystis japonica* Kol 1968 (nom. inval.[1])	Nikko National Park, Japan
= *Cryocystis brevispina* f. *fennoscandia* Kol & Eurola 1973 (nom. inval.[1])	Kilpisjärvi, Finland
***Chloromonas nivalis* (Chodat) Hoham & Mullet 1978**	
≡ *Pteromonas nivalis* Chodat 1902 [basionym]	French mountainous areas near the border with Switzerland
≡ *Scotiella nivalis* (Chodat) F.E. Fritsch 1912	
= *Pteromonas willei* Gain 1912 (nom. inval.[2])	Antarctica
= *Scotiella antarctica* F.E. Fritsch 1912	South Orkney, Antarctica
= *Scotiella cryophila* Chodat 1921	Great St. Bernard Pass, Switzerland
≡ *Pteromonas cryophila* (Chodat) Pascher 1927	
= *Chloromonas cryophila* Hoham & Mullet 1977	Mt. Rainier National Park, Washington, USA

[1]According to Hoham *et al.* (1979).
[2]According to Hoham & Mullet (1978).



Figs 1.1, 1.2. Photographs of coloured snow in Japanese mountainous areas, shown at the same magnification. Scale bar = 2 cm.

Fig. 1.1. Green snow found in Mt. Hakkoda, Aomori Prefecture on 3 June 2014.

Fig. 1.2. Red snow found in Mt. Gassan, Yamagata Prefecture on 11 May 2009.

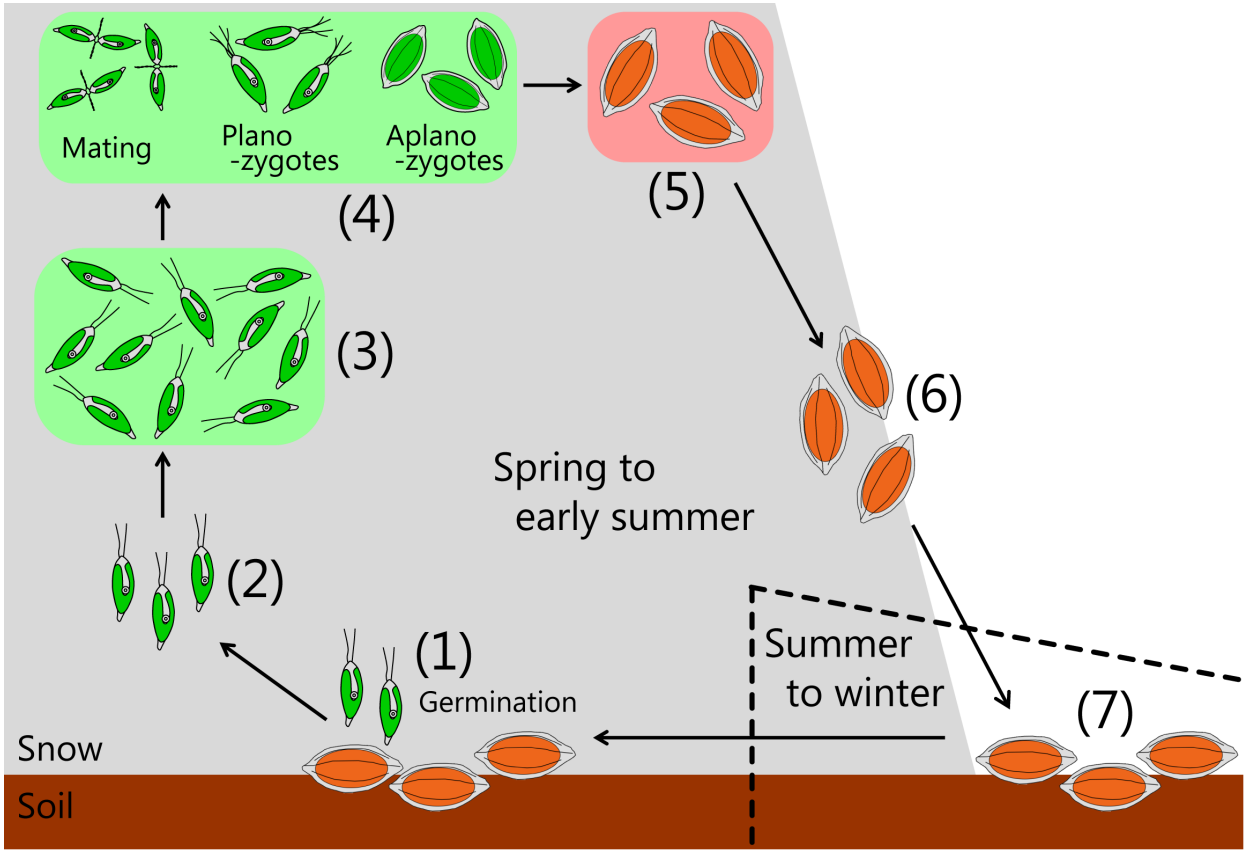


Fig. 1.3. Diagram of possible life history of snow-inhabiting species of *Chloromonas* (based on Hoham & Mullet [1977], Hoham [1980], Gamache [1990], Jones [1991] and Hoham & Duval [2001]).

(1). Meiosis of aplanozygotes and germination of vegetative cells from aplanozygotes.

(2). Active migration of vegetative cells to snow surface through meltwater.

(3). Proliferation of vegetative cells by asexual reproduction, causing green snow.

(4). Sexual reproduction following gamete production.

(5). Accumulation of carotenoid pigments within the protoplast of aplanozygotes, causing red snow.

(6). Passive migration of aplanozygotes to soil surface through melting of snow.

(7). Dormant state.

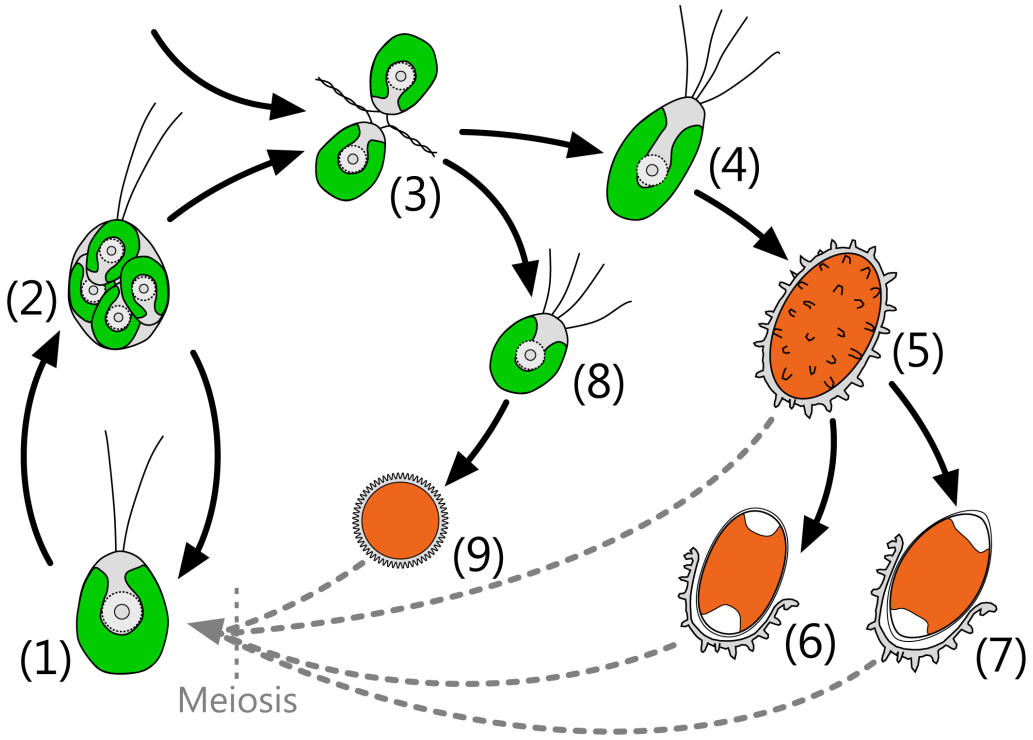


Fig. 1.4. Diagram of possible life cycle of North American *Chloromonas brevispina* (based on Hoham *et al.* [1979]).

(1). Vegetative cell.

(2). Cleavage of vegetative cell which produces zoospores or gametes.

(3). Mating.

(4). Larger planozygote.

(5). Aplanozygote which is identical to *Chodatella* spp. or *Cryocystis* spp. (see Table 1.1).

(6). Aplanozygote which is identical to *Cryodactylon glaciale* after loss of primary wall.

(7). Aplanozygote which is identical to *Oocystis lacustris* f. *nivalis* after loss of primary wall.

(8). Smaller planozygote.

(9). Aplanozygote which is identical to *Trochiscia* spp. (see Table 1.1).

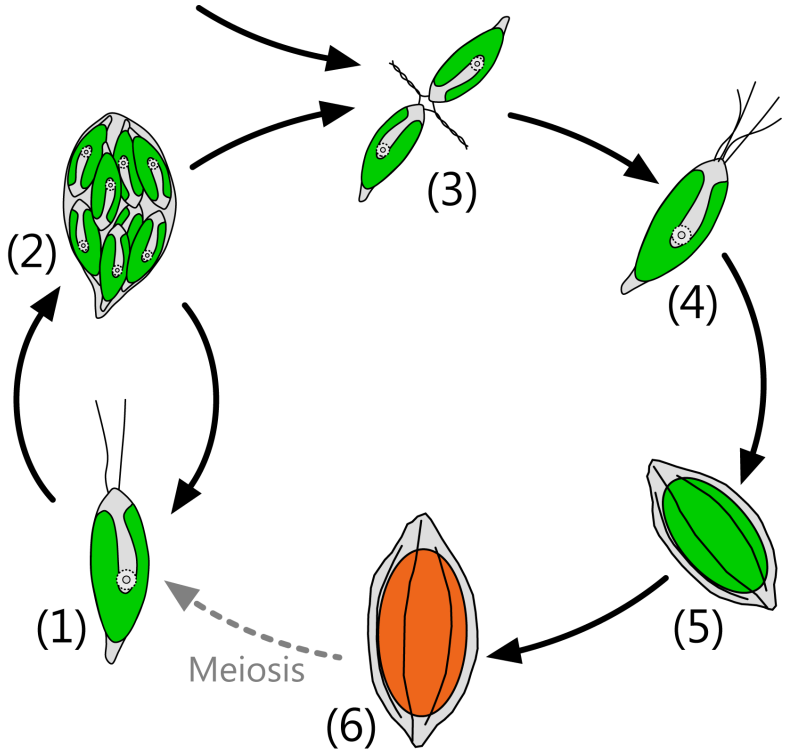


Fig. 1.5. Diagram of possible life cycle of North American *Chloromonas nivalis* (based on Hoham & Mullet [1977, 1978]).

(1). Vegetative cell.

(2). Cleavage of vegetative cell which produces zoospores or gametes.

(3). Mating.

(4). Planozygote.

(5). Aplanozygote which is identical to *Scotiella* spp. (see Table 1.1).

(6). Aplanozygote which enlarges its cell size and accumulates carotenoid pigments within the protoplast.

Chapter 2. Phylogenetic analyses of field-collected aplanozygotes of two "cosmopolitan" species of snow-inhabiting *Chloromonas*

2.1. INTRODUCTION

The life cycles of four snow-inhabiting species of *Chloromonas* (*Chr. brevispina, Chr. hohamii, Chr. nivalis* and *Chr. pichinchae*) were elucidated based on a combination of field and laboratory observation of coloured snow samples collected from various mountainous regions in North America (Hoham 1975; Hoham & Mullet 1977; Hoham *et al.* 1979, 1983). As discussed in chapter 1, *Chr. brevispina* and *Chr. nivalis* are recognised as cosmopolitan species based on only information of aplanozygotes distributed worldwide (e.g. Fukushima 1963; Kol 1968; Hoham & Mullet 1977; Hoham *et al.* 1979). However, the life cycle data demonstrating both vegetative cells and aplanozygotes in the four species have not been reported except for these North American algae (Hoham 1975; Hoham & Mullet 1977; Hoham *et al.* 1979, 1983). Although recent molecular phylogenetic analyses of aplanozygotes of "*Chr. nivalis*" suggested its possible polyphyly (Muramoto *et al.* 2008; Remias *et al.* 2010), their resolutions are not robust possibly due to the single genes analysed.

To solve the robust phylogenetic positions of the *Chloromonas* aplanozygotes collected in snow or ice, I established a new methodology which can provide long sequences of multiple DNA regions from field-collected aplanozygotes. Using the method, robust phylogenetic positions of aplanozygotes of *Chr. brevispina* and *Chr. nivalis* from Japanese field-collected samples were inferred based on three-gene phylogenetic analyses including various strains of snow-inhabiting species of *Chloromonas*.

2.2. MATERIAL AND METHODS

2.2.1. Collection of snow algae samples

Coloured snow samples were collected from the remaining snow in and near the Hakkoda Botanical Garden of Tohoku University, Mt. Hakkoda (Minami-arakawayama, Arakawa, Aomori-shi,

Aomori Prefecture, Japan) and Yamagata Prefectural Nature Conservation Park, Mt. Gassan (Nishikawa-machi, Nishi-murayama-gun, Yamagata Prefecture, Japan) from mid-May 2009 to late-June 2014. Details of the collections are described in Table 2.1. The methods for collection of snow samples and their preservation in the laboratory were essentially the same as those described by Muramoto *et al.* (2010). The coloured snow samples were put in 15-ml or 50-ml Polypropylene Centrifuge Tubes (AGC Techno Glass Corp., Tokyo, Japan) using sterilized stainless-steel or plastic spoon, and stored in a cooler. The samples were carried immediately to the laboratory and maintained at 5°C, with a light:dark cycle of 14 h:10 h under cool-white fluorescent lamps (colour temperature = 5,000 K) at 20–60 μmol m^{-2} s^{-1} intensity. Light microscopic observation of the samples was performed using a BX53 microscope equipped with Nomarski differential interference optics (DIC) or CX21 microscope (Olympus Corp., Tokyo, Japan).

2.2.2. Cultures

For molecular phylogenetic analyses, two Japanese strains of snow-inhabiting *Chloromonas* (*Chloromonas* sp. strains NIES-2379 and NIES-2380) which are closely related to *Chr. miwae* (Fukushima) Muramoto, Nakada, Shitara, Y. Hara & Nozaki (Miwa clade; Muramoto *et al.* 2010) and nine strains of snow-inhabiting *Chloromonas* originating from North America were obtained from the Microbial Culture Collection at the National Institute for Environmental Studies (NIES; Kasai *et al.* 2009) and the UTEX Culture Collection of Algae (Starr & Zeikus 1993), respectively (Table 2.2). In addition, four new culture strains of snow *Chloromonas* species from Japan were included in the phylogenetic analyses (Table 2.2). Cultures were maintained in liquid medium or on 1.5% agar slants containing AF-6 medium (Kato 1982, modified according to Kasai *et al.* 2009) at 5°C, with a light:dark cycle of 14 h:10 h under cool-white fluorescent lamps (colour temperature = 5,000 K) at 20–60 μmol m^{-2} s^{-1} intensity.

2.2.3. DNA extraction

For obtaining a sufficient quantity of DNA sample from field-collected aplanozygotes of a single species, the following method was established in the present study. Before extraction of DNA, the aplanozygotes were cleaned using the modified sterilizing method according to Kawai-Toyooka *et al.* (2004) at room temperature (27°C). The aplanozygotes within a snow sample were collected by centrifugation and sterilized with 150 µl of sterilizing solution (1% [v/v] antiformin containing 0.1% [v/v] Triton X-100) for 30 seconds. The aplanozygotes were then rinsed four times with 150 µl of sterilized Milli-Q water. Fifty morphologically identical aplanozygotes of *Chr. brevispina* or *Chr. nivalis* were isolated from each sample by a micropipette and photographed using a CX21 microscope. The isolated aplanozygotes were washed three times with 500 µl of sterilized Milli-Q water and suspended in 5 µl of sterilized Milli-Q water in a 2.0-ml Graduated Microcentrifuge Tube (Quality Scientific Plastics, California, USA), and the cells were treated by 0.5 µl (10 unit) of Cryonase™ Cold-active Nuclease (Takara Bio Inc., Shiga, Japan) with 5 µl of 25 mM $MgCl_2$ for 30 minutes on ice. Subsequently, the aplanozygotes were subjected to extraction of DNA.

The method for extraction of total DNA from the 50 aplanozygotes (Table 2.1) or culture strain (Table 2.2) was essentially the same as those described in Nakada *et al.* (2008a). Each sample was mixed with 300 µl of Extraction buffer (70 mM Tris-HCl [pH 8.0], 30 mM EDTA [pH 8.0] and 1 M NaCl), 25 µl of 10% CTAB solution including 0.7 M NaCl, and 300 µl of chloroform. The sample was then, shaken with ceramic beads at 25 rotations s^{-1} for 8 minutes using a Mixer Mill MM 300 (Retsch Inc., Haan, Germany). After centrifugation for 2 minutes at 20,400 × g, 200 µl of supernatant containing DNA was transferred to a 1.5-ml Graduated Reaction Tube (Greiner Bio-One Inc., Frickenhausen, Germany). The DNA in the supernatant was extracted using illustra™ blood genomicPrep Mini Spin Kit (GE Healthcare UK Ltd., Buckinghamshire, UK), according to the manufacturer's instructions. Purified DNA was dissolved in 50–250 µl of TE buffer (10 mM Tris-HCl [pH 8.0] and 1 mM EDTA [pH 8.0]) and stored at 4°C.

2.2.4. PCR and sequencing

Partial 18S rDNA, ATP synthase beta subunit (*atp*B), P700 chlorophyll *a* apoprotein A2 (*psa*B) and *rbc*L genes and nuclear rDNA ITS2 region from the total DNA of the specimen of 50 isolated aplanozygotes or the culture strain were amplified by PCR as described by Muramoto *et al.* (2008), with previously and newly designed primers (Table 2.3). For the DNA of the aplanozygote sample, the PCR was carried out in a 30 µl of TaKaRa Taq™ with Mg^{2+} free Buffer (Takara Bio Inc.) reaction mixture prepared according to the manufacturer's protocol. The first reaction was performed in a thermal cycler GeneAmp® PCR System 9700 (Life Technologies Corp., Carlsbad, California, USA) with 35 cycles at 94°C for 2 minutes, 46°C for 3 minutes and 66°C for 3 minutes, followed by 72°C for 15 minutes. The first PCR products were immediately purified using illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare UK Ltd.), and were then used for secondary PCR, carried out as described for the first PCR with 31 cycles. To verify chimera sequences, alternative PCR was run using KOD FX Neo (TOYOBO Corp., Osaka, Japan), according to the manufacturer's instructions with 50 cycles. For the DNA of the culture strain, the PCR was performed as those described in the secondary PCR for the DNA of the specimen. After visualization of the amplified fragment of the PCR products using electrophoresis, the PCR products were purified as described above and sequenced using BigDye® Terminator Cycle Sequencing Ready Reaction Kit v. 3.1 (Life Technologies Corp.).

2.2.5. Phylogenetic analyses based on three-gene sequences

To infer phylogenetic relationships among snow-inhabiting species and field-collected aplanozygotes within the genus *Chloromonas sensu* Pröschold *et al.* or *Chloromonadinia* (Nakada *et al.* 2008b), I used 15 snow algae strains and six specimens of 50 isolated aplanozygotes corresponding to subclade 2 within clade A (Hoham *et al.* 2002, 2006) or SA clade (Matsuzaki *et al.*

2013; ingroup) and 11 strains of mesophilic species (outgroup) belonging to *Chloromonadinia* (Table 2.2). The 18S rDNA sequences were manually aligned according to the previously aligned 18S rDNA sequences (Nozaki *et al.* 2010; available from TreeBASE at http://www.treebase.org/treebase-web/home.html, matrix accession number S10057). The *atp*B and *psa*B exons analysed in the present study corresponded to positions 229–1356 and 274–1665 of the *Chlorella vulgaris* Beijerinck *atp*B and *psa*B genes, respectively (see Nozaki *et al.* 2010). In addition, only the first and second positions of the nucleotides in the *atp*B and *psa*B codons were used to deduce the phylogeny because the third nucleotide position of the codons had an unusual base compositions and markedly higher substitution rates than the 18S rDNA and the first and second positions of the nucleotides in the *atp*B and *psa*B codons (Nozaki *et al.* 2003, 2010; Nakada & Nozaki 2009). Since there was complete identity between the concatenated nucleotide sequences, two strains of *Chr. fukushimae*, two strains of *Chr. krienitzii* and the specimens Gassan-A and Hakkoda-2, three strains of *Chr. tughillensis*, two strains of *Chr. chenangoensis*, the specimens Gassan-B and Hakkoda-3, or *Chloromonas* sp. NIES-2379 and the specimen Gassan-C (Table 2.2) were treated as a single operational taxonomic unit (OTU), respectively.

For comparison, phylogenetic analyses of the 23 OTUs were performed based on 18S rDNA (Fig. 2.1) and the first and second codon positions of *atp*B (Fig. 2.2) and *psa*B (Fig. 2.3), using Bayesian inference (BI), maximum likelihood (ML), maximum parsimony (MP) and neighbour-joining (NJ) analyses, as described by Nakada *et al.* (2008a). BI was carried out using MrBayes 3.2.2 (Ronquist *et al.* 2012) with appropriate substitution model selected by MrModeltest 2.3 (Nylander 2004). Based on hierarchical likelihood ratio tests (hLRT), a general-time-reversible model (Rodrígez *et al.* 1990) estimating the proportion of invariable sites and Γ-distribution (I + G; Gu *et al.* 1995; Waddell & Penny 1996) model was applied for 18S rDNA and for the first and second codon positions of *psa*B gene sequences, and a symmetrical model (Zharkikh 1994) + I + G model was applied for the first and second codon positions of *atp*B gene sequences. Two runs with four chains of Markov chain

Monte Carlo (MCMC) iterations were performed for 1,000,000 generations for alignment of 18S rDNA or the first and second codon positions of *atp*B or *psa*B, respectively. The chain was sampled every 100 generations. The first 25% of the generations were discarded as burn-in, and remaining trees were used to compute a 50% majority-rule tree and the posterior probabilities (PP) of each branch. The average standard deviation of split frequencies of the two MCMC iteration runs was less than 0.01 for each analysis, meaning convergence. ML, MP and NJ analyses were used for estimating bootstrap value (BV; Felsenstein 1985) for individual branches, using PAUP* 4.0b10 (Swofford 2002). On the basis of hLRT using Modeltest 3.7 (Posada & Crandall 1998), the equal-frequency Tamura-Nei model (TrNef; Tamura & Nei 1993) + I + G model was applied for 18S rDNA and for the first and second codon positions of *atp*B and *psa*B genes sequences in ML and NJ analyses. BV for ML and MP analyses was inferred based on 100 and 1,000 replications of full heuristic searches (with the tree bisection-reconnection branch-swapping algorithm), respectively. BV for NJ analysis was calculated based on 1,000 replications. Among these three single-gene trees (Figs 2.1–2.3), a robust discrepancy in the phylogenetic relationships within the SA clade was not detected.

The combined 3,428-bp data matrix for the 18S rDNA and the first and second codon positions of *atp*B and *psa*B genes from the same OTUs (available from TreeBASE, matrix accession number S16764) was subjected to the four phylogenetic methods described above, with two exceptions. For BI, I set three partitions (18S rDNA and first and second codon positions of *atp*B and *psa*B genes) with appropriate substitution models for each partition and applied the “unlink” option. For ML and NJ analyses, the TrNef + I + G model was selected based on hLRT using Modeltest 3.7 (Posada & Crandall 1998) and applied for combined data matrix.

2.2.6. Single-gene phylogeny with wide taxon sampling

Additional OTUs belonging to subclade 2 within clade A (Hoham *et al.* 2002, 2006) or SA clade

(Matsuzaki *et al.* 2013) were selected according to Muramoto *et al.* (2008, 2010), Novis *et al.* (2008), Remias *et al.* (2010, 2013), Raymond (2014) and the website of CCCryo (http://cccryo.fraunhofer.de/web/strains/). Phylogenetic analyses of 18S rDNA or *rbc*L gene sequences including the additional OTUs were carried out by BI, ML, MP and NJ analyses, as described above. For BI of *rbc*L gene sequences, I set three partitions (first, second and third codon positions) as those described by Muramoto *et al.* (2010).

2.2.7. Comparative analysis of nuclear rDNA ITS2

Nuclear rDNA ITS2 regions of each specimen or strain were annotated using Hidden Markov Model-based ITS2 delineation (Keller *et al.* 2009; http://its2.bioapps.biozentrum.uni-wuerzburg.de). Sequences of nuclear rDNA ITS2 regions (Figs 2.4, 2.5) were aligned according to their secondary structures which were predicted using Centroidfold (Hamada *et al.* 2009) and RNAfold at the RNAfold WebServer (Gruber *et al.* 2008; http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) and manually refined (Figs 2.6, 2.7). MP and NJ analyses were performed as described above, with the exception of applying the p-distance for NJ analysis.

2.3. RESULTS

2.3.1. Light microscopy of field-collected aplanozygotes

Aplanozygotes collected from Mt. Gassan and Mt. Hakkoda in Japan could be identified as *Chr. brevispina* and *Chr. nivalis* by the light microscopic characteristics based on the species concepts by Hoham & Mullet (1977, 1978) and Hoham *et al.* (1979). In the aplanozygotes identified as *Chr. brevispina*, two morphological types (*Cryocystis*- and *Trochiscia*-type; Hoham *et al.* 1979) were recognised. The specimen Hakkoda-1 belonged to *Cryocystis*-type (Table 2.1). The aplanozygotes were cylindrical to elongate-ellipsoidal with many large and thick spines on the surface of the cell

wall, measuring 10–14 μm wide and 21–27 μm long (Fig. 2.8). Specimens Gassan-A and Hakkoda-2 were assigned to *Trochiscia*-type (Table 2.1) and could be distinguished from *Cryocystis*-type in having almost spherical cell shape with numerous small spines on the surface of the cell wall. Aplanozygotes of this type were reddish-brown in colour and measured 15–22 μm in diameter (Fig. 2.9). On the other hand, two forms of *Chr. nivalis* aplanozygotes (hereafter, "large type" and "small type") were recognised in the same sample collected in two sites (110517Gs1R and 130630Gs4G) in Mt. Gassan and one site (140603Hk6AR) in Mt. Hakkoda (Table 2.1). The large type was spindle-shaped or ellipsoidal in shape with several flanges developing on the cell wall, measuring 16–24 μm wide and 30–45 μm long. A large quantity of carotenoid pigments was accumulated in the protoplast (Fig. 2.10). The small type was similar to the former type in cell morphology, but cell sizes of the latter type were smaller than those of the former type (measuring 10–16 μm wide and 20–26 μm long). In addition, the latter type lacked visible accumulation of carotenoid pigments within the protoplast (Fig. 2.11). Aplanozygotes of either type were selected as a single specimen or OTU for phylogenetic analysis in the present study (Gassan-B and Hakkoda-3 [the large type] and Gassan-C [the small type]; Table 2.1). Although Hoham & Mullet (1977) reported that the small type aplanozygotes developed into the large type aplanozygotes in North American *Chr. nivalis* (see also Fig. 1.5), the present study could not induce such maturation of aplanozygotes under laboratory conditions.

2.3.2. Multigene phylogenetic analysis of field-collected aplanozygotes

In the phylogenetic analyses based on the combined data set from 18S rDNA and the first and second codon positions of *atp*B or *psa*B gene sequences (Fig. 2.12), two robust monophyletic groups including the present field-collected aplanozygotes were resolved with 1.00 PP in BI and 85–100% BV in ML, MP and NJ analyses (groups A and B). Group A was composed of Miwa clade (Muramoto *et al.* 2010) and *Chr. pichinchae*, and the specimen Gassan-C (the small type

aplanozygotes of *Chr. nivalis*) was positioned in the Miwa clade. Group B was subdivided into two sister lineages; one formed a small clade which included the two strains of *Chr. krienitzii* and the three specimens Gassan-A, Hakkoda-1 and Hakkoda-2 (*Chr. brevispina* aplanozygotes), and the other contained the two specimens Gassan-B and Hakkoda-3 (the large type aplanozygotes of *Chr. nivalis*). Another robust monophyletic group contained *Chr. fukushimae*, *Chr. tughillensis*, *Chr. hohamii* and *Chr. tenuis* (FTH clade), supported with 1.00 PP in BI and 100% BV in ML, MP and NJ analyses. In the present phylogenetic analyses, group A and FTH clade formed a large monophyletic group, although with low support values (50–63% BV in ML, MP and NJ analyses). *Chr. nivalis* strain UTEX SNO66 was sister to this large monophyletic group. Within the FTH clade, *Chr. hohamii* and *Chr. tenuis* were sister to each other (1.00 PP in BI and 63–82% BV in ML, MP and NJ analyses). *Chr. tughillensis* was sister to the clade composed of *Chr. hohamii* and *Chr. tenuis*, and *Chr. fukushimae* was positioned most basally within the FTH clade (52–86% BV in ML, MP and NJ analyses).

To construct phylogenetic trees with wide taxon sampling using published sequence data, I constructed two single-gene trees based on 18S rDNA and *rbc*L gene sequences with additional OTUs (Figs 2.13, 2.14). In both phylogenetic analyses, group B (robustly resolved based on the combined data set from the three genes; see Fig. 2.12) was resolved with moderate to high support values (Figs 2.13, 2.14). Both BI-tree topologies also showed group A (robustly resolved on the basis of the combined data set; see Fig. 2.12). In 18S rDNA-based phylogenetic analysis, group A contained four strains of *Chloromonas* (*Chr.* cf. *alpina* Wille strain CCCryo 032-99, *Chr.* cf. *platystigma* (Pascher) H. Ettl strain CCCryo 020-99, *Chr.* cf. *rostafinskii* (Starmach & Kawecka) Gerloff & H. Ettl strains CCCryo 010-99 and CCCryo 025-99), but its monophyly was not supported with ≥0.95 PP in BI or ≥50% BV in ML, MP or NJ analyses (Fig. 2.13). On the other hand, group A in phylogenetic analysis of *rbc*L gene sequences included the specimen Gassan-4F (field sample of

Chr. miwae; Muramoto *et al.* 2010), and its monophyly was supported with 0.99 PP in BI and 92–98% BV in ML, MP and NJ analyses (Fig. 2.14).

In 18S rDNA-based phylogenetic analysis (Fig. 2.13), the specimens P24/DR4 (aplanozygotes of *Chr. nivalis* from Austrian Alps; Remias *et al.* 2010) and DRAnt023 (cysts of *Chr. polyptera* (F.E. Fritsch) Hoham, Mullet & S.C. Roemer from Antarctica; Remias *et al.* 2013) were included in group B. Within group B, specimens P24/DR4, Gassan-B (the large type aplanozygotes of *Chr. nivalis*), Hakkoda-3 (the large type aplanozygotes of *Chr. nivalis*) and DRAnt023 constituted a monophyletic group, with robust support values (1.00 PP in BI and 81–91% BV in ML, MP and NJ analyses). Within the monophyletic group, the specimen P24/DR4 was sister to the specimen DRAnt023, supported with 64–79% BV in ML, MP and NJ analyses. Since the specimen P24/DR4 (aplanozygotes of *Chr. nivalis* from Austria) was more closely related to the specimen DRAnt023 (*Chr. polyptera* cysts from Antarctica) than the specimens Gassan-B and Hakkoda-3 (the large type aplanozygotes of *Chr. nivalis* from Japan) and branch length between the specimen P24/DR4 and the specimens Gassan-B and Hakkoda-3 was much longer than that between *Chr. hohamii* and *Chr. tenuis* (different species supported by morphological and molecular data; see chapter 3), the two *Chr. nivalis* aplanozygote lineages (specimens P24/DR4 and Gassan-B/Hakkoda-3) should be different species.

In the phylogenetic analysis of *rbc*L gene sequences, the two specimens Gassan-NIV1 and Gassan-NIV2 (*Chr. nivalis* aplanozygotes from Japan; Muramoto *et al.* 2008) were not included in groups A and B (Fig. 2.14), robustly separated from the specimen Gassan-C (the small type aplanozygotes of *Chr. nivalis* from Japan) in group A and the specimens Gassan-B and Hakkoda-3 (the large type aplanozygotes of *Chr. nivalis* from Japan) in group B. Thus, either the specimens Gassan-NIV1 or Gassan-NIV2 should be treated as the different lineages from the specimen Gassan-C and the specimens Gassan-B and Hakkoda-3, respectively. Since phylogenetic positions of

the specimens Gassan-NIV1 and Gassan-NIV2 were not robustly resolved, the separation of these two specimens remained unclear in the present *rbc*L phylogeny.

To obtain further resolution of phylogenetic relationships between field-collected aplanozygotes and their closely related culture strains resolved in my combined data set (Fig. 2.12), phylogenetic analyses within groups A and B were carried out using the rapidly evolving nuclear rDNA ITS2 (Fig. 2.15). Based on the phylogeny of nuclear rDNA ITS2, the specimen Gassan-C (the small type aplanozygotes of *Chr. nivalis*) and *Chloromonas* sp. strain NIES-2379 were sister to each other, and *Chloromonas* sp. strain NIES-2380 was sister to the clade composed of the specimen Gassan-C and *Chloromonas* sp. strain NIES-2379 (100 and 99% BV in NJ and MP analyses, respectively) within group A (Fig. 2.15 top). Within group B (Fig. 2.15 bottom), nuclear rDNA ITS2 sequences of the specimen Gassan-A (*Trochiscia*-type aplanozygotes of *Chr. brevispina*) and *Chr. krienitzii* strain GsCl-54 are identical. *Chr. krienitzii* strain GsCl-49 was sister to the single OTU composed of specimen Gassan-A and *Chr. krienitzii* strain GsCl-54 with low support values (60 and 55% BV in NJ and MP analyses, respectively). The specimens Hakkoda-2 (*Trochiscia*-type aplanozygotes of *Chr. brevispina*) and Hakkoda-1 (*Cryocystis*-type aplanozygotes of *Chr. brevispina*) were most and secondarily basal, respectively, to this small clade, supported with 98 and 75% BV in NJ and MP analyses, respectively. Number of nucleotide differences in nuclear rDNA ITS2 between the specimen Gassan-C and *Chloromonas* sp. strain NIES-2379 is at most one (Fig. 2.6), and that among the specimens Gassan-A and Hakkoda-2 and two strains of *Chr. krienitzii* is zero to two (Fig. 2.7).

2.4. DISCUSSION

My new method to extract DNA from 50 light microscopically identical aplanozygotes from a single snow sample provided more than 4,000 nucleotides of 18S rDNA, *atp*B and *psa*B genes and full length of nuclear rDNA ITS2 region. Based on the present phylogenetic analyses of multiple

genes, the specimens of *Chr. brevispina* aplanozygotes (Gassan-A, Hakkoda-1 and Hakkoda-2) formed a robust clade with two strains of *Chr. krienitzii* (Fig. 2.12). On the contrary, the specimens of *Chr. nivalis* aplanozygotes (Gassan-B, Gassan-C and Hakkoda-3) were apparently polyphyletic forming separate two lineages that were positioned in groups A and B (Fig. 2.12). Moreover, the specimen P24/DR4 (*Chr. nivalis* aplanozygotes from Austria; Remias *et al.* 2010) was positioned within group B in 18S rDNA-based phylogeny (Fig. 2.13) but this specimen was separated from the specimens Gassan-B and Hakkoda-3. In addition, the specimens Gassan-NIV1 and Gassan-NIV2 (*Chr. nivalis* aplanozygotes from Japan; Muramoto *et al.* 2008) were positioned outside of groups A and B in the present phylogenetic analysis of *rbc*L gene sequences (Fig. 2.14). Therefore, aplanozygotes of *Chr. nivalis* from Austria and Japan possibly contain at least four different lineages or cryptic species within the clade composed entirely of snow species of *Chloromonas* (SA clade; Matsuzaki *et al.* 2013).

The specimen Gassan-C (the small type aplanozygotes of "*Chr. nivalis*" from Japan; Fig. 2.11) and *Chloromonas* sp. strain NIES-2379 formed a small clade (Figs 2.12, 2.15). Genetic differences in nuclear rDNA ITS2 between the specimen and the strain are 0.0–0.4% (Fig. 2.6). On the other hand, nucleotide differences in nuclear rDNA ITS2 between strains of *Chr. reticulata* (Goroschankin) Gobi, a monophyletic morphological species of mesophilic *Chloromonas* (Matsuzaki *et al.* 2012), are 3.4–4.1% and that between *Chlamydomonas reinhardtii* P.A. Dangeard strains SAG 11-32a and NIES-2463, which can cross and produce zygotes (Nakada *et al.* 2010a), is 3.3%. Thus, these aplanozygotes and strain should fall within the range of the same biological species on the basis of comparing nuclear rDNA ITS2 sequences. The vegetative cells of the strain NIES-2379 are almost spherical (Muramoto *et al.* 2010), whereas those of *Chr. nivalis* from North America are tear-shaped with a posterior tail (Hoham & Mullet 1977). In addition, the vegetative cell length of the strain NIES-2379 (9–15 μm; Muramoto *et al.* 2010) is significantly shorter than that of North American *Chr. nivalis* (17–34 μm; Hoham & Mullet 1977). Therefore, the specimen Gassan-C and *Chr. nivalis*

from North America (Hoham & Mullet 1977) should be treated as the different species, although the phylogenetic position of the latter is still unknown.

The specimens Gassan-A and Hakkoda-2 (*Trochiscia*-type aplanozygotes of *Chr. brevispina*; Fig. 2.9) were closely related to culture strains of *Chr. krienitzii* (Figs 2.12, 2.15). Since genetic differences in nuclear rDNA ITS2 among the two specimens and the two cultures are 0.0–0.7% (Fig. 2.7) and much smaller than the intraspecific genetic differences in the region of mesophilic *Chloromonas* and *Chlamydomonas* discussed above, specimens Gassan-A and Hakkoda-2 (*Trochiscia*-type aplanozygotes of *Chr. brevispina*) and *Chr. krienitzii* strains can be considered the same biological species. Although the phylogenetic position of *Chr. brevispina* from North America (Hoham *et al.* 1979) has been unclear because of lacking sequence information, *Chr. krienitzii* can be distinguished from North American *Chr. brevispina* in absence of ovoid or pyriform vegetative cells (Hoham *et al.* 1979) (see chapter 3). Thus, this Japanese species (composed of specimens Gassan-A and Hakkoda-2) should be different from the North American specimen of *Chr. brevispina* studied by Hoham *et al.* (1979).

In conclusion, Japanese aplanozygotes identified as *Chr. brevispina* or *Chr. nivalis* are no longer considered the same species reported from North America. Thus, both *Chr. brevispina* and *Chr. nivalis* which were thought to be cosmopolitan based only on aplanozygote morphology actually contain multiple species.

2.5. TABLES AND FIGURES

Table 2.1. List of specimens of field-collected aplanozygotes examined in the present study.

Taxon	Specimen designation	Origin of specimen	Date
Chloromonas brevispina[1]	Gassan-A (*Trochiscia*-type)	Reddish snow sample collected from the site 140628Gs1R in Mt. Gassan, Yamagata, Japan (38°30’25’’N, 139°59’56’’E)	28 Jun 2014
	Hakkoda-1 (*Cryocystis*-type)	Green snow sample collected from the site 090603Hk4G-2 in Mt. Hakkoda, Aomori, Japan (40°39’22’’N, 140°51’1’’E)	3 Jun 2009
	Hakkoda-2 (*Trochiscia*-type)	Reddish snow sample collected from the site 140602Hk2A-2R in Mt. Hakkoda, Aomori, Japan (40°38’51’’N, 140°51’4’’E)	2 Jun 2014
Chloromonas nivalis[2]	Gassan-B (large type)	Reddish snow sample collected from the site 110517Gs1R in Mt. Gassan, Yamagata, Japan (38°30’16’’N, 139°59’52’’E)	17 May 2011
	Gassan-C (small type)	Green snow sample collected from the site 130630Gs4G in Mt. Gassan, Yamagata, Japan (38°30’24’’N, 139°59’52’’E)	30 Jun 2013
	Hakkoda-3 (large type)	Reddish snow sample collected from the site 140603Hk6AR in Mt. Hakkoda, Aomori, Japan (40°39’16’’N, 140°50’59’’E)	3 Jun 2014

[1]Identified based on Hoham *et al.* (1979).
[2]Identified based on Hoham & Mullet (1977).

Table 2.2. List of taxa/specimens/strains in the present phylogenetic analyses (Figs 2.1–2.3, 2.12–2.14) and DDBJ/EMBL/GenBank accession numbers of nuclear-encoded 18S ribosomal DNA (rDNA), ATP synthase beta subunit (*atp*B), P700 chlorophyll *a* apoprotein A2 (*psa*B) and the large subunit of RuBisCO (*rbc*L) genes.

Taxon	Specimen/strain designation	Accession number			
		18S rDNA	*atp*B	*psa*B	*rbc*L
Snow-inhabiting species (ingroup)					
Chloromonas brevispina	Gassan-A[1]	LC012709[2]	LC012717[2]	LC012725[2]	LC012733[2]
	Hakkoda-1[1]	LC012710[2]	LC012718[2]	LC012726[2]	LC012734[2]
	Hakkoda-2[1]	LC012711[2]	LC012719[2]	LC012727[2]	LC012735[2]
Chloromonas chenangoensis[3]	UTEX[4] SNO150[5]	AB906341[2]	AB906360[2]	AB906371[2]	LC012736[2]
	UTEX SNO143	AB734113	AB734114	AB734115	AB434264
					LC012737[2]
Chloromonas fukushimae sp. nov.[3]	GsCl-11[5, 6]	AB906342[2]	AB906361[2]	AB906372[2]	LC012738[2]
	HkCl-65[5, 6]	AB906343[2]	AB906362[2]	AB906373[2]	LC012739[2]
Chloromonas hohamii[3]	UTEX SNO67[5]	AB906344[2]	AB906363[2]	AB906374[2]	AB434265,
					LC012742[2]
Chloromonas krienitzii sp. nov.[3]	GsCl-54[5, 6]	LC012712[2]	LC012720[2]	LC012728[2]	LC012740[2]
	GsCl-49[5, 6]	LC012713[2]	LC012721[2]	LC012729[2]	LC012741[2]
Chloromonas nivalis	Gassan-B[1]	LC012714[2]	LC012722[2]	LC012730[2]	LC012743[2]
	Gassan-C[1]	LC012715[2]	LC012723[2]	LC012731[2]	LC012744[2]
	Hakkoda-3[1]	LC012716[2]	LC012724[2]	LC012732[2]	LC012745[2]
	UTEX SNO66[5]	AB906345[2]	AB906364[2]	AB906375[2]	AB434272
Chloromonas pichinchae[3]	UTEX SNO33[5]	AB906346[2]	AB906365[2]	AB906376[2]	AB434266,
					LC012746[2]
Chloromonas tenuis sp. nov.[3]	UTEX SNO132[5]	AB906347[2]	AB906366[2]	AB906377[2]	AB434263
Chloromonas tughillensis[3]	UTEX SNO91[5]	AB906348[2]	AB906367[2]	AB906378[2]	LC012747[2]
	UTEX SNO88	AB734116	AB734117	AB734118	AB434273
					LC012748[2]
					LC012749[2]
	UTEX SNO92[5]	AB906349[2]	AB906368[2]	AB906379[2]	LC012750[2]
Chloromonas sp.	NIES[7]-2379[5]	AB906350[2]	AB906369[2]	AB906380[2]	AB434271
	NIES-2380[5]	AB906351[2]	AB906370[2]	AB906381[2]	AB434270
Mesophilic species (outgroup)					
Chloromonas asteroidea	SAG[8] 11-47	U70783	AB084808	AB084342	AB022225
Chloromonas augustae	SAG 5.73	AJ410452	AB504757	AB504769	AB504764
Chloromonas carrizoensis	SAG 46.72 (= UTEX 968)	AJ410446	AB101503	AB101514	AB101508

Table 2.2. Continued

Chloromonas kasaiae	NIES-2862	AB734109	AB734110	AB734111	LC012751[2]
Chloromonas pseudoplatyrhyncha	NIES-2563	AB548689	AB548690	AB548691	LC012752[2]
Chloromonas radiata	UTEX 966	U57697	AB084311	AB084345	AJ001878
Chloromonas reticulata	SAG 29.83 (= UTEX 1970)	U70791, AB624560	AB084312	AB084346, AB084347	AB022534
Chloromonas rubrifilum	SAG 3.85	AJ410455	AB504758	AB504770	AB504765
Chloromonas serbinowii	UTEX 492 (= SAG 11.84)	U70795, AB624568, AB624569	AB084317	AB084354	AJ001879
Chloromonas typhlos	SAG 26.86 (= UTEX 1969)	AB624566	AB084307	AB084341	AB022228
Gloeomonas lateperforata	NIES-464	AB504779	AB504761	AB504773	AB504768

[1]See Table 2.1.
[2]Sequenced in the present study.
[3]For taxonomy of the species, see chapter 3.
[4]Culture Collection of Algae at the University of Texas at Austin (Starr & Zeikus 1993).
[5]Total DNA of the strain was extracted in the present study.
[6]For origin and establishment of the strain, see chapter 3.
[7]Microbial Culture Collection at the National Institute for Environmental Studies (Kasai *et al.* 2009).
[8]Sammlung von Algenkulturen at the University of Göttingen (Schlösser 1994).

Table 2.3. Primers used for amplifications and sequencing of nuclear-encoded 18S ribosomal DNA (rDNA), ATP synthase beta subunit (*atp*B), P700 chlorophyll *a* apoprotein A2 (*psa*B), the large subunit of RuBisCO (*rbc*L) genes and nuclear rDNA internal transcribed spacer 2 (ITS2) region.

Designation	Position[1]	Sequence (5'–3')
18S rDNA		
FA[2]	1–21	AACCTGGTTGATCCTGCCAGT
FC[3]	458–478	GGGAGGTAGTGACAAIAAATA
RD[2]	570–550[4]	GCTGGCACCAGACTTGCCCTC
FE[2]	1112–1132	GGGAGTATGGTCGCAAGGCTG
RF[2]	1202–1182[4]	CCCGTGTTGAGTCAAATTAAG
RB[2]	1799–1774[4]	TGATCCTTCTGCAGGTTCACCTAC
*atp*B		
F1[5]	180–200	TGTTACTTGTGAAGTTCAACA
breviZFa[6]	206–227	TTGGTGACAATTGTGTTCGTGC
nivZF[6]	208–233	GGTGACAATTGTGTACGTGCTGTATC
Snow-F1[6]	290–314	TAGGWCGTATTTTTAACGTTCTTGG
Snow-F2[6]	359–380	TACCYATTCAYCGTACWGCYCC
R5[5]	662–643[4]	ACICCAGATTCTTTCATTTC
breviZFb[6]	721–741	GAACCACCAGGAGCAAGAATG
F2[5]	721–741	GAACCACCAGGTGCTCGTATG
R12[5]	857–835[4]	GAAACTTCWGCWCCRGCTTGAAC
R3[5]	895–875[4]	GGTAACCTACAGCTGATGGCA
Snow-R3[6]	1139–1117[4]	ACRATCCAWGGYTGWARCATWGT
Snow-R4[6]	1286–1267[4]	TCWGCWACRAARAAAGGTTG
breviZR[6]	1377–1356[4]	AAGCTGTTTAGTTCACCAGAGA
nivZR[6]	1380–1356[4]	GGTAAGCTGTCTAGTTCACCAGAGA
R4[5]	1433–1412[4]	CCTACTAAGTAGAATGCTTGTT
*psa*B		
F1[7]	205–224	GCITGGCARGGIAAYTTYGA
breviZFa[6]	247–269	CATGTGCGCCCTATTGCTCATGC
Snow-F0[6]	247–269	CATGTACGCCCAATWGCKCATGC
GsCl54F[6]	258–279	TATTGCTCATGCCATTTGGGAC
Snow-F1[6]	274–296	TGGGAYCCWCAYTTTGGYCAACC

Table 2.3. Continued

nivZF[6]	304–326	ATATCACACGTACCGCCACGACCAGGAC
GsCl54Ra[6]	512–491[4]	GCATCTTTAAACCACGATAACG
Snow-F4[6]	496–518	TCWTGGTTTAAAGAYGCAGAATC
nivalis-F[6]	601–626	GAATCACGTGGACAACACGTTGGTTG
Snow-R4[6]	807–784[4]	CCATAARCTTTGTGTTTGTGGATG
Snow-R5[6]	821–796[4]	GCCATATCAGTTAACCATAARCTTTG
Snow-R6[6]	851–829[4]	AAAATMACGGCAATTGCTAAATG
breviZFb[6]	940–962	GGTTTAGGAGCAGGACACAAAGG
F5[7]	989–1010	TACAYTTCCAATTAGGYTTAGC
HkCl65F[6]	989–1010	TTCATTTCCAACTAGGGTTAGC
nivalis-R[6]	1085–1060[4]	GCAAGAAAAGCATAAGGCGGCATAGC
GsCl54Rb[6]	1121–1100[4]	TGAGTATAAAGTGCAGCTTGTG
R2[7]	1133–1114[4]	ATRTAYTGRTGRTGIGTRTA
Snow-R7[6]	1388–1364[4]	ATCCATTGTGCAAAAACWGGYTCAA
Snow-R9[6]	1634–1615[4]	TTWGARCCACGYGCRTCTAA
nivZR[6]	1709–1682[4]	ATATCACACGTACCGCCACGACCAGGAC
breviZR[6]	1716–1691[4]	CGCTGAAATATCACATGTTCCTCCAC
HkCl65R[6]	1724–1703[4]	GCGTCATAAGCTGAAATATCAC
R6[7]	1760–1741[4]	ATIGTRTTIARCATCCARAA
*rbc*L		
F1[8]	1–20	ATGGTTCCACAAACAGAAAC
Snow-F0[6]	38–65	TTAAAGCTGGTGTWAAAGAYTAYCGTTT
Snow-F1[9]	178–200	GAATCTTCWACWGGTACTTGGAC
Snow-F2[9]	211–233	ACTGATGGTTTAACAAGTCTTGA
Snow-F3[6]	466–489	CAAGTWGAACGTGACAAATTAAAC
Snow-F4[6]	472–494	GAACGTGACAAATTAAACAAATA
Snow-R2[9]	596–574[4]	AARTCTAAWCCACCACGTAAACA
Snow-R3[9]	656–634[4]	ATRAAACGGTCTCTCCAACGCAT
Snow-R12[6]	1030–1006[4]	CTAAAGTAACTTCACGTTCTCCTTC
R8[8]	1181–1160[4]	AAGATTTCAACTAAAGCTGGCA
R42AE[10]	1403–1384[4]	TCRAAYTTRATYTCYTTCCA
R3[8]	1421–1402[4]	TTGTCAATAGTATCAAATTC

Table 2.3. Continued

nuclear rDNA ITS2		
Fc[11]	30–49[12]	GCATCGATGAAGAACGCAGC
Fcr[6]	30–51[12]	GCAACGATGAAGAACGCAGCGA
Rb[11]	50–23[4, 13]	GGGATCCATATGCTTAAGTTCAGCGGGT

[1]Coordinate numbers from the *Chlorella vulgaris* 18S rDNA (Huss & Sogin 1990), *atp*B or *psa*B genes (Wakasugi *et al.* 1997), the *Chlorella ellipsoidea rbc*L gene (Yoshinaga *et al.* 1988) or the *Didymogenes soliella* 5.8S or LSU rDNA (Hoshina & Fujiwara 2013), respectively.
[2]Primer sourced from Nakazawa & Nozaki (2004).
[3]Primer sourced from Nakada *et al.* (2007).
[4]Reverse primer.
[5]Primer sourced from Nozaki *et al.* (1999).
[6]Primer designed in the present study.
[7]Primer sourced from Nozaki *et al.* (2000).
[8]Primer sourced from Nozaki *et al.* (1995).
[9]Primer sourced from Muramoto *et al.* (2008).
[10]Primer sourced from Shimada *et al.* (1995).
[11]Primer sourced from Coleman (1994).
[12]Position in 5.8S rDNA.
[13]Position in LSU rDNA.

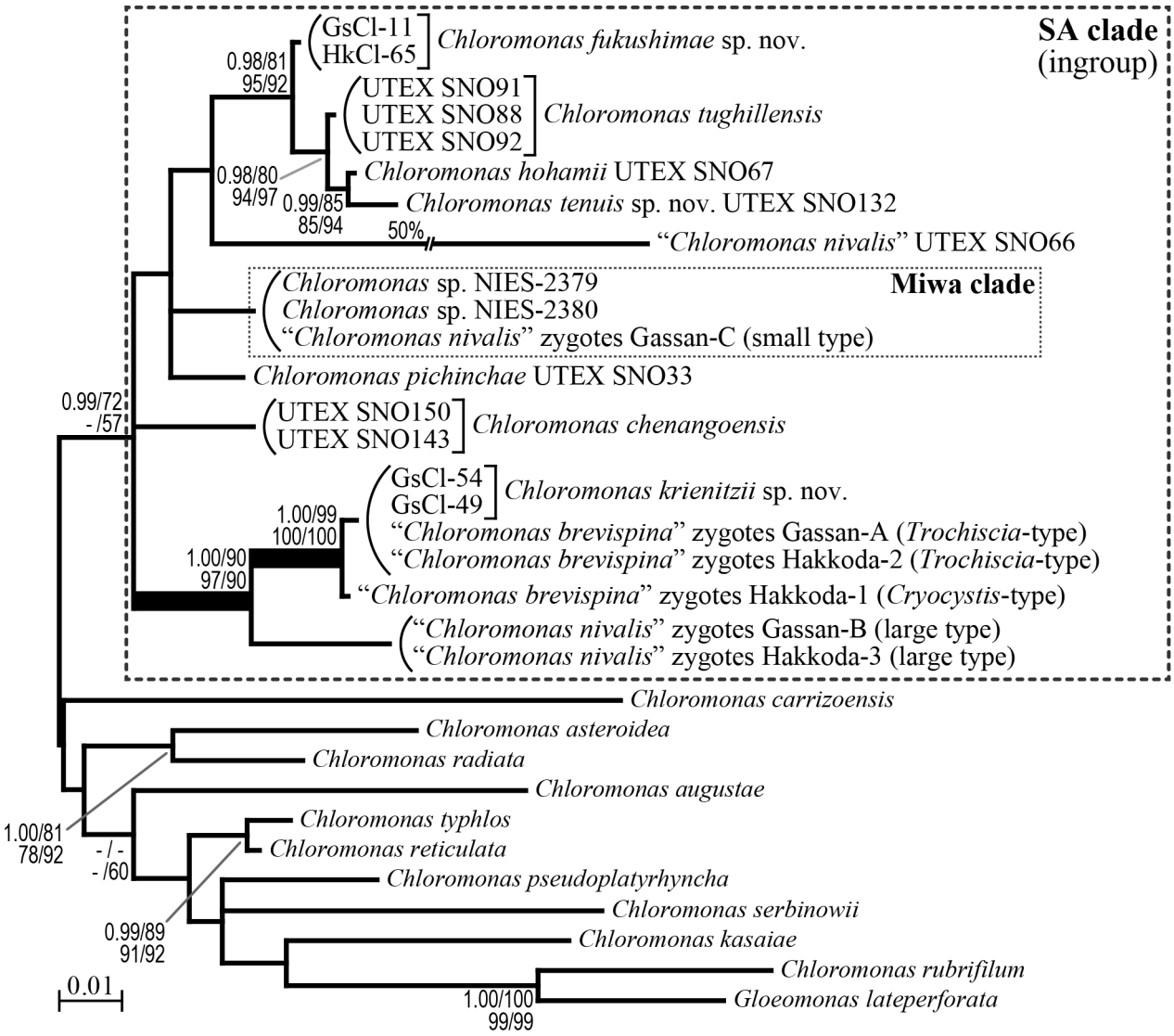


Fig. 2.1. Bayesian phylogenetic tree based on 18S ribosomal DNA sequences. The corresponding posterior probabilities (PP) (0.95 or more) are shown (top left). Numbers shown in top right, bottom left and bottom right indicate bootstrap values (BV) (50% or more) from maximum likelihood (ML), maximum parsimony (MP) and neighbour-joining (NJ) analyses. Branches recovered at the level (1.00 PP and 90% or more BV from ML, MP and NJ analyses respectively) within the SA clade are shown by thicker lines.

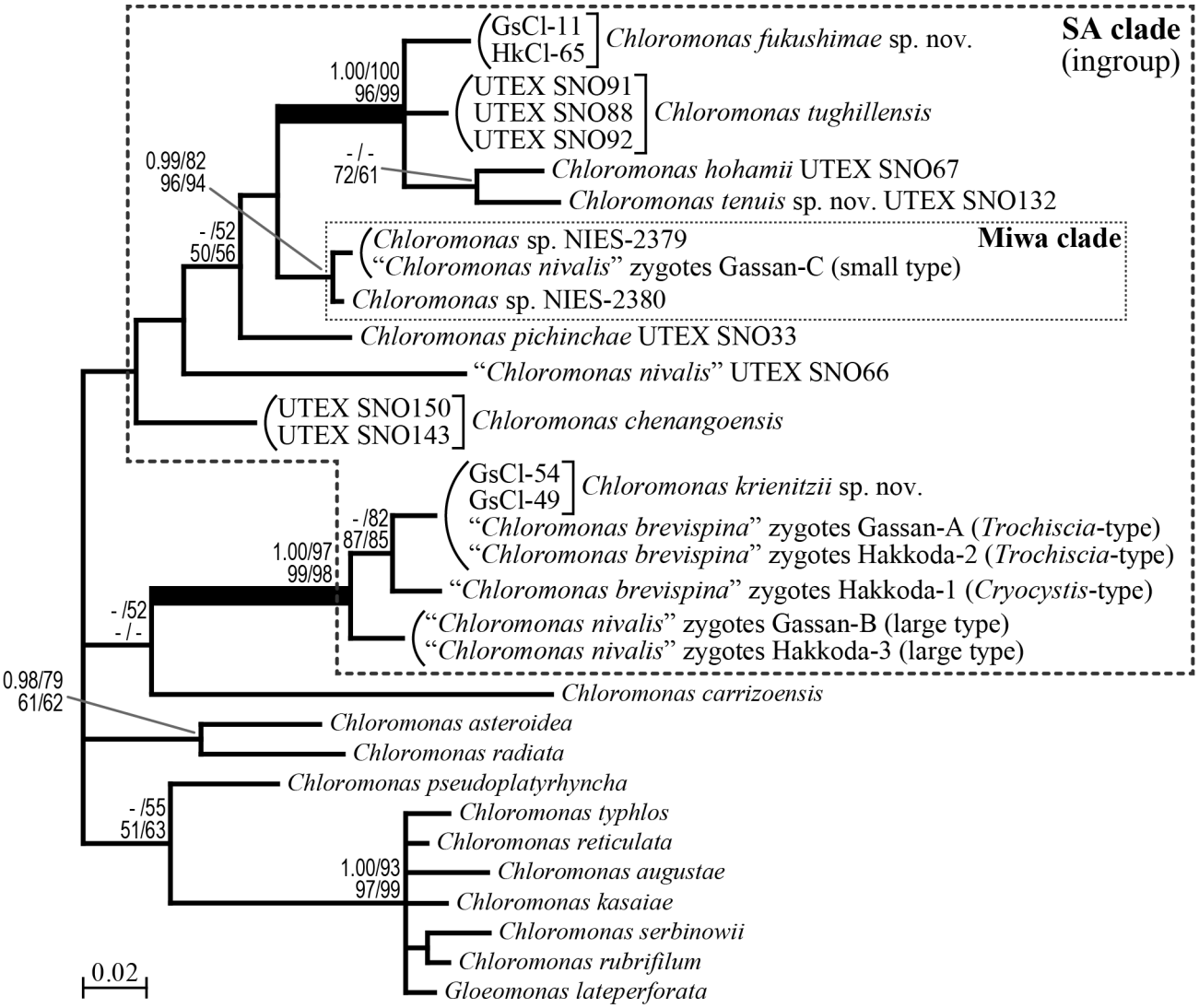


Fig. 2.2. Bayesian phylogenetic tree based on the first and second codon positions of *atp*B gene sequences. The corresponding posterior probabilities (PP) (0.95 or more) are shown (top left). Numbers shown in top right, bottom left and bottom right indicate bootstrap values (BV) (50% or more) from maximum likelihood (ML), maximum parsimony (MP) and neighbour-joining (NJ) analyses. Branches recovered at the level (1.00 PP and 90% or more BV from ML, MP and NJ analyses respectively) within the SA clade are shown by thicker lines.

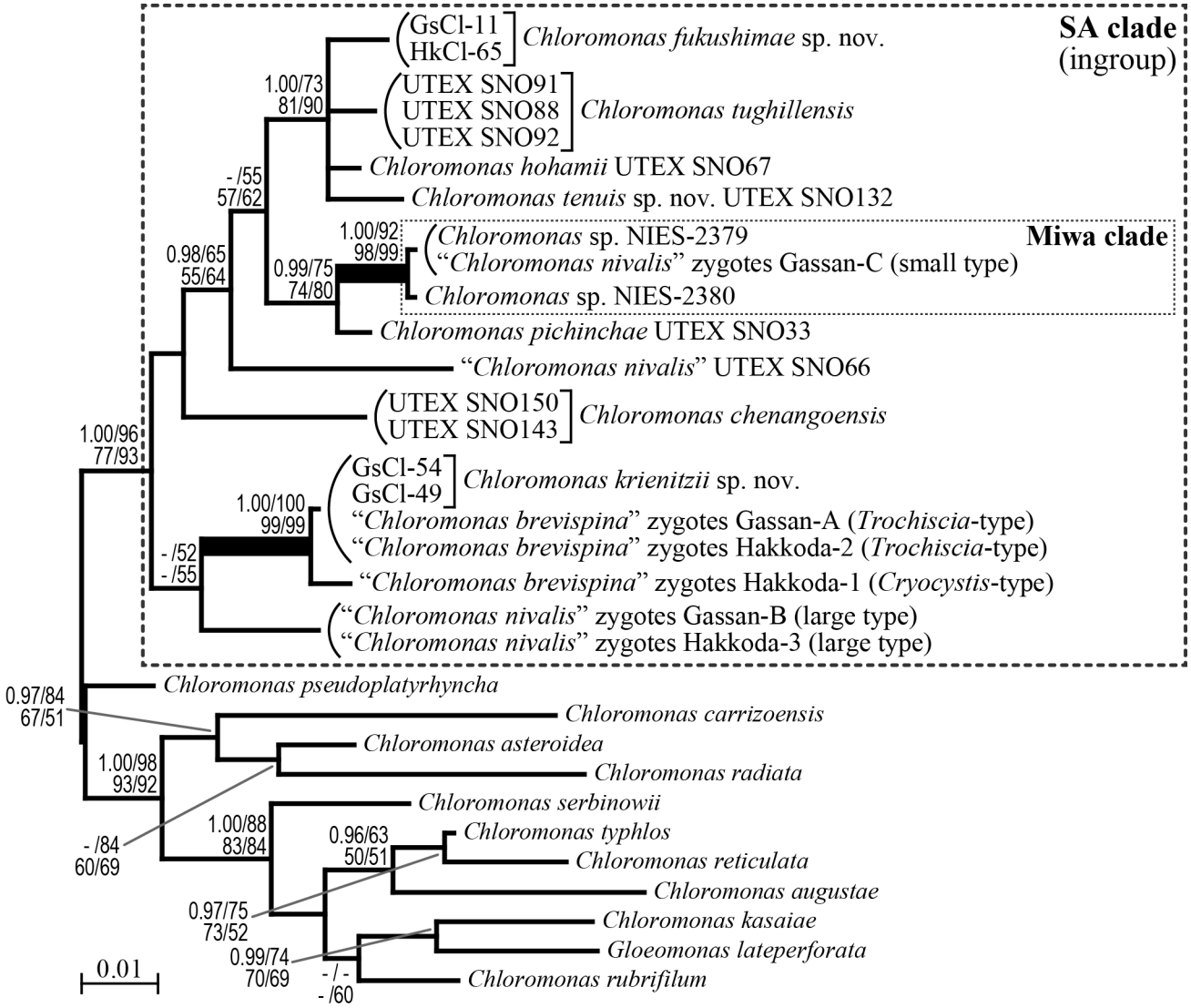


Fig. 2.3. Bayesian phylogenetic tree based on the first and second codon positions of *psa*B gene sequences. The corresponding posterior probabilities (PP) (0.95 or more) are shown (top left). Numbers shown in top right, bottom left and bottom right indicate bootstrap values (BV) (50% or more) from maximum likelihood (ML), maximum parsimony (MP) and neighbour-joining (NJ) analyses. Branches recovered at the level (1.00 PP and 90% or more BV from ML, MP and NJ analyses respectively) within the SA clade are shown by thicker lines.

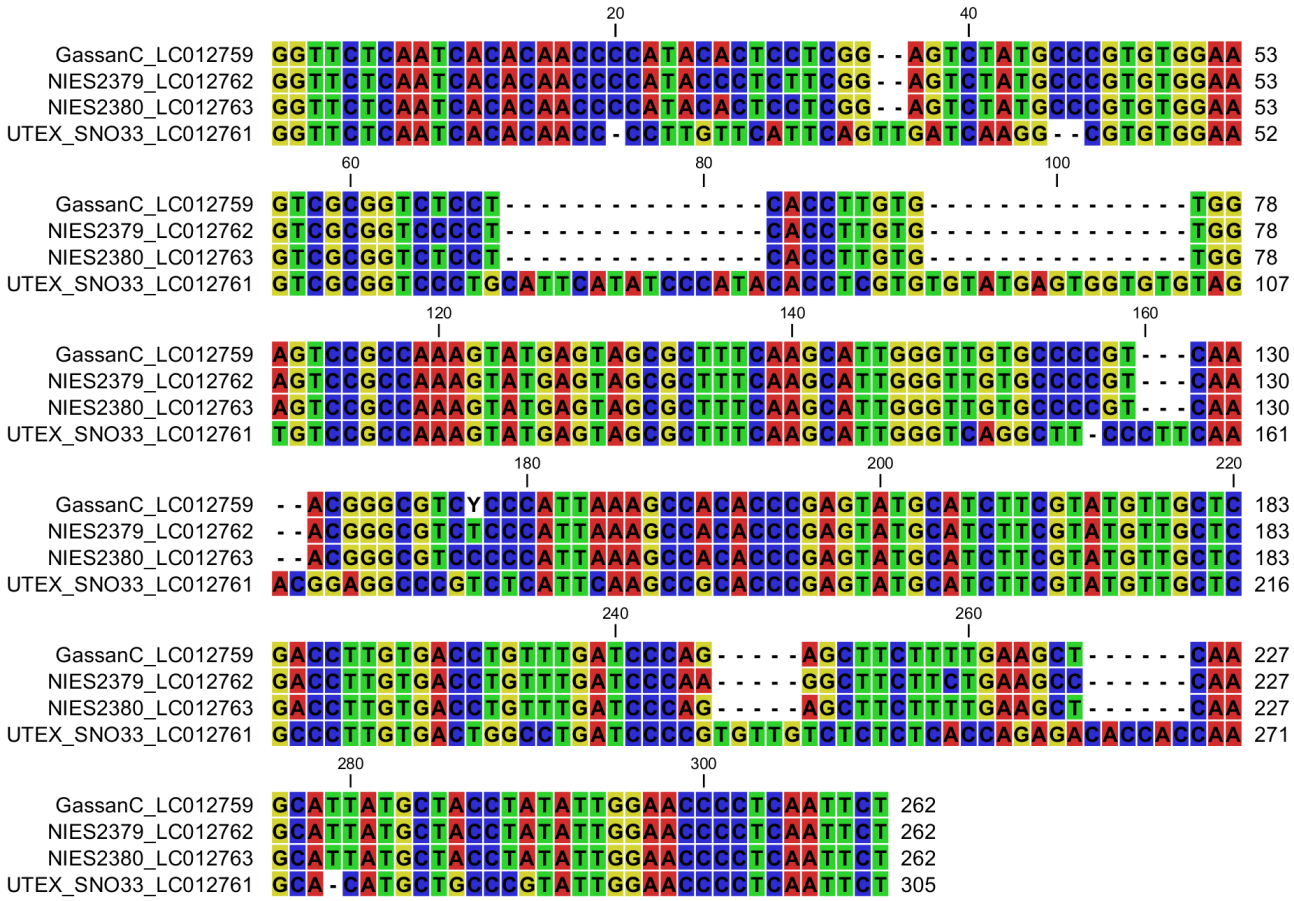


Fig. 2.4. Alignment of full-length of nuclear ribosomal DNA internal transcribed spacer 2 from the specimen Gassan-C (aplanozygotes of *Chloromonas nivalis*), *Chr. pichinchae* strain UTEX SNO33 and *Chloromonas* sp. strains NIES-2379 and NIES-2380 (available from TreeBASE, matrix accession number S16764). The sequences are arranged according to Fig. 2.15. Accession numbers of each strain/specimen are described in the right of the OTU name.

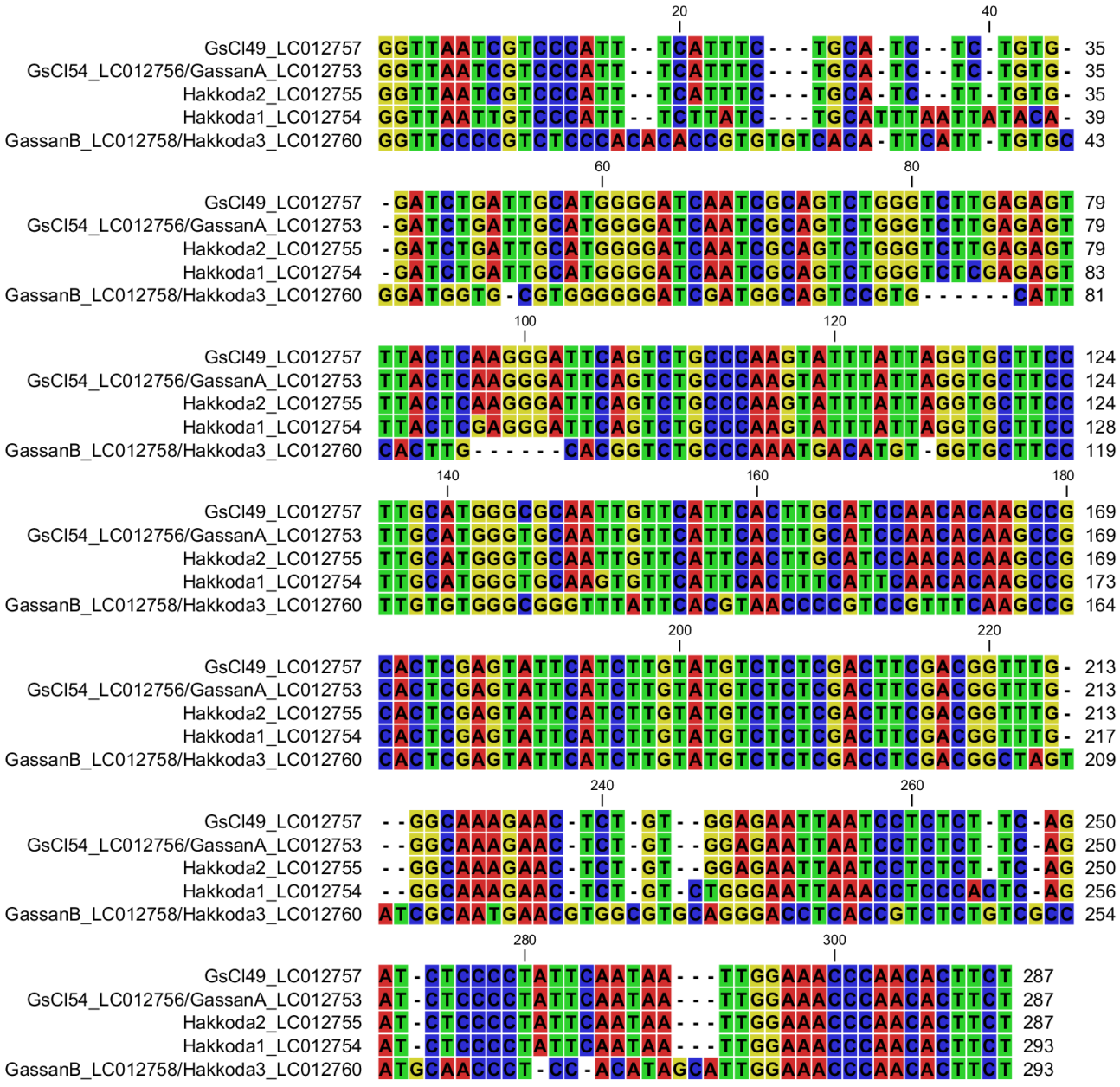


Fig. 2.5. Alignment of full length of nuclear ribosomal DNA internal transcribed spacer 2 from the specimens Gassan-A, Hakkoda-1 and Hakkoda-2 (aplanozygotes of *Chloromonas brevispina*), the specimens Gassan-B and Hakkoda-3 (aplanozygotes of *Chr. nivalis*) and *Chr. krienitzii* strains GsCl-54 and GsCl-49 (available from TreeBASE, matrix accession number S16764). The sequences are arranged according to Fig. 2.15. Note that the sequences from the specimen Gassan-A and *Chr. krienitzii* strain GsCl-54 or the specimens Gassan-B and Hakkoda-3 are completely identical, respectively. Accession numbers of each strain/specimen are described in the right of the OTU name.

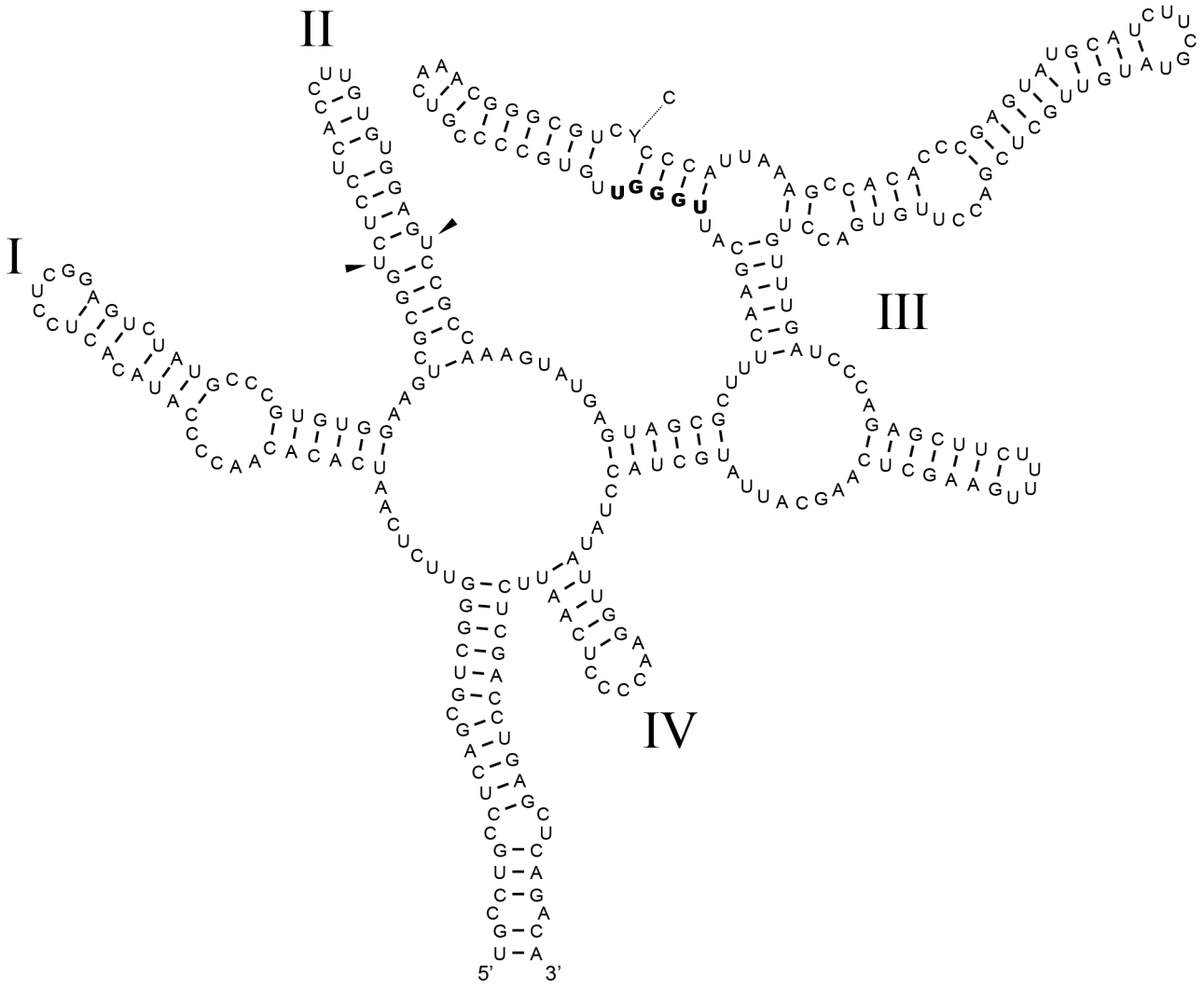


Fig. 2.6. The secondary structure of nuclear ribosomal DNA internal transcribed spacer 2 transcript of the specimen Gassan-C (the small type aplanozygotes of *Chloromonas nivalis*). Difference between the specimen Gassan-C and *Chloromonas* sp. strain NIES-2379 is shown by a character just outside the secondary structure. Note the U-U mismatch in helix II (arrowheads) and the YGGY motif on the 5' side near the apex of helix III (bold faces). Secondary structures of nuclear rDNA ITS2 were drawn using VARNA version 3.9 (Darty *et al.* 2009).

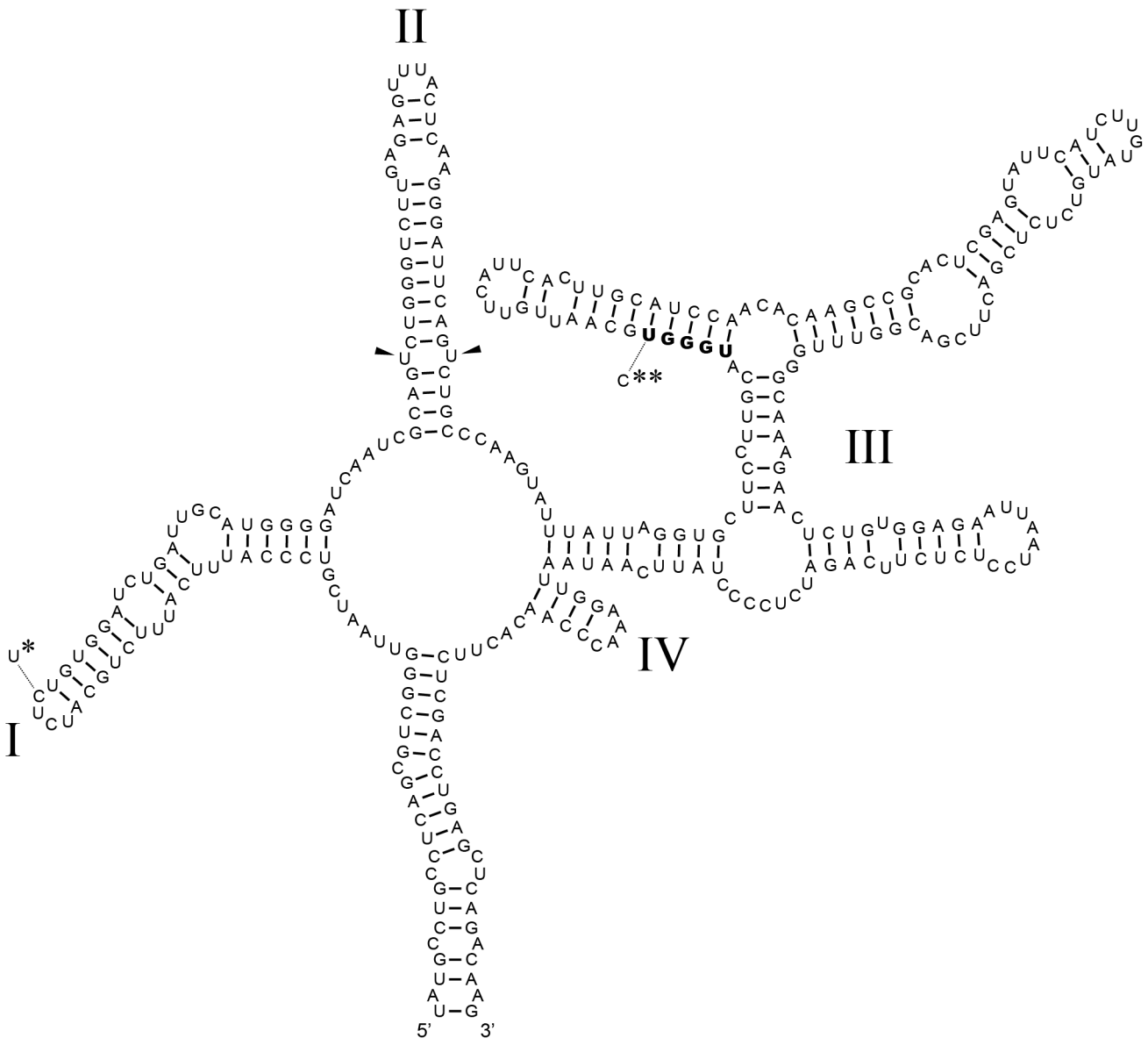
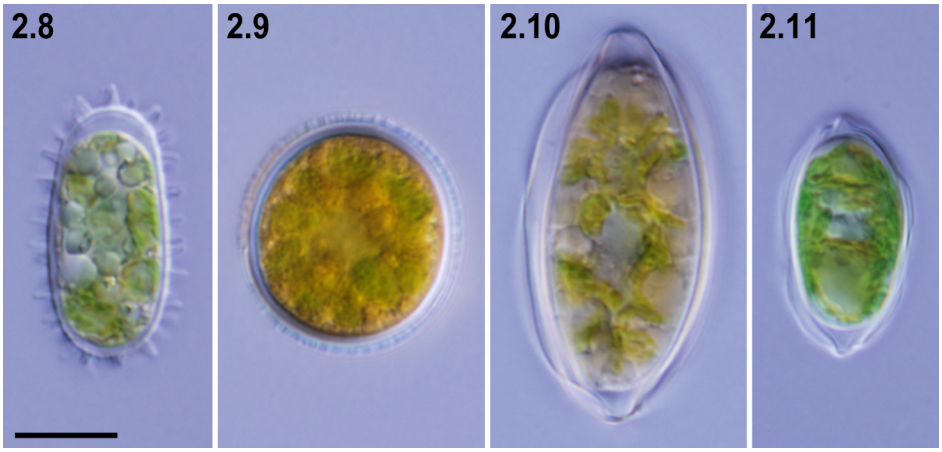


Fig. 2.7. The secondary structure of nuclear ribosomal DNA (rDNA) internal transcribed spacer 2 (ITS2) transcript of the specimen Gassan-A (*Trochiscia*-type aplanozygotes of *Chloromonas brevispina*; Hoham *et al.* 1979). Nuclear rDNA ITS2 sequences of the specimen Gassan-A and *Chr. krienitzii* strain GsCl-54 are identical. Differences between the specimen Gassan-A and the specimen Hakkoda-2 (*Trochiscia*-type aplanozygotes of *Chr. brevispina*) or the *Chr. krienitzii* strain GsCl-49 are shown by characters just outside the secondary structure. Single asterisk means that the difference was detected only in the specimen Hakkoda-2, and double asterisks imply that the difference was detected only in *Chr. krienitzii* strain GsCl-49. Note the U-U mismatch in helix II (arrowheads) and the YGGY motif on the 5’ side near the apex of helix III (bold faces). Secondary structures of nuclear rDNA ITS2 were drawn using VARNA version 3.9 (Darty *et al.* 2009).



Figs 2.8–2.11. Nomarski differential interference micrographs of optical section of field-collected aplanozygotes of *Chloromonas brevispina* and *Chr. nivalis*, shown at the same magnification throughout. For information of collection sites, see Table 2.1. Scale bar = 10 μm.

Fig. 2.8. *Chr. brevispina* aplanozygote (*Cryocystis*-type) from the site 090603Hk4G-2 in Mt. Hakkoda.

Fig. 2.9. *Chr. brevispina* aplanozygote (*Trochiscia*-type) from the site 140628Gs1R in Mt. Gassan.

Fig. 2.10. *Chr. nivalis* aplanozygote (large type) from the site 140603Hk6AR in Mt. Hakkoda.

Fig. 2.11. *Chr. nivalis* aplanozygote (small type) from the site 130630Gs4G in Mt. Gassan.

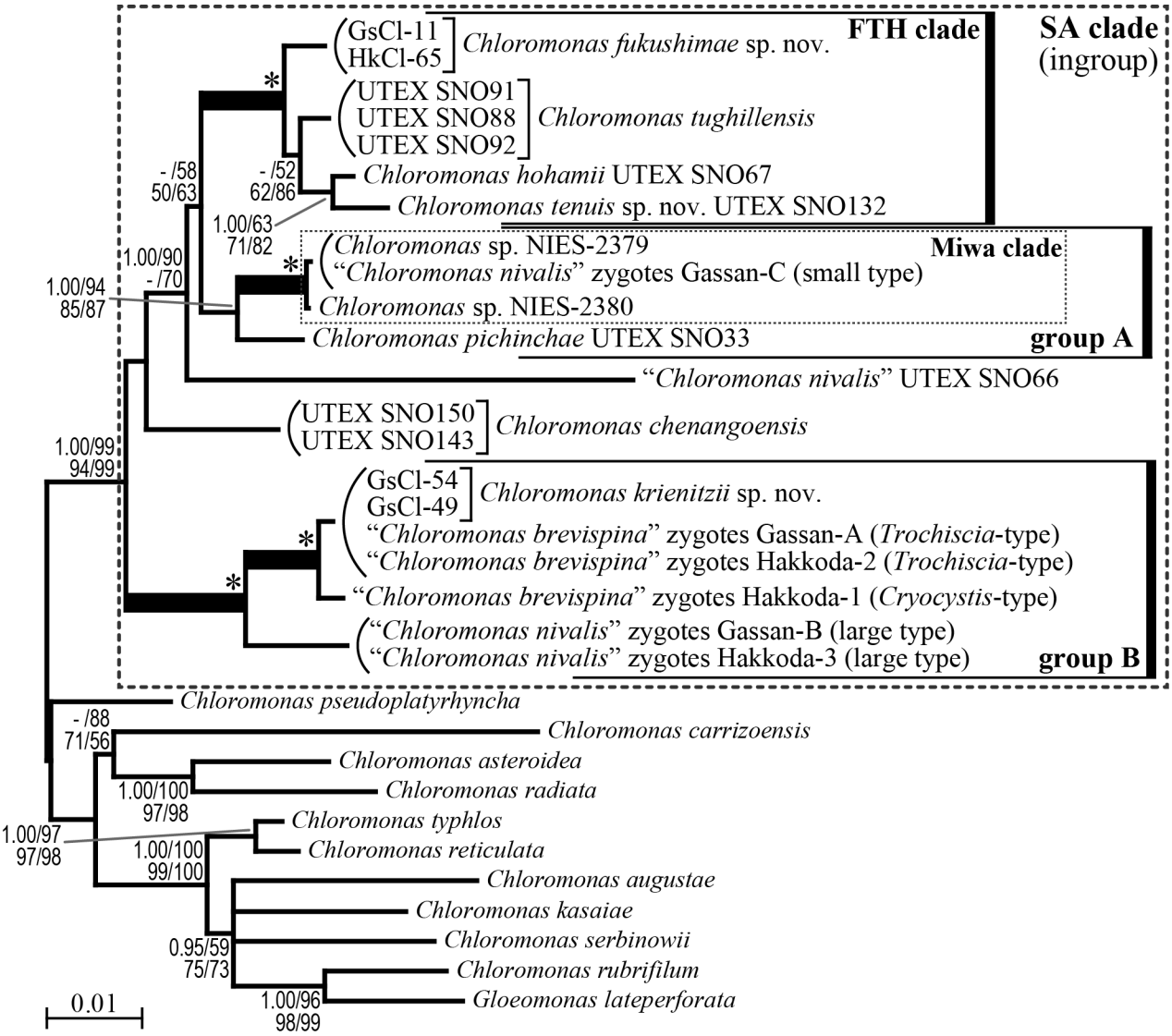


Fig. 2.12. Bayesian phylogenetic tree based on combined 18S rDNA, *atp*B and *psa*B gene sequences (first, second codons only) where the three partitions were unlinked. The corresponding posterior probabilities (PP) (0.95 or more) are shown (top left). Numbers shown in top right, bottom left and bottom right indicate bootstrap values (BV) (50% or more) from maximum likelihood (ML), maximum parsimony (MP) and neighbour-joining (NJ) analyses based on the combined 18S rDNA and first and second codon positions of *atp*B and *psa*B genes, respectively. Branches recovered at the level (1.00 PP and 90% or more BV from ML, MP and NJ analyses respectively) within the SA clade are shown by thicker lines. Asterisk indicates 1.00 PP and 100% BV in ML, MP and NJ analyses.

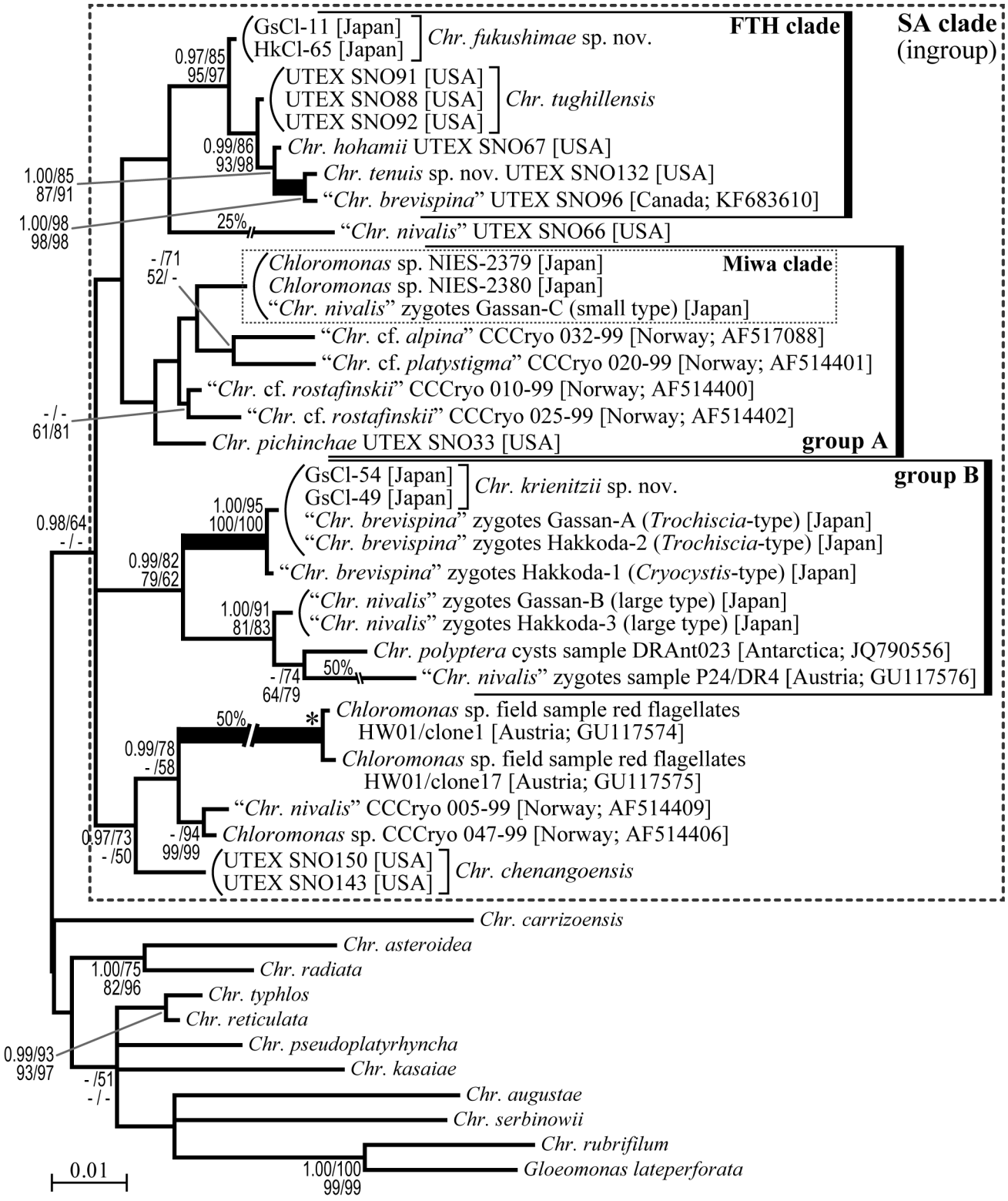


Fig. 2.13. Bayesian phylogenetic tree based on 18S ribosomal DNA sequences. The 1,748-bp data matrix for the 18S rDNA gene from 33 OTUs is available from TreeBASE (matrix accession number S16764). *Chr.* = *Chloromonas*. FTH clade, groups A and B, and Miwa clade are indicated on the basis of Fig. 2.12. The corresponding posterior probabilities (PP) (0.95 or more) are shown (top left). Numbers shown in top right, bottom left and bottom right indicate bootstrap values (BV) (50% or more) from maximum likelihood (ML), maximum parsimony (MP) and neighbour-joining (NJ)

analyses. Branches recovered at the level (1.00 PP and 90% or more BV from ML, MP and NJ analyses respectively) within the SA clade are shown by thicker lines. Asterisk indicates 1.00 PP and 100% BV in ML, MP and NJ analyses. Within the SA clade, origins and accession numbers of each strain/specimen not included in Table 2.2 are described in brackets. The species name enclosed in double quotation marks indicates that accuracy of species identification for the strain/specimen has not been confirmed (based on Pröschold *et al.* [2001], Hoham *et al.* [2006], Matsuzaki *et al.* [2010, 2012, 2013], Muramoto *et al.* [2010], Nozaki *et al.* [2010], Remias *et al.* [2010, 2013], the website of Culture Collection of Algae at the University of Texas at Austin [Starr & Zeikus 1993] and Culture Collection of Cryophilic Algae at the Fraunhofer Institute for Cell Therapy and Immunology [http://cccryo.fraunhofer.de/web/strains/], and the present study).

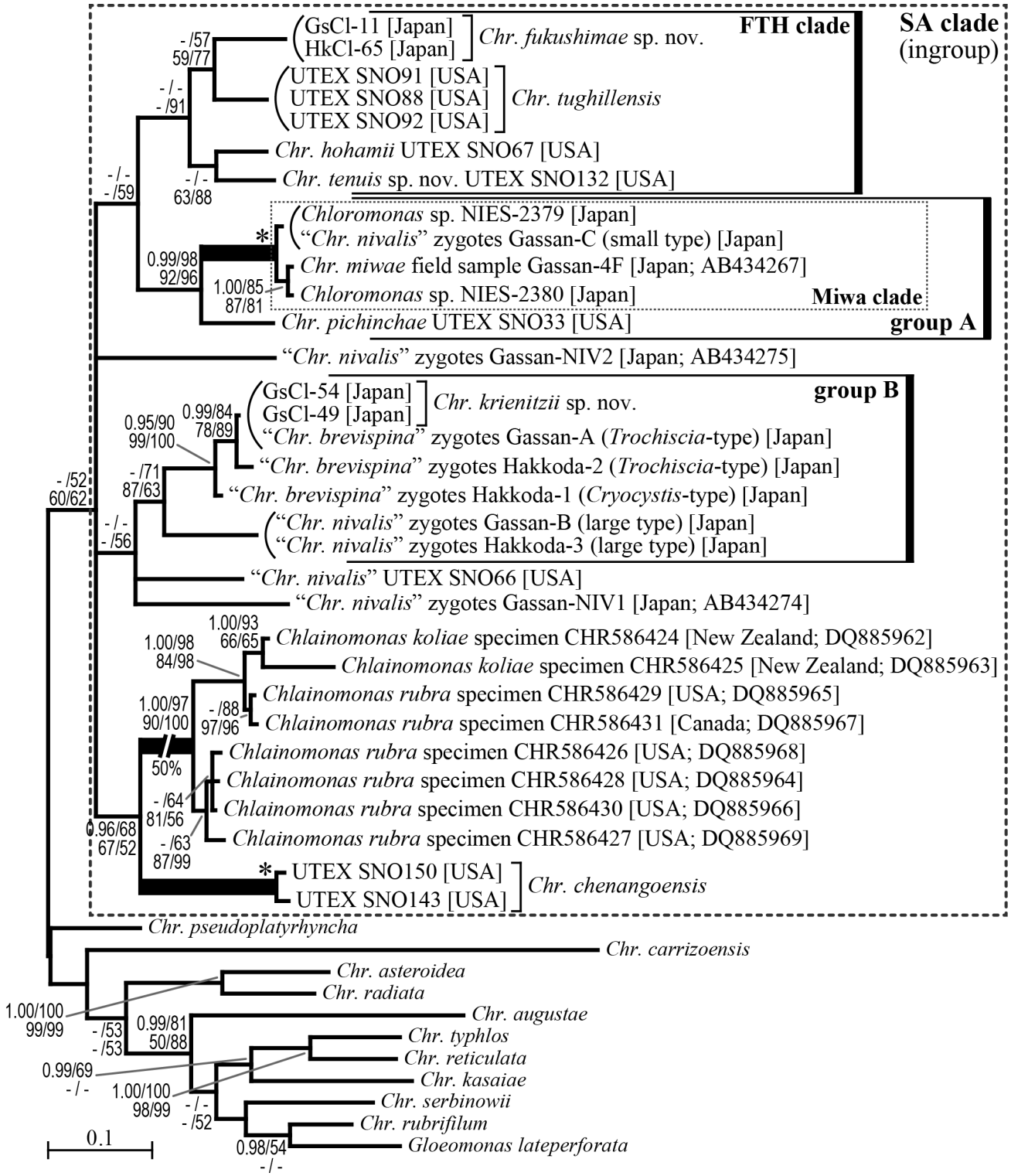


Fig. 2.14. Bayesian phylogenetic tree based on *rbc*L gene sequences. The 1,128-bp data matrix for the *rbc*L gene from 36 OTUs is available from TreeBASE (matrix accession number S16764). *Chr.* = *Chloromonas*. FTH clade, groups A and B, and Miwa clade are indicated on the basis of Fig. 2.12. The corresponding posterior probabilities (PP) (0.95 or more) are shown (top left). Numbers shown in top right, bottom left and bottom right indicate bootstrap values (BV) (50% or more) from maximum likelihood (ML), maximum parsimony (MP) and neighbour-joining (NJ) analyses. Branches recovered at the level (1.00 PP and 90% or more BV from ML, MP and NJ analyses

respectively) within the SA clade are shown by thicker lines. Asterisk indicates 1.00 PP and 100% BV in ML, MP and NJ analyses. Within the SA clade, origins and accession numbers of each strain/specimen not included in Table 2.2 are described in brackets. The species name enclosed in double quotation marks within the SA clade indicates that accuracy of species identification for the strain/specimen has not been confirmed (based on Pröschold *et al.* [2001], Hoham *et al.* [2006], Muramoto *et al.* [2008, 2010], Novis *et al.* [2008], Matsuzaki *et al.* [2010, 2012, 2013], Nozaki *et al.* [2010], the website of Culture Collection of Algae at the University of Texas at Austin [Starr & Zeikus 1993], and the present study).

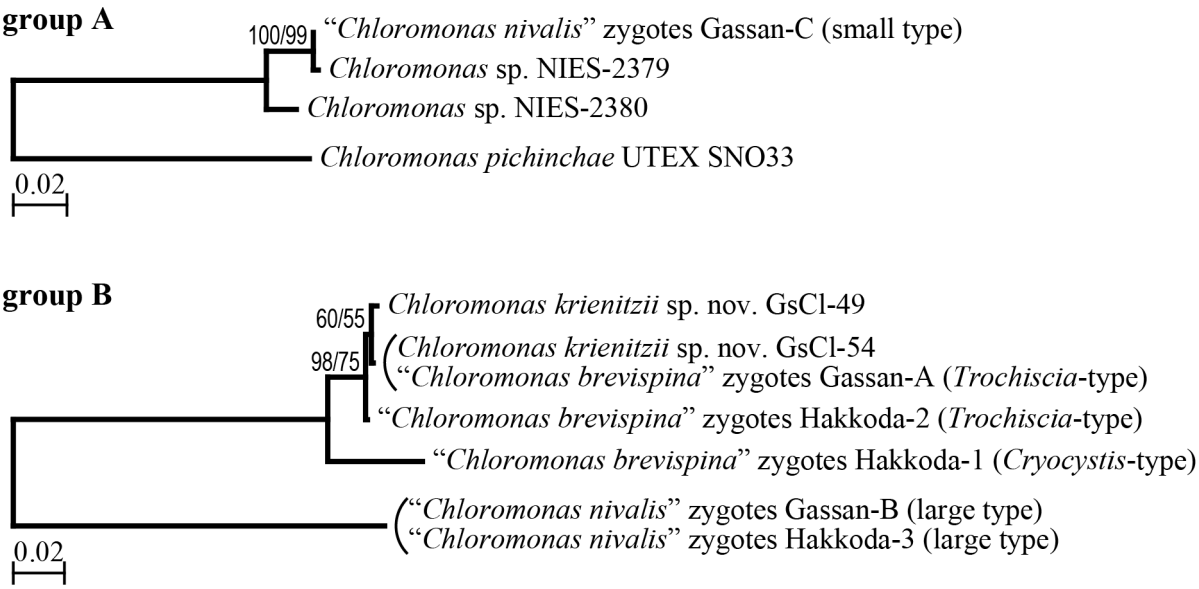


Fig. 2.15. Phylogenetic trees based on neighbour-joining (NJ) analysis, using nuclear ribosomal DNA internal transcribed spacer 2 regions of group A (top) or group B (bottom) in Fig. 2.12. Numbers indicate bootstrap values (50% or more) from NJ (right) and maximum parsimony analyses (left).

Chapter 3. Combined analysis of comparative light and electron microscopy and molecular data of cultured material of snow-inhabiting species of *Chloromonas*

3.1. INTRODUCTION

In chapter 2, I demonstrated that the two snow-inhabiting species of *Chloromonas* which were thought to be cosmopolitan based only on aplanozygote morphology (Hoham & Mullet 1977; Hoham *et al.* 1979) actually contain multiple cryptic species. This result clearly indicates that the species diversity of snow *Chloromonas* would have been underestimated for a long time.

Based on the previous taxonomic studies of snow-inhabiting *Chloromonas* (Hoham 1975; Hoham & Mullet 1977, 1978, Hoham *et al.* 1979, 1983, 2006), aplanozygote morphology is judged to be an important criterion (Remias *et al.* 2010, 2013). However, vegetative cells within the field-collected material generally lack information of aplanozygote morphology and thus, species identification of the cells cannot be carried out easily (e.g. Stein & Amundsen 1967; Hoham *et al.* 1993). Induction of aplanozygote formation in culture was only achieved in strains of *Chr. tughillensis* (Hoham *et al.* 1998, 2000, 2006). In addition, induction of vegetative cells from field-collected or laboratory-induced aplanozygotes of snow-inhabiting *Chloromonas* has not been reported (Remias *et al.* 2010).

Recently, several taxonomic studies based on the combination of LM, TEM and molecular analysis (i.e. polyphasic methods; Pröschold & Leliaert 2007) demonstrated that such polyphasic approaches using cultured material are useful for natural classification of green microalgae at species level (e.g. Nozaki *et al.* 1998; Nakazawa *et al.* 2001, 2004; Nakada *et al.* 2007, 2010b). Very recently, Matsuzaki *et al.* (2012, 2013) delineated five mesophilic species of *Chloromonas* based on comparative LM, TEM, multigene phylogeny and comparison of secondary structures of nuclear rDNA ITS2. However, no snow-inhabiting *Chloromonas* species have been classified using comparative analyses of LM, TEM and molecular data of culture strains.

The present study evaluated the species concept of snow-inhabiting *Chloromonas* based on LM, TEM and molecular data using cultured material. On the basis of my polyphasic analyses, eight North American and four Japanese strains were placed into seven species with descriptions of three

new species, *Chr. fukushimae*, *Chr. krienitzii* and *Chr. tenuis*.

3.2. MATERIAL AND METHODS

3.2.1. Cultures and morphological observation

For comparative light and electron microscopic examinations,12 strains of snow *Chloromonas* species having elongate or ellipsoidal vegetative cells with a rounded posterior end (majority of snow species of *Chloromonas*; see Muramoto *et al.* 2010), originating from North America and Japan, were used (Table 3.1). Cultures were maintained as described in chapter 2. Light and epifluorescence microscopy were performed using a BX53 microscope and a BX60 microscope (Olympus Corp.) equipped with DIC. TEM was performed as essentially the same as those described in Muramoto *et al.* (2010). Cells for TEM were fixed in a final concentration of 0.5% osmium tetroxide (OsO_4) and 0.625% glutaraldehyde (GA). GA was prepared as a 2.5% solution in 0.025M sodium cacodylate buffer (pH 7.3) and mixed immediately before use with the same volume of 2% OsO_4 in 0.025M sodium cacodylate buffer (pH 7.3). Cultures were mixed with the same volume of the GA/OsO_4 fixative and fixed for 3 h on ice. The fixed materials were then dehydrated through an ethanol series, substituted with propylene oxide and embedded in Spurr's resin (Spurr 1969). Sections were cut with a diamond knife (Diatome Corp., Biel, Switzerland) on an Ultracut UCT (Leica Microsystems Corp., Wetzlar, Germany), and stained with 4% samarium chloride solution in distilled water and lead citrate (Venable & Coggeshall 1965). Sections were viewed under a JEM-1010 TEM (JEOL Corp., Tokyo, Japan). Cells in actively growing 4- to 10-day-old cultures were used for these observations.

3.2.2. PCR and sequencing

The nuclear-encoded 26S rDNA and nuclear rDNA ITS2 from eight strains (Table 3.2) were determined by direct sequencing of PCR products as described in chapter 2 with newly designed

specific primers (Table 3.3), excluding nuclear rDNA ITS2 of four strains (GsCl-11, UTEX SNO91, UTEX SNO88 and UTEX SNO92). Since the direct sequencing methodology for the four strains resulted in ambiguous data, possibly due to the suppression of concerted evolution of the ITS region within nuclear rDNA cistrons, the PCR products were cloned into pCR™4-TOPO® vector (Life Technologies Corp.) and sequenced using BigDye™ Terminator Cycle Sequencing Ready Reaction Kit v. 3.1 (Life Technologies Corp.), according to the manufacturer's instructions. Based on 12 cloned products for each strain, two or three variants were recognised (Figs 3.1, 3.2). Such variants within a strain were observed in helix III or II, respectively.

3.2.3. Estimation of genetic differences

To estimate genetic differences (p-distance) between closely related species, nuclear-encoded 18S and 26S rDNA sequences and plastid-encoded *atp*B and *psa*B genes sequences of four snow species among FTH clade (Fig. 2.12) and two mesophilic species, *Chr. chlorococcoides* (H. Ettl & K. Schwarz) Matsuzaki, Y. Hara & Nozaki (DDBJ/EMBL/GenBank accession numbers AJ410449 and AB624555, AB624580, and AB624595 for 18S rDNA, *atp*B, and *psa*B, respectively) and *Chr. reticulata* (Matsuzaki *et al.* 2012), were used (Tables 2.2, 3.2). For alignment of nuclear-encoded 18S rDNA, see chapter 2. 26S rDNA gene sequences of the four snow species and the two mesophilic species of *Chloromonas* (Table 3.2) were aligned according to a consensus secondary structure of nuclear-encoded large subunit rDNA of eukaryotes (Schnare *et al.* 1996).

3.2.4. Construction of secondary structures of nuclear rDNA ITS2

Annotation and prediction of secondary structures of nuclear rDNA ITS2 regions of *Chr. fukushimae* strains GsCl-11 and HkCl-65, *Chr. tughillensis* strains UTEX SNO91, UTEX SNO88 and UTEX SNO92, *Chr. hohamii* and *Chr. tenuis* were performed as described in chapter 2. Secondary structures of nuclear rDNA ITS2 were drawn using VARNA version 3.9 (Darty *et al.*

2009). The consensus structure among the four species was generated using 4SALE (Seibel *et al.* 2006, 2008).

3.3. RESULTS

3.3.1. Comparison of secondary structures of nuclear rDNA ITS2 and genetic differences of nuclear- and plastid-encoded genes among the FTH clade

The phylogenetic positions of the seven species of snow *Chloromonas* were shown in the previous chapter (Fig. 2.12). *Chr. fukushimae*, *Chr. tughillensis*, *Chr. hohamii* and *Chr. tenuis* formed a robust monophyletic group (FTH clade), supported with 1.00 PP in BI and 100% BV in ML, MP and NJ analyses (Fig. 2.12). Within the FTH clade, predicted secondary structure models of nuclear rDNA ITS2 of *Chr. fukushimae* (Fig. 3.1), *Chr. tughillensis* (Fig. 3.2), *Chr. hohamii* (Fig. 3.3) and *Chr. tenuis* (Fig. 3.4) had four helices and a U-U mismatch in helix II (arrowheads in Figs 3.1–3.4), as well as common structural hallmarks of eukaryotic nuclear rDNA ITS2 secondary structures (e.g. Coleman 2003; Schultz *et al.* 2005). The branched helix III of nuclear rDNA ITS2 was highly conserved among the four species (Fig. 3.5). The YGGY motif on the 5' side near the apex of nuclear rDNA ITS2 helix III (modified as CGGY, UGGU or CGGC; Coleman 2007) was observed in the nuclear rDNA ITS2 secondary structures of *Chr. fukushimae*, *Chr. tughillensis* and *Chr. tenuis* (boldface in Figs 3.1, 3.2, 3.4, 3.6). In *Chr. hohamii*, a possible GYU motif (modified as GUU), which is a common motif on the 5' side near the tip of nuclear rDNA ITS2 helix III of Chlorophyceae and Ulvophyceae (Caisová *et al.* 2013), was found (dotted brackets in Figs 3.3, 3.6). Comparing the alignable region in the tip of nuclear rDNA ITS2 helix III encompassing the modified YGGY or GYU motif, at least one to three compensatory base changes (CBCs) were found among the four species (asterisks in Fig. 3.6).

Genetic differences in nuclear-encoded 18S and 26S rDNA and plastid-encoded *atp*B and *psa*B

genes between species within the FTH clade were assessed and compared with those of *Chr. chlorococcoides* and *Chr. reticulata*, a sister mesophilic species having at least two CBCs in the nuclear rDNA ITS2 helix III (Matsuzaki *et al.* 2012). Nucleotide differences in each of these four-gene sequences between species within the FTH clade were almost identical to or larger than between *Chr. chlorococcoides* and *Chr. reticulata* (Figs 3.7–3.10).

3.3.2. Comparison of morphological characteristics of cultures

Based on light and epifluorescence microscopic studies of cultures, all strains had elongate or ellipsoidal vegetative cells with a rounded posterior end, two contractile vacuoles near the base of the flagella, a single nucleus and a single chloroplast (Figs 3.11–3.45). The chloroplast lacked an eyespot and pyrenoids (Figs 3.11, 3.12, 3.15, 3.16, 3.19, 3.20, 3.23, 3.24, 3.27, 3.28, 3.31, 3.32, 3.35, 3.36, 3.39–3.45). A prominent anterior papilla was not observed (Figs 3.12, 3.16, 3.20, 3.24, 3.28, 3.32, 3.36, 3.39–3.45). The flagella were as long as the cell length (Figs 3.46–3.52), as described in Hoham (1975) for *Chr. pichinchae* and Hoham *et al.* (1983, 2006) for *Chr. tughillensis*, *Chr. hohamii* and *Chr. chenangoensis*.

Despite the general similarities for these strains, there were specific differences in cell shape, vegetative chloroplast morphology, number of zoospores within the parental cell and presence or absence of cell aggregates in old cultures; seven morphological species were distinguished based upon the differences (Table 3.4). Four types of vegetative cell shapes were recognised. The first type had a parietal chloroplast on the dorsal side, and the nucleus was located on the ventral side; the cell outline was elongate-kidney- to elongate-bean shaped (Fig. 3.53) (*Chr. fukushimae* and *Chr. krienitzii*; Figs 3.11, 3.12, 3.35, 3.36, 3.39, 3.45). The other three types had a cup-shaped chloroplast with a centrally located nucleus (Fig. 3.54), and they were distinguished by cell outlines: elongate-ellipsoidal (*Chr. tughillensis* and *Chr. hohamii*; Figs 3.15, 3.16, 3.19, 3.20, 3.40, 3.41), elongate-cylindrical (*Chr. tenuis*; Figs 3.23, 3.24, 3.42) and ellipsoidal (*Chr. pichinchae* and *Chr.*

chenangoensis; Figs 3.27, 3.28, 3.31, 3.32, 3.43, 3.44). The chloroplast of *Chr. fukushimae* and *Chr. krienitzii* was parietal, occupying the dorsal side of the cell (Fig. 3.53). The chloroplast of *Chr. krienitzii* differed from that of *Chr. fukushimae* in the presence of the chloroplast filling in the posterior end of the protoplast (Figs 3.11–3.14, 3.35–3.38, 3.39, 3.45). The other five species had cup-shaped chloroplasts (Figs 3.15–3.34, 3.40–3.44, 3.54). Furthermore, the cup-shaped chloroplasts in the latter five species were subdivided into two types. *Chr. tughillensis*, *Chr. hohamii* and *Chr. tenuis* contained chloroplasts apparently composed of elongate-ovoid or elongate-cylindrical platelets (Figs 3.15, 3.17, 3.19, 3.21, 3.23, 3.25, 3.40–3.42). In contrast, the chloroplasts of the other two species (*Chr. pichinchae* and *Chr. chenangoensis*) appeared to be composed of discs (Figs 3.27, 3.29, 3.31, 3.33, 3.43, 3.44).

Asexual reproduction of the present seven species of *Chloromonas* was accomplished through zoospore formation (Figs 3.55–3.65), as described in Hoham (1975) for *Chr. pichinchae* and Hoham *et al.* (1983, 2006) for *Chr. tughillensis*, *Chr. hohamii* and *Chr. chenangoensis*. Immediately before the first cell division, the protoplast rotated, and the parental contractile vacuoles moved to the equator of the parent cells (Figs 3.55, 3.60, 3.64). The protoplast then divided transversely. Generally, two, three or four successive divisions occurred to form four (*Chr. tenuis* [Fig. 3.61], *Chr. pichinchae* [Fig. 3.62], *Chr. chenangoensis* [Fig. 3.63] and *Chr. krienitzii*, [Fig. 3.65]), eight (*Chr. fukushimae* [Fig. 3.57] and *Chr. hohamii* [Fig. 3.59]) or 16 (*Chr. tughillensis* [Fig. 3.58]) biflagellate zoospores, respectively, within the parental cell wall (see Table 3.4; Hoham 1975; Hoham *et al.* 1983, 2006). Sexual reproduction or hypnospore formation was not observed in the present cultured materials of the seven species.

Aggregates of 16 or more cells, which resulted from repeated divisions of daughter cells and were retained within the parental cell wall, were observed in old cultures of *Chr. tughillensis* (Fig. 3.66) and *Chr. pichinchae* (Fig. 3.67) after 2 to 3 months in 1.5% agar slants. However, such an aggregate was not observed in the other five species.

Ultrastructural features of cellular organelles were essentially identical among the seven species (Figs 3.68–3.88). In all seven species, the cell had a nucleus in the middle (Figs 3.68, 3.71, 3.74, 3.77, 3.80, 3.83, 3.86) and chloroplast profiles near the cell periphery (Figs 3.70, 3.73, 3.76, 3.79, 3.82, 3.85, 3.88). The chloroplasts lacked pyrenoid matrices, and mitochondrial profiles were observed mainly in the cytoplasm between the chloroplast and the nucleus or sometimes in the cytoplasm outside the chloroplast (Figs 3.68, 3.70, 3.71, 3.73, 3.74, 3.76, 3.77, 3.79, 3.80, 3.82, 3.83, 3.85, 3.86, 3.88). However, the chloroplast shape and position of mitochondria differed among species. In *Chr. fukushimae* and *Chr. krienitzii*, several chloroplast profiles were on the dorsal side of the protoplast (Figs 3.68, 3.70, 3.86, 3.88; see also Fig. 3.53), but the chloroplasts of the other five species were cup-shaped and found throughout the peripheral region of the protoplast, with the exception of the anterior region (Figs 3.71, 3.73, 3.74, 3.76, 3.77, 3.79, 3.80, 3.82, 3.83, 3.85; see also Fig. 3.54). The chloroplasts composed of elongated platelets under the LM were clearly distinguished in the TEM from those composed of discs. In tangential cell sections of *Chr. tughillensis*, *Chr. hohamii* and *Chr. tenuis*, chloroplast profiles appeared to be elongate-ovoid or elongate-cylindrical (Figs 3.72, 3.75, 3.78). On the contrary, chloroplast profiles of *Chr. pichinchae* and *Chr. chenangoensis* were almost angular in shape (Figs 3.81, 3.84). In *Chr. fukushimae*, mitochondrial profiles were also observed in the posterior region of the protoplast (asterisks in Fig. 3.68). However, such posterior mitochondria were not observed in the other six species of *Chloromonas* (Figs 3.71, 3.74, 3.77, 3.80, 3.83, 3.86).

With regard to growth in culture, the cells of the seven snow species did not grow at 20°C after cultivation for 2 weeks.

3.3.3. Taxonomic treatments

***Chloromonas fukushima*e Matsuzaki *sp. nov.*[†]**

Figs 3.11–3.14, 3.39, 3.46, 3.55–3.57, 3.68–3.70

[†]The name of this new taxon is not validly published since the present thesis does not constitute effective publication and the type of the name is not designated (see Articles 30.8, 32.1 and 40.1 of International Code of Nomenclature for algae, fungi, and plants [ICN]; McNeill *et al.* 2012).

DESCRIPTION: Vegetative cells solitary, having two flagella, without prominent anterior papilla. Cells elongate, kidney-shaped or bean-shaped; 4–9 μm wide and 15–23 μm long. Cell with a single nucleus in the ventral half of the cell. A single, laminate parietal chloroplast in the dorsal side of the cell, not filling in the posterior end of the protoplast; chloroplast with irregular incisions on the surface, lacking an eyespot and pyrenoids. Posterior region of the protoplast containing mitochondria. Asexual reproduction by formation of two, four or eight zoospores within the parental cell, with rotation of the protoplast before the first cell division. Cell aggregates not observed in old cultures.

TYPE LOCALITY: 38°30’27’’N, 140°0’1’’E; Yamagata Prefectural Nature Conservation Park, Mt. Gassan, Nishikawa-machi, Nishimurayama-gun, Yamagata Prefecture, Japan.

OTHER DISTRIBUTIONS: 40°38’52’’N, 140°51’6’’E; the Hakkoda Botanical Garden of Tohoku University, Mt. Hakkoda, Minami-arakawayama, Arakawa, Aomori-shi, Aomori Prefecture, Japan.

AUTHENTIC STRAIN: GsCl-11. This strain was deposited as NIES-3389 at the Microbial Culture Collection at NIES (Kasai *et al.* 2009).

SECOND STRAIN EXAMINED: HkCl-65. This strain was deposited as NIES-3390 at the Microbial Culture Collection at NIES (Kasai *et al.* 2009).

ETYMOLOGY: The species epithet ‘*fukushimae*’ is in honour of Dr. Hiroshi Fukushima, who has contributed greatly to the systematics, life histories and physiological ecology of Japanese snow algae (e.g. Fukushima 1963).

***Chloromonas krienitzii* Matsuzaki *sp. nov.*[†]**

Figs 3.35–3.38, 3.45, 3.52, 3.64, 3.65, 3.86–3.88

DESCRIPTION: Vegetative cells solitary, having two flagella, without prominent anterior papilla. Cells elongate, kidney-shaped or bean-shaped; 7–11 μm wide and 17–24 μm long. Cell with a single nucleus in the ventral half of the cell. A single, parietal chloroplast in the dorsal side of the cell, filling in the posterior end of the protoplast; chloroplast with irregular incisions on the surface, lacking an eyespot and pyrenoids. Posterior region of the protoplast lacking mitochondria. Asexual reproduction by formation of two or four zoospores within the parental cell, with rotation of the protoplast before the first cell division. Cell aggregates not observed in old cultures.

TYPE LOCALITY: 38°30’50’’N, 139°59’27’’E; Yamagata Prefectural Nature Conservation Park, Mt. Gassan, Nishikawa-machi, Nishimurayama-gun, Yamagata Prefecture, Japan.

OTHER DISTRIBUTIONS: 38°30’25’’N, 139°59’56’’E; Yamagata Prefectural Nature Conservation Park, Mt. Gassan, Nishikawa-machi, Nishimurayama-gun, Yamagata Prefecture, Japan. 40°38’51’’N, 140°51’4’’E; the Hakkoda Botanical Garden of Tohoku University, Mt. Hakkoda, Minami-arakawayama, Arakawa, Aomori-shi, Aomori Prefecture, Japan.

AUTHENTIC STRAIN: GsCl-54.

SECOND STRAIN EXAMINED: GsCl-49.

ETYMOLOGY: The species epithet ‘*krienitzii*’ is in honour of Dr. Lothar Krienitz, who has

[†]The name of this new taxon is not validly published since the present thesis does not constitute effective publication and the type of the name is not designated (see Articles 30.8, 32.1 and 40.1 of ICN; McNeill *et al.* 2012).

contributed greatly to the taxonomy of green microalgae (e.g. Krienitz & Bock 2012).

REMARKS: On the basis of the present phylogenetic analyses of multiple DNA regions, the specimens Gassan-A and Hakkoda-2 can be considered the aplanozygotes of *Chr. krienitzii* (chapter 2). Based on the observation of North American materials, Hoham *et al.* (1979) suggested that *Chr. brevispina* has two types of aplanozygotes (*Cryocystis*- and *Trochiscia*-type; see also Fig. 1.4). Although the specimens Gassan-A and Hakkoda-2 can be assigned to the *Trochiscia*-type aplanozygotes (Fig. 2.9), vegetative cells of *Chr. krienitzii* (elongate-bean-shaped; Figs 3.36, 3.45) are morphologically different from those of North American *Chr. brevispina* (ovoid or pyriform in shape; Hoham *et al.* 1979) (see Key).

***Chloromonas tenuis* Matsuzaki *sp. nov.*[†]**

Figs 3.23–3.26, 3.42, 3.49, 3.60, 3.61, 3.77–3.79

DESCRIPTION: Vegetative cells solitary, having two flagella, without a prominent anterior papilla. Cells elongate-cylindrical, 5–9 μm wide and 15–23 μm long. Cells with a single central nucleus and a single cup-shaped chloroplast. Chloroplast apparently composed of elongate-ovoid or elongate-cylindrical platelets, showing irregular incisions on the surface, lacking an eyespot and pyrenoids. Posterior region of the protoplast lacking mitochondria. Asexual reproduction by formation of generally two or four zoospores, with rotation of the protoplast before the first cell division. Cell aggregates not observed in old cultures.

TYPE LOCALITY: Jay Peak, Vermont, USA (Hoham *et al.* 2002).

[†]The name of this new taxon is <u>not</u> validly published since the present thesis does not constitute effective publication and the type of the name is not designated (see Articles 30.8, 32.1 and 40.1 of ICN; McNeill *et al.* 2012).

AUTHENTIC STRAIN: UTEX SNO132.

ETYMOLOGY: The species epithet is derived from the Latin adjective *tenuis*, meaning ‘slender’ in reference to the elongate-cylindrical vegetative cells.

REMARKS: The strain UTEX SNO132 was previously designated as *Chr. brevispina* (Hoham *et al.* 2002, 2006). However, vegetative cells of *Chr. brevispina* are ovoid or pyriform in shape (Hoham *et al.* 1979), while only elongate-cylindrical vegetative cells were observed in *Chr. tenuis* strain UTEX SNO132 (Figs 3.24, 3.42).

Chloromonas tughillensis Hoham, Berman, H.S. Rogers, Felio, Ryba & P.R. Miller. *Phycologia* 45: 322 (2006)

Figs 3.15–3.18, 3.40, 3.47, 3.58, 3.66, 3.71–3.73

DESCRIPTION: Vegetative cells solitary, having two flagella, without a prominent anterior papilla. Cells elongate-ellipsoidal, 6–12 μm wide and 16–23 μm long. Cells with a single central nucleus and a single cup-shaped chloroplast. Chloroplast apparently composed of elongate-ovoid or elongate-cylindrical platelets, showing irregular incisions on the surface, lacking an eyespot and pyrenoids. Posterior region of the protoplast lacking mitochondria. Asexual reproduction by formation of generally two, four, eight or 16 zoospores, with rotation of the protoplast before the first cell division. Cell aggregates observed in old cultures.

DISTRIBUTION: Whetstone Gulf State Park, New York, USA (Hoham *et al.* 2006).

STRAINS EXAMINED: UTEX SNO91 (authentic *Chr. tughillensis* strain), UTEX SNO88 and UTEX

SNO92.

REMARKS: Hoham *et al.* (2006) described the chloroplast as being composed of mostly parietal bands and discs with or without extensions that may or may not be connected. However, the chloroplast of the present strains (including the authentic strain) was cup-shaped, apparently composed of elongate-ovoid to elongate-cylindrical platelets (Figs 3.15–3.18, 3.40, 3.71–3.73).

Chloromonas hohamii H.U. Ling & Seppelt. *Polar Biology* 20: 323 (1998)

Figs 3.19–3.22, 3.41, 3.48, 3.59, 3.74–3.76

"*Chloromonas polyptera*" Hoham, Mullet & S.C. Roemer 1983 *non* (F.E. Fritsch) Hoham, Mullet & S.C. Roemer [basionym: *Scotiella polyptera* F.E. Fritsch]

DESCRIPTION: Vegetative cells solitary, having two flagella, without a prominent anterior papilla. Cells elongate-ellipsoidal, 5–10 μm wide and 12–25 μm long. Cells with a single central nucleus and a single cup-shaped chloroplast. Chloroplast apparently composed of elongate-ovoid or elongate-cylindrical platelets, showing irregular incisions on the surface, lacking an eyespot and pyrenoids. Posterior region of the protoplast lacking mitochondria. Asexual reproduction by formation of generally two, four or eight zoospores, with rotation of the protoplast before the first cell division. Cell aggregates not observed in old cultures.

DISTRIBUTION: Arizona, Colorado, Montana, New Mexico and Washington in USA (Hoham *et al.* 1983).

STRAIN EXAMINED: UTEX SNO67.

REMARKS: Although no authentic strain of *Chr. hohamii* exists, the vegetative morphology of the strain UTEX SNO67 was identical to that of *Chr. hohamii*, as described by Hoham *et al.* (1983) and Ling & Seppelt (1998) (Figs 3.19–3.22, 3.41, 3.74–3.76). This strain originated from Arizona, USA, where the original material of *Chr. hohamii* was collected (Hoham *et al.* 1983). According to Hoham *et al.* (1983) and Ling & Seppelt (1998), *Chr. hohamii* possesses or lacks an eyespot. In the present study, *Chr. hohamii* strain UTEX SNO67 lacks an eyespot in all stages of the vegetative phase and asexual reproduction (Figs 3.19, 3.41).

Chloromonas pichinchae Wille. *Nyt Magazin for Naturvidenskaberne* 41: 152 (1903)

Figs 3.27–3.30, 3.43, 3.50, 3.62, 3.67, 3.80–3.82

Chlamydomonas tingens A. Braun var. *nivalis* Lagerheim, *Chlamydomonas pichinchae* (Wille) Gerloff

DESCRIPTION: Vegetative cells solitary, having two flagella, without a prominent anterior papilla. Cells elongate-ovoid or ellipsoidal, 8–15 μm wide and 18–26 μm long. Cells with a single central nucleus and a single cup-shaped chloroplast. Chloroplast apparently composed of discs, showing irregular incisions on the surface, lacking an eyespot and pyrenoids. Posterior region of the protoplast lacking mitochondria. Asexual reproduction by formation of generally two or four zoospores, with rotation of the protoplast before the first cell division. Cell aggregates observed in old cultures.

DISTRIBUTION: Mt. Pichincha, Ecuador (Lagerheim 1892) and Washington, USA (Hoham 1975).

STRAIN EXAMINED: UTEX SNO33.

REMARKS: In the original description of *Chr. pichinchae*, this species was found in permanent

snow on Mt. Pichincha, Ecuador, and was previously identified as *Chlamydomonas tingens* var. *nivalis* (Lagerheim 1892). After taxonomic revision by Wille (1903) and others (Gerloff 1940; Huber-Pestalozzi 1961; Kol 1968), a detailed vegetative morphology was reported based on a combination of field and laboratory observations of field materials collected in several mountainous regions in the state of Washington, USA (Hoham 1975). The vegetative morphology of the strain UTEX SNO33 is almost identical to that described by Wille (1903) and Hoham (1975) (Figs 3.27–3.30, 3.43, 3.80–3.82).

Chloromonas chenangoensis Hoham, Berman, H.S. Rogers, Felio, Ryba & P.R. Miller. *Phycologia* 45: 321 (2006)

Figs 3.31–3.34, 3.44, 3.51, 3.63, 3.83–3.85

DESCRIPTION: Vegetative cells solitary, having two flagella, without a prominent anterior papilla. Cells ellipsoidal, 7.5–17.5 μm wide and 14–25 μm long. Cells with a single central nucleus and a single cup-shaped chloroplast. Chloroplast apparently composed of discs, showing irregular incisions on the surface, lacking an eyespot and pyrenoids. Posterior region of the protoplast lacking mitochondria. Asexual reproduction by formation of generally two or four zoospores, with rotation of the protoplast before the first cell division. Cell aggregates not observed in old cultures.

DISTRIBUTION: Chenango Valley, Hamilton, New York, USA (Hoham *et al.* 2006).

STRAINS EXAMINED: UTEX SNO150 (authentic *Chr. chenangoensis* strain) and UTEX SNO143.

REMARKS: The vegetative morphology of the strains used in the present study (including the authentic strain) was consistent with the *Chr. chenangoensis* description by Hoham *et al.* (2006) in

having ellipsoidal or spherical vegetative cells lacking a prominent anterior papilla and a cup-shaped chloroplast without pyrenoids, which appeared to be composed of discs (Figs 3.31–3.34, 3.44, 3.83–3.85).

3.4. DISCUSSION

The present light, epifluorescence and electron microscopic observations clearly distinguished the 12 strains into seven morphological species based on differences in cell morphology, chloroplast shape, number of zoospores formed within the parental cell and presence or absence of cell aggregates in old cultures (Table 3.4). *Chr. fukushimae*, *Chr. tughillensis*, *Chr. hohamii* and *Chr. tenuis* formed the FTH clade with robust support, and *Chr. pichinchae*, *Chr. chenangoensis* and *Chr. krienitzii* were separate from the FTH clade (Fig. 2.12). To verify the species boundaries, I compared secondary structures of nuclear rDNA ITS2 between morphological species within the FTH clade (Figs 3.1–3.6). According to Coleman (2009), structural differences in the nuclear rDNA ITS2 are correlated with the separation of biological species; organisms that differ by even one CBC in the most conserved region of nuclear rDNA ITS2 (30 consecutive nucleotides on the 5' side and its complementary *c.* 40 nucleotides on the 3' side near the apex of helix III) cannot cross. In my predicted models, among the four species at least one CBC was recognised on the tip of nuclear rDNA ITS2 helix III, encompassing the modified YGGY or GYU motif (conserved hallmarks on the 5' side near the tip of helix III of eukaryotic or chlorophycean and ulvophycean nuclear rDNA ITS2 secondary structures, respectively; Coleman 2007; Caisová *et al.* 2013) (Fig. 3.6). The separation of these four morphological species was further examined by comparing their genetic differences with those of morphologically and genetically delineated species of mesophilic *Chloromonas*, *Chr. chlorococcoides* and *Chr. reticulata*. These two sister species are clearly distinguished from each other based on differences in vegetative cell shape and presence or absence of pyrenoid in the

chloroplast as well as by having at least two CBCs in nuclear rDNA ITS2 helix III (Matsuzaki *et al.* 2012). Genetic differences in nuclear-encoded 18S and 26S rDNA and plastid-encoded *atp*B and *psa*B genes between species within the FTH clade were almost identical to or larger than those between *Chr. chlorococcoides* and *Chr. reticulata* (Figs 3.7–3.10). Therefore, *Chr. fukushimae*, *Chr. tughillensis*, *Chr. hohamii* and *Chr. tenuis* are four separate entities at or above the biological species level when compared to the mesophilic *Chloromonas* spp.

Chr. fukushimae, *Chr. krienitzii* and *Chr. tenuis* contain a pyrenoid-lacking chloroplast under LM and TEM and are robustly positioned within *Chloromonadinia*, consisting of both traditional (e.g. Ettl 1970, 1983) and recently revised (Pröschold *et al.* 2001) generic concepts of *Chloromonas*. I compared my new species, *Chr. fukushimae*, *Chr. krienitzii* and *Chr. tenuis*, with traditional *Chloromonas* species that grow only on snow or ice (based on Hoham 1975; Hoham & Mullet 1977, 1978; Hoham *et al.* 1979, 1983, 2002, 2006; Ettl 1983; Ling & Seppelt 1993, 1998; Muramoto *et al.* 2010). *Chr. fukushimae*, *Chr. krienitzii* and *Chr. tenuis* resembled four species that I re-examined in this study (*Chr. tughillensis*, *Chr. hohamii*, *Chr. pichinchae* and *Chr. chenangoensis*) as well as two other species, *Chr. rostafinskii* and *Chr. polyptera* (see Key and Table 3.4). Among these nine snow species, *Chr. fukushimae* and *Chr. krienitzii* are very similar to *Chr. rostafinskii* and *Chr. polyptera* in having elongate-kidney- to elongate-bean-shaped vegetative cells (Key; Table 3.4; Starmach & Kawecka 1965; Ling & Seppelt 1998). However, the chloroplast morphology differs between *Chr. fukushimae* or *Chr. krienitzii* and the latter two species. The chloroplast of *Chr. fukushimae* and *Chr. krienitzii* is parietal occupying the dorsal side of the cell (Figs 3.11–3.14, 3.35–3.38, 3.39, 3.45); whereas, the chloroplast is cup-shaped in *Chr. rostafinskii* (Starmach & Kawecka 1965). A longitudinal slit is present in the anterior half of the chloroplast in *Chr. polyptera* (Ling & Seppelt 1998); whereas, such a slit is absent in *Chr. fukushimae* and *Chr. krienitzii* (Figs 3.11–3.14, 3.35–3.38, 3.39, 3.45). *Chr. fukushimae* and *Chr. krienitzii* also differ from *Chr. polyptera* because it does not produce cell aggregates in old cultures (Table 3.4; Ling & Seppelt 1998). Furthermore, the

phylogenetic position of cysts of *Chr. polyptera* collected from Antarctica (Remias *et al.* 2013) differed from that of *Chr. fukushimae* and *Chr. krienitzii* in my phylogenetic analyses based on 18S rDNA gene sequences (Fig. 2.13). *Chr. fukushimae* and *Chr. krienitzii* are different from each other in differences of chloroplast shape and number of zoospores formed within the parental cell, as described above (Table 3.4). In contrast, among the nine species of snow algae *Chloromonas*, *Chr. tenuis* is very similar to the four species re-examined in this study in lacking elongate-kidney- to elongate-bean-shaped vegetative cells (see Key). However, *Chr. tenuis* can be distinguished from these four species based on differences in cell morphology, chloroplast shape, number of zoospores formed within the parental cell and presence or absence of cell aggregates in old cultures, as described above (Table 3.4).

Although *Chr. fukushimae*, *Chr. krienitzii* and *Chr. tenuis* did not grow at 20°C, the morphological characteristics of these two species were compared with mesophilic species of traditional *Chloromonas sensu* Ettl (1970, 1983). *Chr. fukushimae* and *Chr. krienitzii* are similar to four mesophilic species of traditional *Chloromonas* in having a parietal chloroplast on the dorsal surface and elongate vegetative cells, including *Chr. chlorogoniopsis* (H. Ettl) Gerloff & H. Ettl, *Chr. lunulatiformis* (L.Ş. Péterfi) Gerloff & H. Ettl, *Chr. prona* (H. & O. Ettl) P.C. Silva and *Chr. pumilio* H. Ettl (based on Ettl 1970, 1983). However, *Chr. fukushimae* and *Chr. krienitzii* lack an eyespot in the chloroplast (Figs 3.11, 3.35, 3.39, 3.45); whereas, the eyespot is present in the chloroplasts of these four mesophilic species (Ettl 1958, 1970, 1983; Ettl & Ettl 1959; Péterfi & Péterfi 1966). Furthermore, vegetative cell length for *Chr. fukushimae* is 15–23 μm and that for *Chr. krienitzii* is 17–24 μm (Table 3.4); whereas, the maximum vegetative cell length for *Chr. chlorogoniopsis*, *Chr. lunulatiformis* and *Chr. pumilio* is less than 15 μm (Ettl 1958, 1970, 1983; Péterfi & Péterfi 1966). The mesophilic *Chr. prona* overlaps in cell size with *Chr. fukushimae* and *Chr. krienitzii*, but it has a prominent anterior papilla and acute posterior cell end (Ettl & Ettl 1959; Ettl 1970, 1983); such characteristics were not observed in the vegetative cell of *Chr. fukushimae* and *Chr. krienitzii* (Figs

3.12, 3.36, 3.39, 3.45).

Chr. tenuis resembles five mesophilic species having elongate-cylindrical vegetative cells with a rounded posterior end (based on Ettl 1970, 1983): *Chr. angustissima* (H. Ettl) Gerloff & H. Ettl, *Chr. canaliformis* (H. Ettl) Gerloff & H. Ettl, *Chr. infirma* (Gerloff) P.C. Silva, *Chr. nasus* (Pascher) Gerloff & H. Ettl and *Chr. rosae* (H. & O. Ettl) H. Ettl. However, an eyespot is absent in the chloroplast of *Chr. tenuis* (Figs 3.23, 3.42); whereas, the chloroplasts of these five mesophilic species possess eyespots (Pascher 1930; Anakhin 1931; Ettl 1958; Ettl & Ettl 1959). In addition, the vegetative cell length of *Chr. tenuis* (15–23 μm) is significantly longer than that of *Chr. angustissima* (7–12 μm; Ettl 1958), *Chr. canaliformis* (10–12 μm; Ettl 1958) and *Chr. infirma* (8–14.5 μm; Anakhin 1931).

Thus, after careful evaluation of all described species of *Chloromonas*, I conclude that *Chr. fukushimae*, *Chr. krienitzii* and *Chr. tenuis* are new species. My molecular and morphological data support this conclusion. These results exhibit that a polyphasic approach could be used to identify snow species of *Chloromonas* even when aplanozygote morphology is lacking.

3.5. KEY TO SPECIES OF SNOW-INHABITING *CHLOROMONAS*

Key to species only growing on snow or ice in the genus *Chloromonas sensu* Ettl (1970, 1983), based on Ettl (1983), Ling & Seppelt (1993), Muramoto *et al.* (2010), and the present study. Species that have been studied using cultured material are marked with asterisks.

1. Cell not elongate or ellipsoidal with a rounded posterior end ...2

1. Cell elongate or ellipsoidal with a rounded posterior end ...5

2. Cell with prominent anterior papilla ...3

2. Cell without prominent anterior papilla ...4

3. Cell spherical to ovoid; two contractile vacuoles positioned near the base of the flagella; anterior papilla without concave top face ...*Chr. miwae*

3. Cell kidney-shaped with a flattened anterior end; four contractile vacuoles positioned near the base of flagella; anterior papilla with concave top face ...*Chr. rubroleosa*

4. Cell tear-shaped with a posterior tail ...*Chr. nivalis*

4. Cell ovoid or pyriform ...*Chr. brevispina*

5. Maximum cell length more than 40 μm ...*Chr. bolyaiana*

5. Maximum cell length less than 40 μm ...6

6. Maximum cell length less than 15 μm ...*Chr. alpina*

6. Maximum cell length more than 15 μm ...7

7. Cell elongate-kidney- to elongate-bean-shaped ...8

7. Cell elongate-ellipsoidal, elongate-cylindrical or ellipsoidal ...11

8. Chloroplast cup-shaped ...*Chr. rostafinskii*

8. Chloroplast parietal on the dorsal side of the cell ...9

9. Chloroplast with a longitudinal slit in the anterior half of the chloroplast; cell aggregates (more than 16 cells) formed in old cultures ...*Chr. polyptera**

9. Chloroplast without a longitudinal slit in the anterior half of the chloroplast; cell aggregates not formed in old cultures ...10

10. Chloroplast not filling in the posterior end of the protoplast; zoospores formed within the parental cell up to eight ...*Chr. fukushimae* sp. nov.*

10. Chloroplast filling in the posterior end of the protoplast; zoospores formed within the parental cell up to four ...*Chr. krienitzii* sp. nov.*

11. Chloroplast apparently composed of discs ...12

11. Chloroplast apparently composed of elongate-ovoid or elongate-cylindrical platelets ...13

12. Cell aggregates formed in old cultures ...*Chr. pichinchae**

12. Cell aggregates not formed in old cultures ...*Chr. chenangoensis**

13. Cell aggregates formed in old cultures ...*Chr. tughillensis**

13. Cell aggregates not formed in old cultures ...14

14. Cell elongate-ellipsoidal; zoospores formed within the parental cell up to eight ...*Chr. hohamii**

14. Cell elongate-cylindrical; zoospores formed within the parental cell up to four ...*Chr. tenuis* sp. nov.*

3.6. TABLES AND FIGURES

Table 3.1. List of strains and species of snow-inhabiting *Chloromonas* examined in this study.

Species	Strain designation	Locality	Date	Reference
Chloromonas chenangoensis	UTEX[1] SNO150 (authentic strain)	Hamilton, New York, USA	9 Apr 2001	Hoham *et al.* (2006), the website of the UTEX Culture Collection of Algae
	UTEX SNO143	Hamilton, New York, USA	9 Apr 2001	Hoham *et al.* (2006), the website of the UTEX Culture Collection of Algae
Chloromonas fukushimae	GsCl-11[2] (authentic strain)	Mt. Gassan, Yamagata, Japan (38°30’27’’N, 140°0’1’’E)	4 Jun 2008	the present study
	HkCl-65[3]	Mt. Hakkoda, Aomori, Japan (40°38’52’’N, 140°51’6’’E)	15 May 2012	the present study
Chloromonas hohamii	UTEX SNO67	Mt. Lemon, Arizona, USA	26 Apr 1987	The website of the UTEX Culture Collection of Algae
Chloromonas krienitzii	GsCl-54[2] (authentic strain)	Mt. Gassan, Yamagata, Japan (38°30’50’’N, 139°59’27’’E)	30 Jun 2013	the present study
	GsCl-49[2]	Mt. Gassan, Yamagata, Japan (38°30’50’’N, 139°59’27’’E)	30 Jun 2013	the present study
Chloromonas pichinchae	UTEX SNO33	Wenatchee Mts., Washington, USA	5 Jun 1977	Hoham *et al.* (2002), the website of the UTEX Culture Collection of Algae

Table 3.1. Continued

Chloromonas tenuis	UTEX SNO132 (authentic strain)	Jay Peak, Vermont, USA	17 May 1992	Hoham *et al.* (2002), the website of the UTEX Culture Collection of Algae
Chloromonas tughillensis	UTEX SNO91 (authentic strain)	Whetstone Gulf State Park, New York, USA	15 Apr 1988	Hoham *et al.* (2006), the website of the UTEX Culture Collection of Algae
	UTEX SNO88	Whetstone Gulf State Park, New York, USA	15 Apr 1988	Hoham *et al.* (2006), the website of the UTEX Culture Collection of Algae
	UTEX SNO92	Whetstone Gulf State Park, New York, USA	15 Apr 1988	Hoham *et al.* (2006), the website of the UTEX Culture Collection of Algae

[1]Culture Collection of Algae at University of Texas at Austin (UTEX) (Starr & Zeikus 1993).
[2]Established using the pipette-washing method (Pringsheim 1946).
[3]Established using the spread plate method (Segawa *et al.* 2011).

Table 3.2. List of taxa/strains in the comparison of secondary structures of nuclear ribosomal DNA (rDNA) internal transcribed spacer 2 (ITS2) (Figs 3.1–3.6) and genetic differences (Figs 3.7–3.10), and DDBJ/EMBL/GENBANK accession numbers of nuclear rDNA ITS2 and nuclear-encoded 26S rDNA. For information of nuclear-encoded 18S rDNA and plastid-encoded *atp*B and *psa*B genes, see Table 2.2.

Taxon	Strain designation	Accession number	
		nuclear rDNA ITS2	26S rDNA
Chloromonas fukushimae sp. nov.	GsCl-11	AB906382–3[1]	AB906352[1]
	HkCl-65	AB906384[1]	AB906353[1]
Chloromonas hohamii	UTEX[2] SNO67	AB906394[1]	AB906354[1]
Chloromonas tenuis sp. nov.	UTEX SNO132	AB906395[1]	AB906355[1]
Chloromonas tughillensis	UTEX SNO91	AB906385–7[1]	AB906356[1]
	UTEX SNO88	AB906388–90[1]	AB906357[1]
	UTEX SNO92	AB906391–3[1]	AB906358[1]
Chloromonas chlorococcoides	SAG[3] 15.82		AB906359[1]
Chloromonas reticulata	SAG 29.83 (= UTEX 1970)		AF395508

[1]Sequenced in the present study.
[2]Culture Collection of Algae at the University of Texas at Austin (Starr & Zeikus 1993).
[3]Sammlung von Algenkulturen at the University of Göttingen (Schlösser 1994).

Table 3.3. Primers used for amplifications and sequencing of nuclear-encoded 26S ribosomal DNA gene.

Designation	Position[1]	Sequence (5'–3')
F1	77–101	ATTCCCCTAGTAACGGCGAGCGAAC
F2	804–826	CGAAAGATGGTGAACTATGCCTG
R1	882–860[2]	TGCACGTCAGCACATCTACGAGC
F3	1501–1521	CAGCAATTGGACATGGGTTAG
R2	1726–1702[2]	CTATCGCCGGATGATCCGATTCCAG
R3	2159–2139[2]	CCTTTAGGACCATCACAATGC

[1]Coordinate numbers from the *Oryza sativa* 25S ribosomal DNA (Takaiwa *et al.* 1985).
[2]Reverse primer.

Table 3.4. Comparison of morphological characteristics of 11 snow algae species having elongate or ellipsoidal vegetative cells with a rounded posterior end, in the genus *Chloromonas sensu* Ettl (1970, 1983).

	***Chr. fukushimae* sp. nov.**	***Chr. tughillensis***	***Chr. hohamii***
Strain(s) used in this study	GsCl-11, HkCl-65	UTEX SNO91, UTEX SNO88, UTEX SNO92	UTEX SNO67
Cell shape	elongate-bean- to elongate-kidney-shaped	elongate-ellipsoidal	elongate-ellipsoidal
Cell width × cell length (μm)	4–9 × 15–23	6–12 × 16–23	5–10 × 12–25
Eyespot	absent	absent	absent or generally present[1]
Chloroplast shape	laminate parietal in the dorsal side of the cell, not filling in the posterior end of the protoplast	cup-shaped, apparently composed of elongate-ovoid or elongate-cylindrical platelets	cup-shaped, apparently composed of elongate-ovoid or elongate-cylindrical platelets
Longitudinal slit of the chloroplast in the anterior half of the cell	not observed	not observed	not observed
Cell aggregates in old cultures	not observed	observed	not observed
Number of zoospores formed within the parental cell	two, four or eight	two, four, eight or 16	two, four or eight
Aplanozygote shape	−	spherical	ellipsoidal, elongate or somewhat fusiform, with longitudinal ridges not spiralled
References	the present study	Hoham *et al.* (2006), the present study	Hoham *et al.* (1983), the present study

[1]Based on Hoham *et al.* (1983).

[2]See chapter 2

Table 3.4. Extended

***Chr. tenuis* sp. nov.**	***Chr. pichinchae***	***Chr. chenangoensis***	***Chr. krienitzii* sp. nov.**
UTEX SNO132	UTEX SNO33	UTEX SNO150, UTEX SNO143	GsCl-54, GsCl-49
elongate-cylindrical	elongate-ovoid to ellipsoidal	ellipsoidal	elongate-bean- to elongate-kidney-shaped
5–9 × 15–23	8–15 × 18–26	7.5–17.5 × 14–25	7–11 × 17–24
absent	absent	absent	absent
cup-shaped, apparently composed of elongate-ovoid or elongate-cylindrical platelets	cup-shaped, apparently composed of discs	cup-shaped, apparently composed of discs	parietal in the dorsal side of the cell, filling in the posterior end of the protoplast
not observed	not observed	not observed	not observed
not observed	observed	not observed	not observed
generally two or four	generally two or four	generally two or four	two or four
–	ellipsoidal with usually nine to 11 irregular ridges extending over the wall from one cell pole to the other	–	almost spherical with numerous short spines on the surface of the cell wall[2]
the present study	Wille (1903), Hoham (1975), the present study	Hoham *et al.* (2006), the present study	the present study

Table 3.4. Extended

Chr. alpina	*Chr. bolyaiana*	*Chr. polyptera*	*Chr. rostafinskii*
–	–	–	–
ellipsoidal to ovoid	ellipsoidal	elongate-bean- to elongate-kidney-shaped	elongate-bean- to elongate-kidney-shaped or ellipsoidal
4–7 × 9–12	46–52 × 52–72	8–14 × 20–27	6.6–13.2 × 11–22.5
present	absent	generally absent	absent
parietal, composed of numerous discoid-lobes	asteroid-shaped	parietal in the dorsal side of the cell	cup-shaped
not observed	not observed	observed	not observed
–	–	observed	–
two or four	two, four or eight	two or four	two or four
–	spherical, having three layered membrane of which the middle layer is ornamented as star-shaped	–	ellipsoidal with thick, warty cell wall
Wille (1903), Ettl (1970, 1983)	Kol (1947), Ettl (1970, 1983)	Ling & Seppelt (1998)	Starmach & Kawecka (1965), Kawecka (1983/1984), Ettl (1970, 1983)

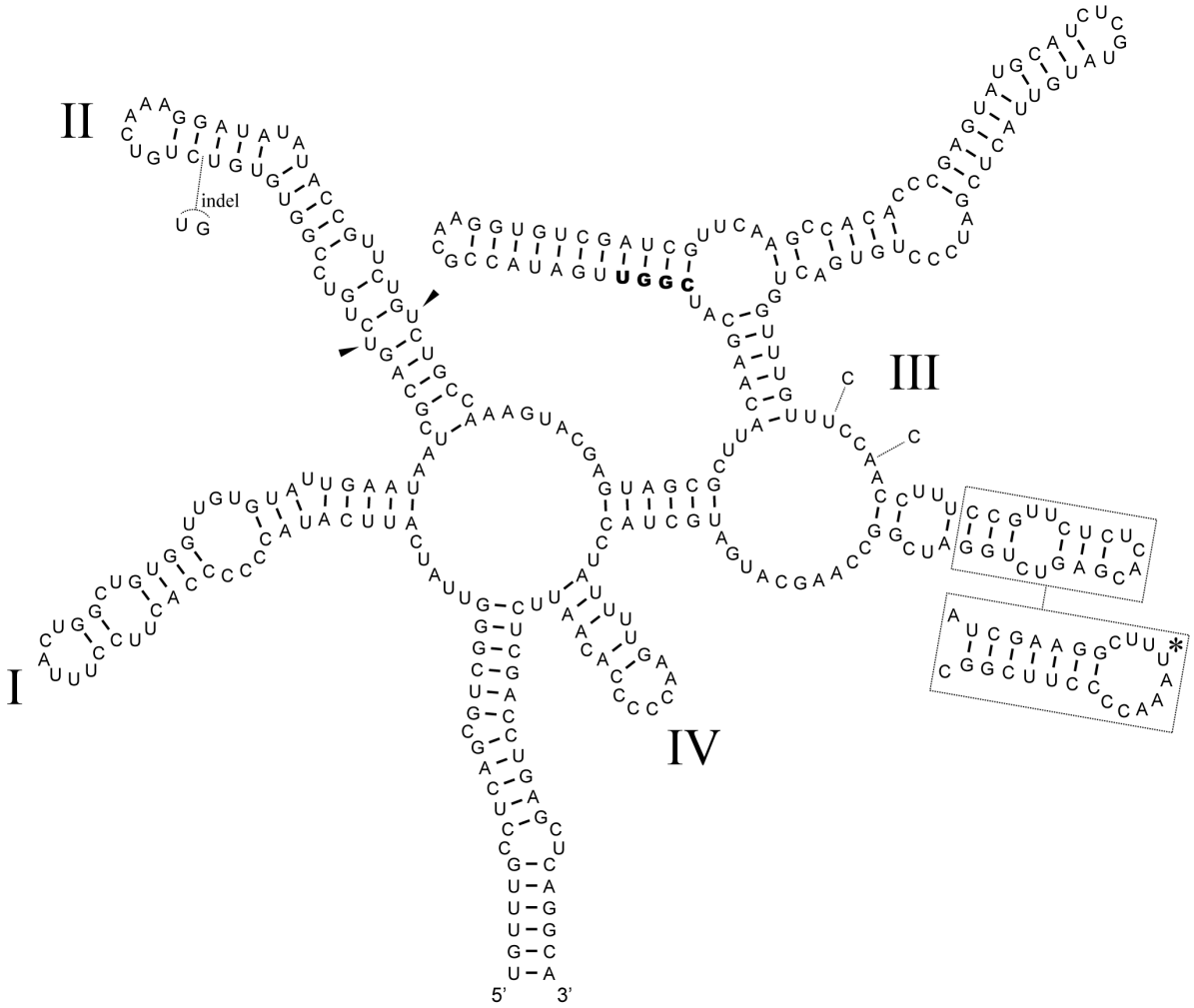


Fig. 3.1. The secondary structure of nuclear ribosomal DNA internal transcribed spacer 2 transcript of *Chloromonas fukushimae* strain GsCl-11. Differences within *Chr. fukushimae* strains (vs. HkCl-65) are also shown by characters just outside the secondary structures. Dotted box with asterisk indicates the structural variation due to the sequence variant in the tip of helix III, only detected in the strain GsCl-11. Note the U-U mismatch in helix II (arrowheads) and the YGGY motif on the 5' side near the apex of helix III (bold faces).

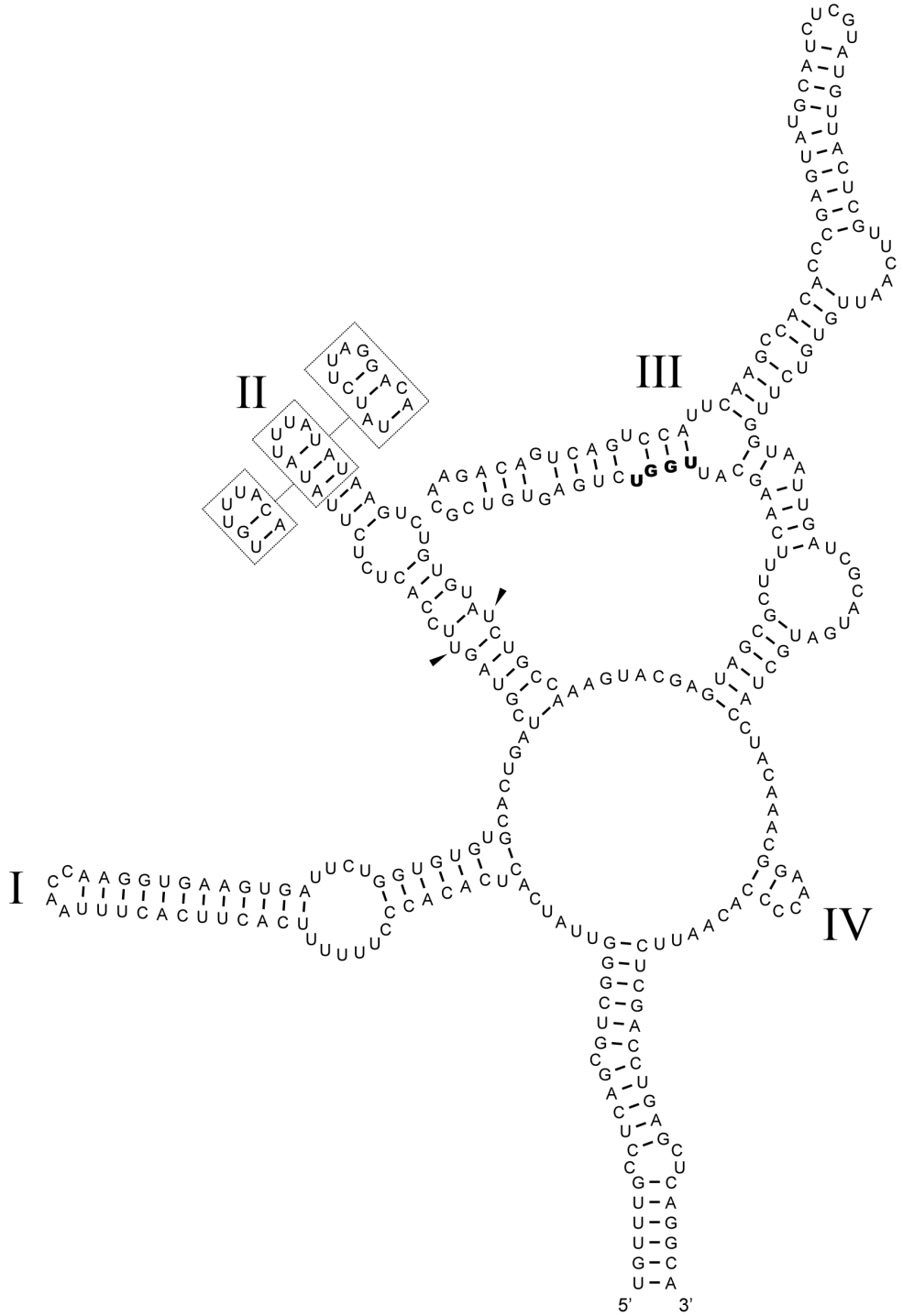


Fig. 3.2. The secondary structure of nuclear ribosomal DNA (rDNA) internal transcribed spacer 2 (ITS2) transcript of *Chloromonas tughillensis* strain UTEX SNO91. Nuclear rDNA ITS2 sequences of *Chr. tughillensis* strain UTEX SNO91, UTEX SNO88 and UTEX SNO92 are identical. Structural variations due to the sequence variants found in the tip of helix II are shown in the dotted boxes. Note the U-U mismatch in helix II (arrowheads) and the YGGY motif on the 5' side near the apex of helix III (bold faces).

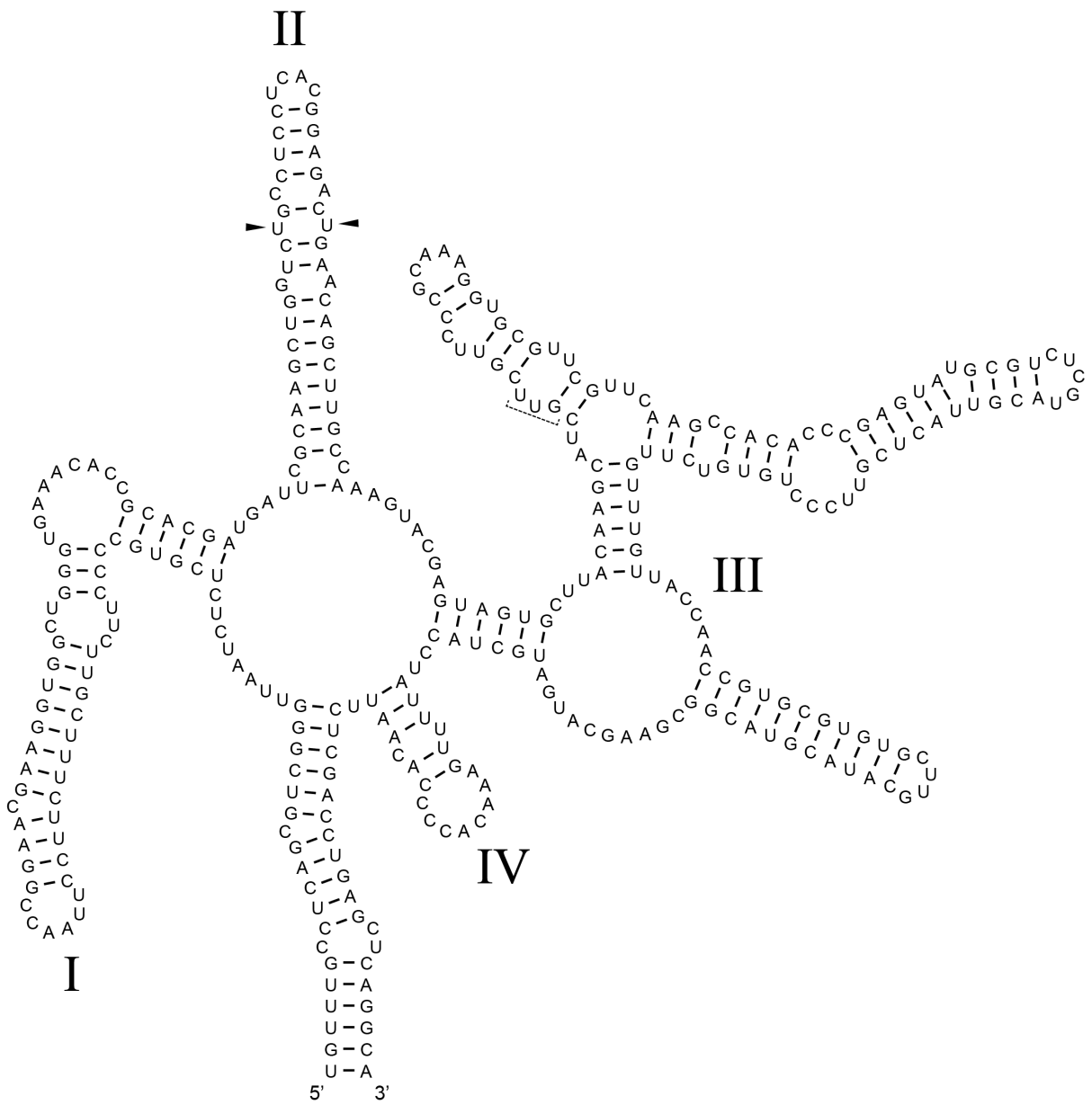


Fig. 3.3. The secondary structure of nuclear ribosomal DNA internal transcribed spacer 2 transcript of *Chloromonas hohamii* strain UTEX SNO67. Note the U-U mismatch in helix II (arrowheads) and a possible GYU motif on the 5' side near the apex of helix III (dotted bracket).

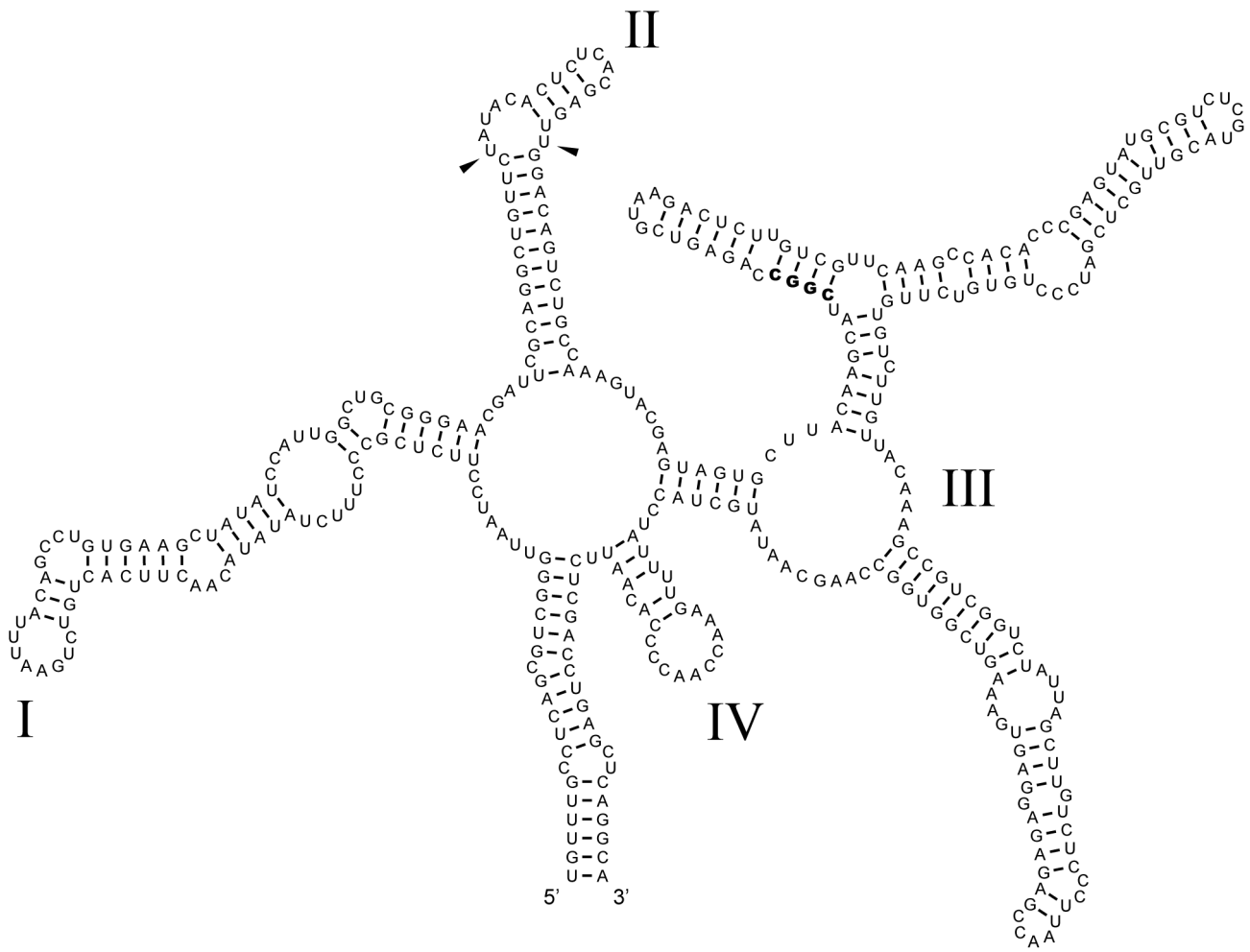


Fig. 3.4. The secondary structure of nuclear ribosomal DNA internal transcribed spacer 2 transcript of *Chloromonas tenuis* strain UTEX SNO132. Note the U-U mismatch in helix II (arrowheads) and the YGGY motif on the 5' side near the apex of helix III (bold faces).

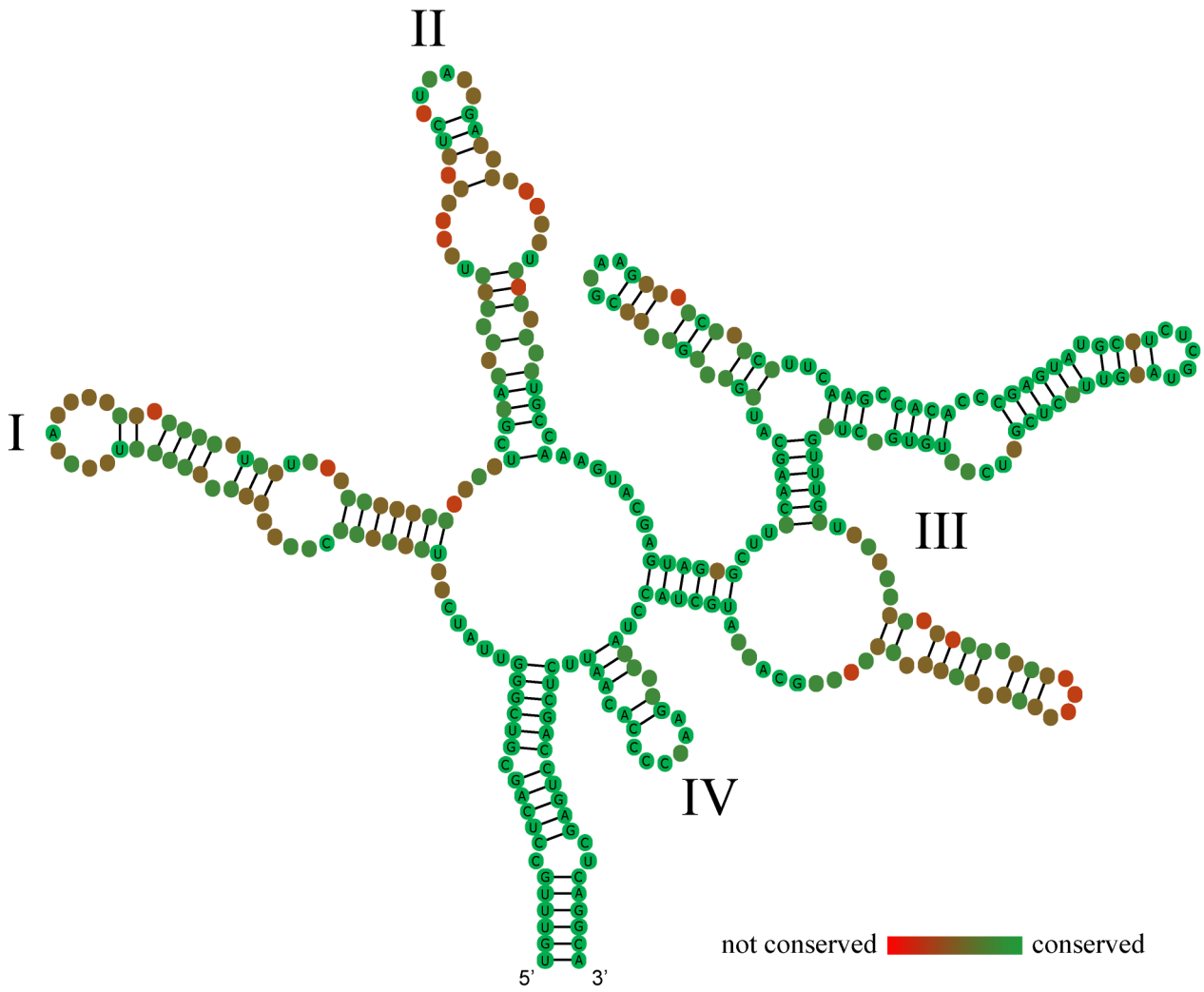


Fig. 3.5. Sequence conservation of nuclear ribosomal DNA (rDNA) internal transcribed spacer 2 (ITS2) transcripts among *Chloromonas fukushimae*, *Chr. tughillensis*, *Chr. hohamii* and *Chr. tenuis*. The consensus secondary structure of the nuclear rDNA ITS2 transcripts among the four species shows the common four helices with branched helix III. Sequence conservation is visualised via colour interpolation between red (not conserved) and green (conserved).

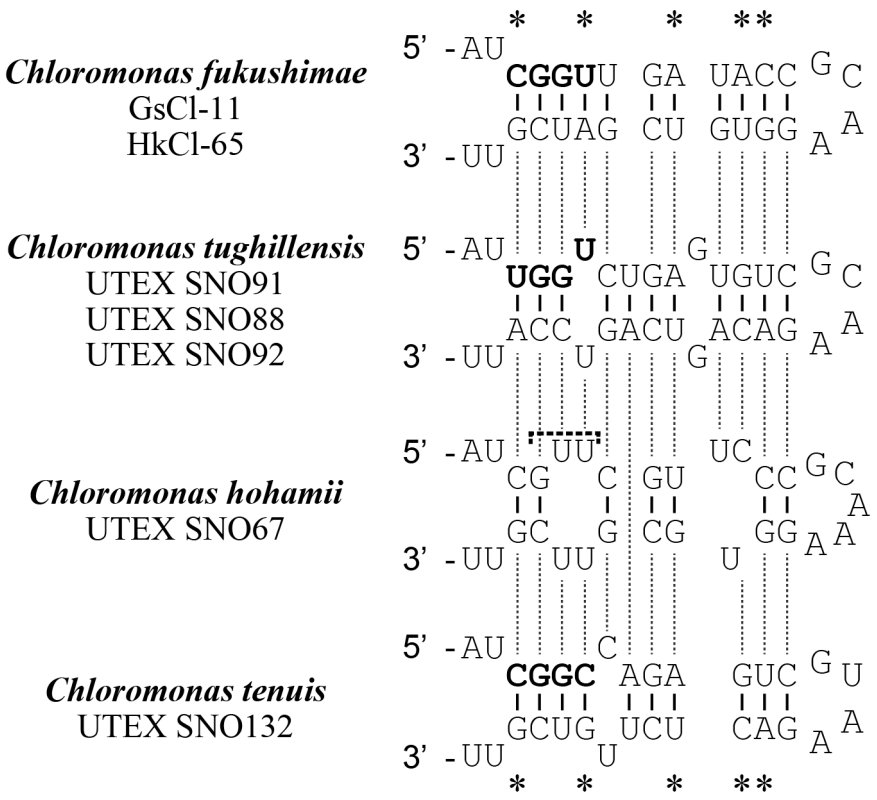
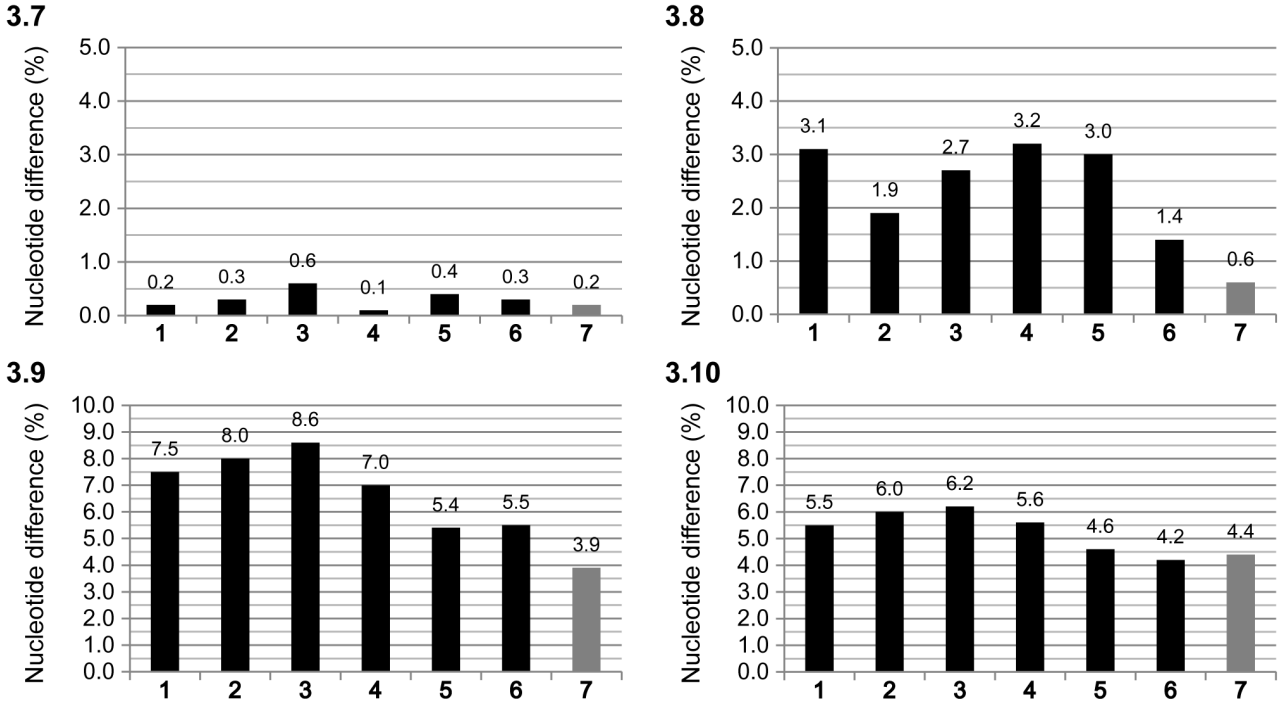


Fig. 3.6. Comparison of the tip of helix III of the nuclear ribosomal DNA (rDNA) internal transcribed spacer 2 (ITS2) secondary structures of *Chloromonas fukushimae* (top), *Chr. tughillensis* (upper middle), *Chr. hohamii* (lower middle) and *Chr. tenuis* (bottom). Note the YGGY motif (boldface) and the possible GYU motif (dotted bracket). The base pair position, including interspecific compensatory base change among the four species, is indicated by an asterisk. For complete nuclear rDNA ITS2 secondary structures, see Figs 3.1–3.4.



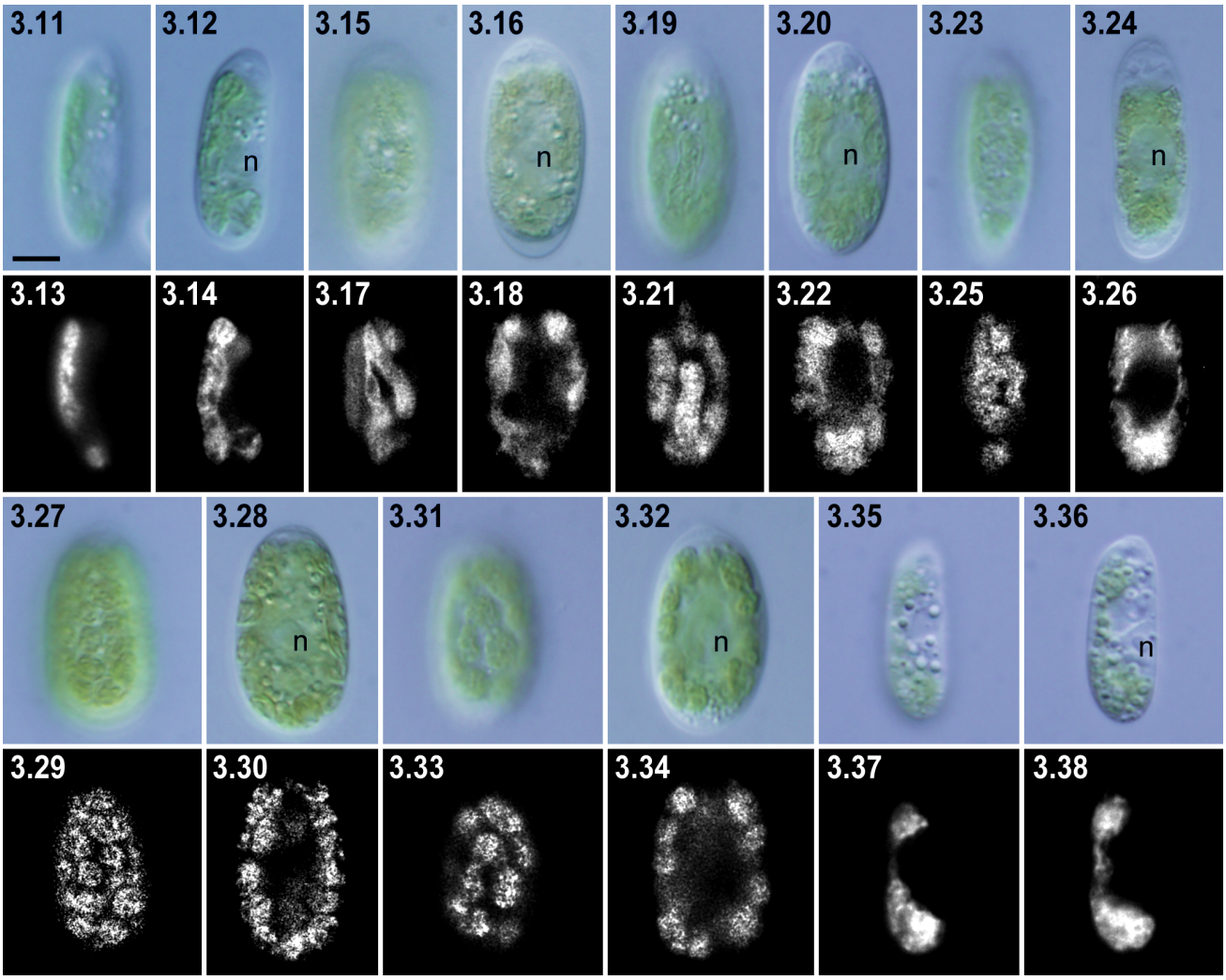
Figs 3.7–3.10. Nucleotide differences (%) between (1) *Chloromonas fukushimae* and *Chr. tughillensis*, (2) *Chr. fukushimae* and *Chr. hohamii*, (3) *Chr. fukushimae* and *Chr. tenuis*, (4) *Chr. tughillensis* and *Chr. hohamii*, (5) *Chr. tughillensis* and *Chr. tenuis*, (6) *Chr. hohamii* and *Chr. tenuis* and (7) *Chr. chlorococcoides* and *Chr. reticulata*. Note that the nucleotide sequences of nuclear-encoded 18S and 26S ribosomal DNA (rDNA), *atp*B and *psa*B genes of the two strains of *Chr. fukushimae* or the three strains of *Chr. tughillensis* are identical, respectively.

Fig. 3.7. 1,746 bases of 18S rDNA gene.

Fig. 3.8. 2,020 bases of 26S rDNA gene.

Fig. 3.9. 1,128 bases of *atp*B gene.

Fig. 3.10. 1,392 bases of *psa*B gene.



Figs 3.11–3.38. Vegetative cells of seven snow-inhabiting species of *Chloromonas* having elongate or ellipsoidal cell shapes with a rounded posterior end, shown at the same magnification throughout. Scale bar = 5 μm. n = nucleus.

Figs 3.11–3.14. *Chr. fukushimae* strain GsCl-11. Dorsal to the left, ventral to the right.

Fig. 3.11. DIC. Surface view of a cell.

Fig. 3.12. DIC. Optical section of a cell.

Fig. 3.13. Fluorescence image of Fig. 3.11.

Fig. 3.14. Fluorescence image of Fig. 3.12.

Figs 3.15–3.18. *Chr. tughillensis* strain UTEX SNO88.

Fig. 3.15. DIC. Surface view of a cell.

Fig. 3.16. DIC. Optical section of a cell.

Fig. 3.17. Fluorescence image of Fig. 3.15.

Fig. 3.18. Fluorescence image of Fig. 3.16.

Figs 3.19–3.22. *Chr. hohamii* strain UTEX SNO67.

Fig. 3.19. DIC. Surface view of a cell.

Fig. 3.20. DIC. Optical section of a cell.

Fig. 3.21. Fluorescence image of Fig. 3.19.

Fig. 3.22. Fluorescence image of Fig. 3.20.

Figs 3.23–3.26. *Chr. tenuis* strain UTEX SNO132.

Fig. 3.23. DIC. Surface view of a cell.

Fig. 3.24. DIC. Optical section of a cell.

Fig. 3.25. Fluorescence image of Fig. 3.23.

Fig. 3.26. Fluorescence image of Fig. 3.24.

Figs 3.27–3.30. *Chr. pichinchae* strain UTEX SNO33.

Fig. 3.27. DIC. Surface view of a cell.

Fig. 3.28. DIC. Optical section of a cell.

Fig. 3.29. Fluorescence image of Fig. 3.27.

Fig. 3.30. Fluorescence image of Fig. 3.28.

Figs 3.31–3.34. *Chr. chenangoensis* strain UTEX SNO143.

Fig. 3.31. DIC. Surface view of a cell.

Fig. 3.32. DIC. Optical section of a cell.

Fig. 3.33. Fluorescence image of Fig. 3.31.

Fig. 3.34. Fluorescence image of Fig. 3.32.

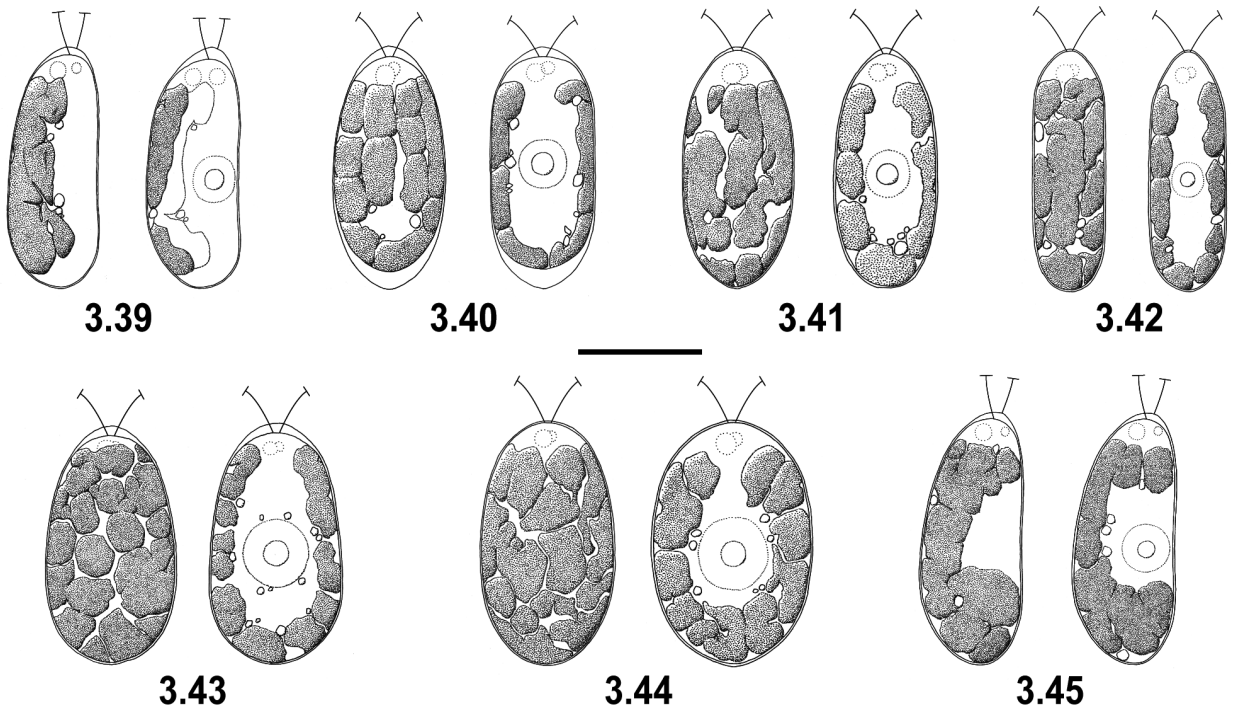
Figs 3.35–3.38. *Chr. krienitzii* strain GsCl-54. Dorsal to the left, ventral to the right.

Fig. 3.35. DIC. Surface view of a cell.

Fig. 3.36. DIC. Optical section of a cell.

Fig. 3.37. Fluorescence image of Fig. 3.35.

Fig. 3.38. Fluorescence image of Fig. 3.36.



Figs 3.39–3.45. Line drawings of vegetative cells, shown at the same magnification throughout. Left, surface view. Right, optical section. Scale bar = 10 μm.

Fig. 3.39. *Chloromonas fukushimae* strain GsCl-11. Dorsal to the left, ventral to the right.

Fig. 3.40. *Chr. tughillensis* strain UTEX SNO88.

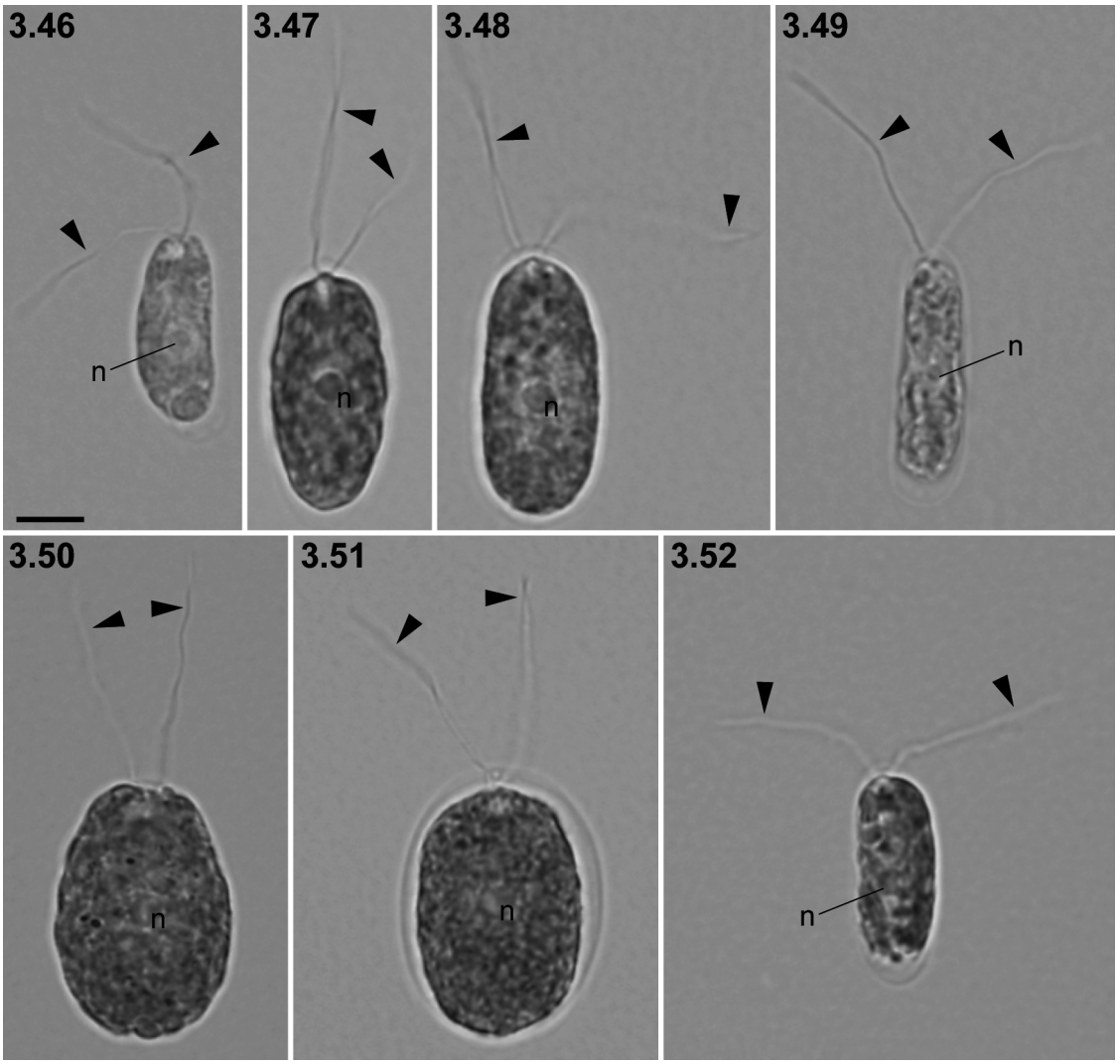
Fig. 3.41. *Chr. hohamii* strain UTEX SNO67.

Fig. 3.42. *Chr. tenuis* strain UTEX SNO132.

Fig. 3.43. *Chr. pichinchae* strain UTEX SNO33.

Fig. 3.44. *Chr. chenangoensis* strain UTEX SNO143.

Fig. 3.45. *Chr. krienitzii* strain GsCl-54. Dorsal to the left, ventral to the right.



Figs 3.46–3.52. Bright-field micrographs of vegetative cells with two flagella (arrowheads), shown at the same magnification throughout. Vegetative cells were fixed with approximately 0.4 w/v% iodine solution (Meiji Seika Pharma Co., Ltd., Tokyo, Japan). n = nucleus. Scale bar = 5 μm.

Fig. 3.46. *Chloromonas fukushimae* strain GsCl-11.

Fig. 3.47. *Chr. tughillensis* strain UTEX SNO88.

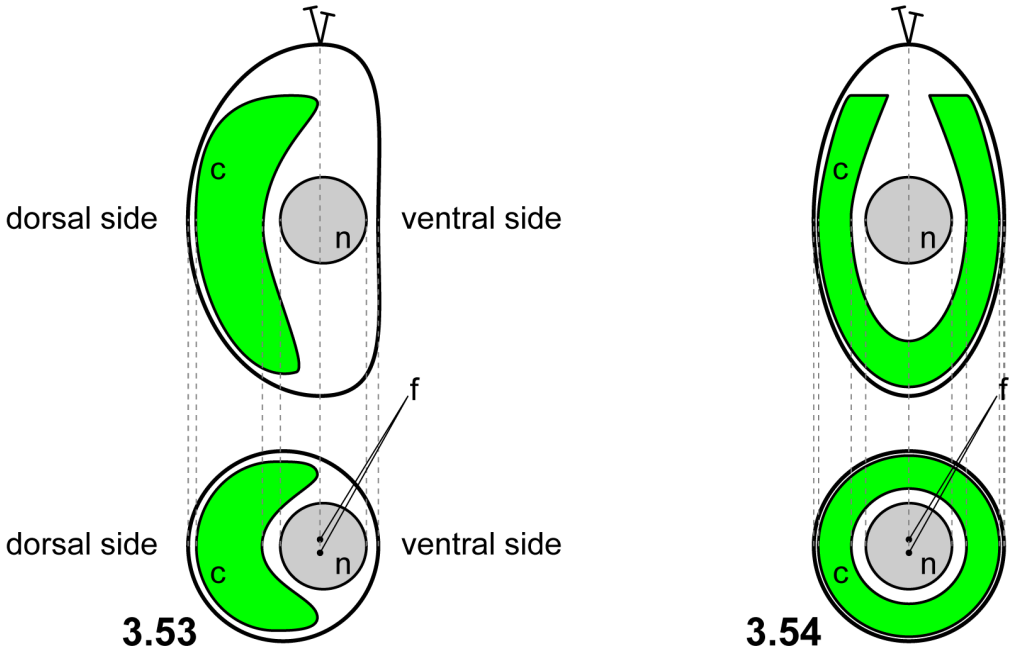
Fig. 3.48. *Chr. hohamii* strain UTEX SNO67.

Fig. 3.49. *Chr. tenuis* strain UTEX SNO132.

Fig. 3.50. *Chr. pichinchae* strain UTEX SNO3.

Fig. 3.51. *Chr. chenangoensis* strain UTEX SNO143.

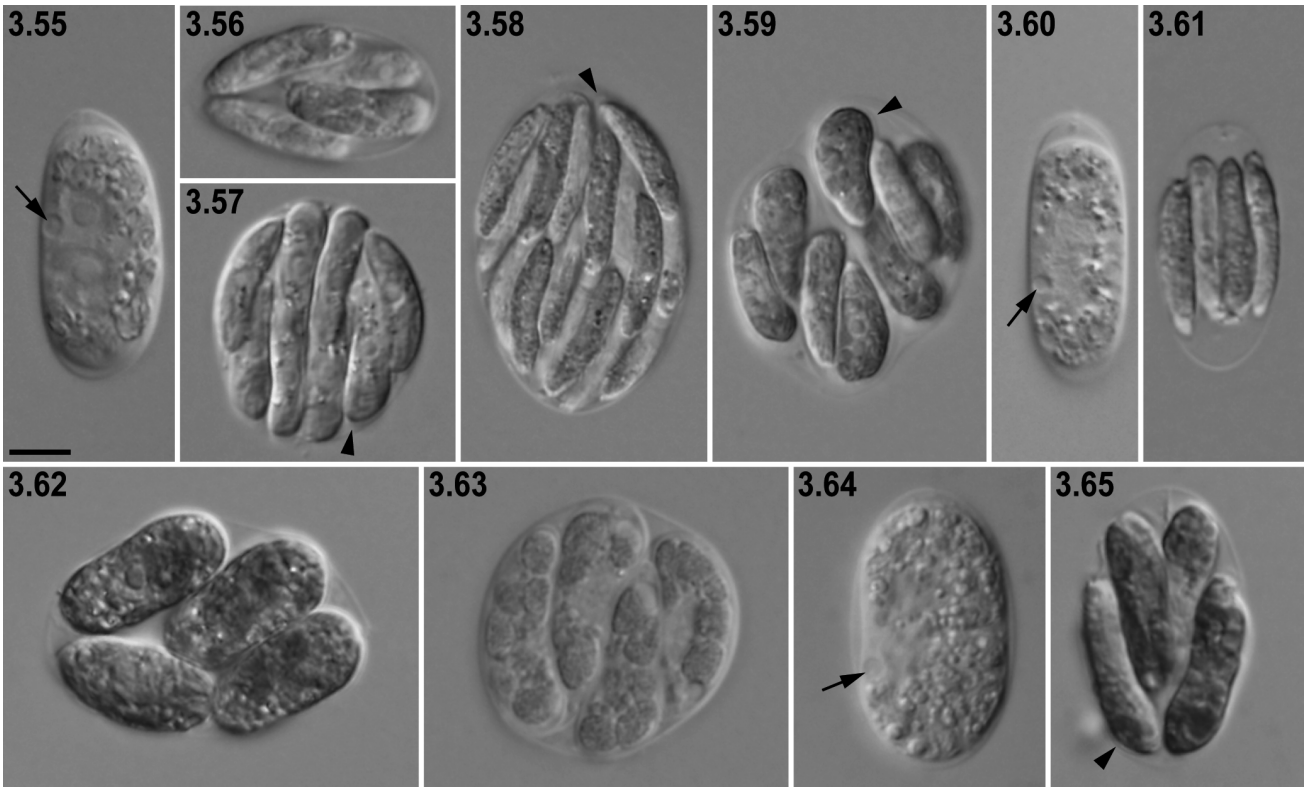
Fig. 3.52. *Chr. krienitzii* strain GsCl-54.



Figs 3.53, 3.54. Diagrams showing cell morphology of seven snow-inhabiting species of *Chloromonas*. Top, longitudinal cell section. Bottom, the cell viewed from the anterior end toward the posterior end. c = chloroplast; f = flagellum; n = nucleus.

Fig. 3.53. *Chr. fukushimae* and *Chr. krienitzii*.

Fig. 3.54. *Chr. tughillensis*, *Chr. hohamii*, *Chr. tenuis*, *Chr. pichinchae* and *Chr. chenangoensis*.



Figs 3.55–3.65. Nomarski differential interference micrographs of asexual reproduction, shown at the same magnification throughout. Scale bar = 5 μm.

Figs 3.55–3.57. *Chloromonas fukushimae* strain GsCl-11.

Fig. 3.55. First transverse division, showing the position of a contractile vacuole (arrow) that originated from the parental cell.

Fig. 3.56. Four daughter cells within the parental cell wall, fixed with approximately 0.4 w/v% iodine solution (Meiji Seika Pharma Co., Ltd., Tokyo, Japan).

Fig. 3.57. Eight daughter cells within the parental cell wall, fixed with approximately 0.4 w/v% iodine solution. Note the cell wall of the daughter cell (arrowhead).

Fig. 3.58. *Chr. tughillensis* strain UTEX SNO88; 16 daughter cells within the parental cell wall, fixed with approximately 0.4 w/v% iodine solution. Note the cell wall of the daughter cell (arrowhead).

Fig. 3.59. *Chr. hohamii* strain UTEX SNO67; eight daughter cells within the parental cell wall, fixed with approximately 0.4 w/v% iodine solution. Note the cell wall of the daughter cell (arrowhead).

Figs 3.60, 3.61. *Chr. tenuis* strain UTEX SNO132.

Fig. 3.60. First transverse division, showing the position of a contractile vacuole (arrow) that originated from the parental cell.

Fig. 3.61. Four daughter cells within the parental cell wall, fixed with approximately 0.4 w/v% iodine solution.

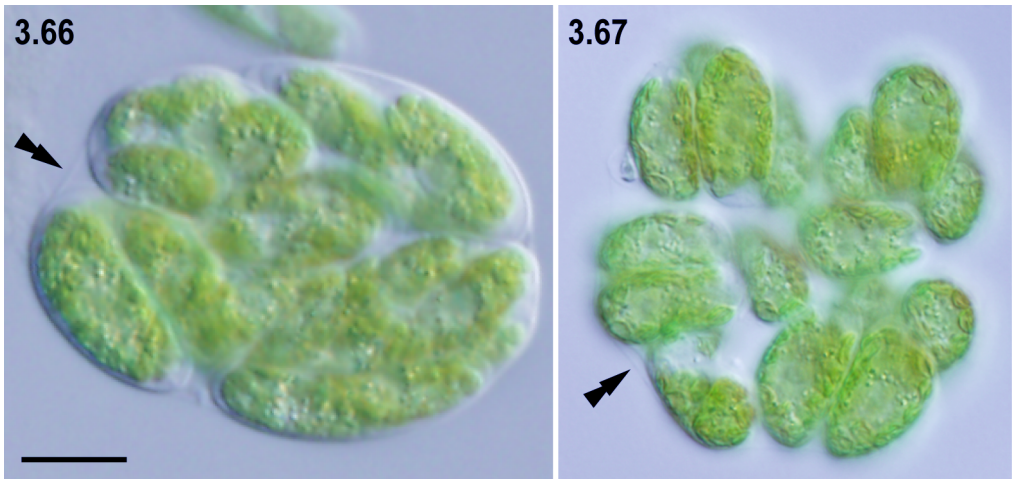
Fig. 3.62. *Chr. pichinchae* strain UTEX SNO33; four daughter cells within the parental cell wall, fixed with approximately 0.4 w/v% iodine solution.

Fig. 3.63. *Chr. chenangoensis* strain UTEX SNO143; four daughter cells within the parental cell wall.

Figs 3.64, 3.65. *Chr. krienitzii* strain GsCl-54.

Fig. 3.64. First transverse division, showing the position of a contractile vacuole (arrow) that originated from the parental cell.

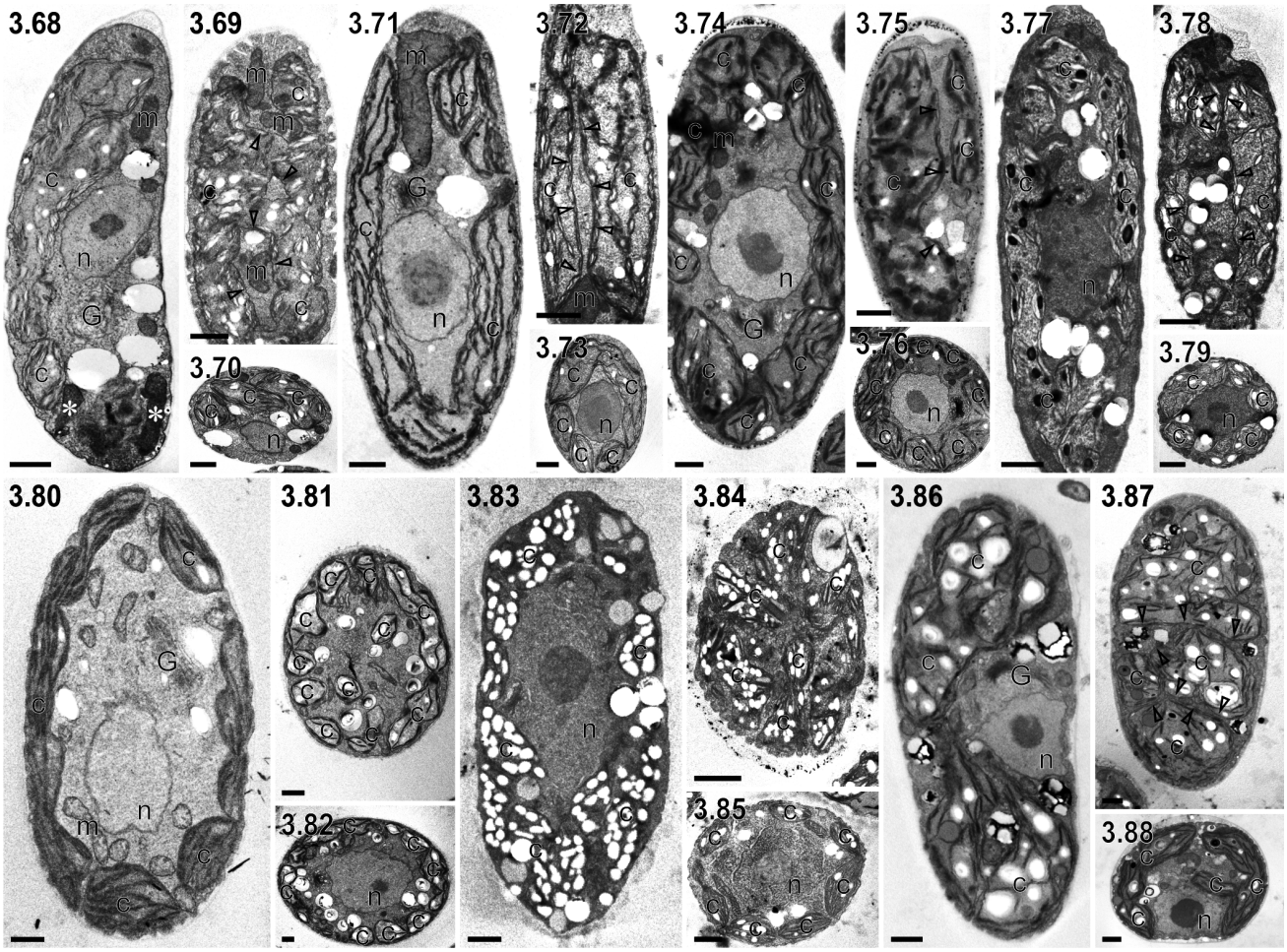
Fig. 3.65. Four daughter cells within the parental cell wall, fixed with approximately 0.4 w/v% iodine solution. Note the cell wall of the daughter cell (arrowhead).



Figs 3.66, 3.67. Nomarski differential interference micrographs of an aggregate of cells resulting from repeated divisions of daughter cells retained in the parental cell wall (double arrowheads) in old cultures, shown at the same magnification. Scale bar = 10 μm.

Fig. 3.66. *Chloromonas tughillensis* strain UTEX SNO91 after 2 months on 1.5% agar slant of AF-6 medium.

Fig. 3.67. *Chr. pichinchae* strain UTEX SNO33 after 2 months on 1.5% agar slant of AF-6 medium.



Figs 3.68–3.88. Transmission electron micrographs of vegetative cells. Scale bars = 1 μm. c = chloroplast; G = Golgi body; m = mitochondrion; n = nucleus.

Figs 3.68–3.70. *Chloromonas fukushimae* strain GsCl-11.

Fig. 3.68. Longitudinal cell section. Note the posterior mitochondrion (asterisk).

Fig. 3.69. Tangential cell section. Open arrowheads indicate chloroplast membranes.

Fig. 3.70. Transverse cell section.

Figs 3.71–3.73. *Chr. tughillensis* strain UTEX SNO88.

Fig. 3.71. Longitudinal cell section.

Fig. 3.72. Tangential cell section. Open arrowheads indicate chloroplast membranes.

Fig. 3.73. Transverse cell section.

Figs 3.74–3.76. *Chr. hohamii* strain UTEX SNO67.

Fig. 3.74. Longitudinal cell section.

Fig. 3.75. Tangential cell section. Open arrowheads indicate chloroplast membranes.

Fig. 3.76. Transverse cell section.

Figs 3.77–3.79. *Chr. tenuis* strain UTEX SNO132.

Fig. 3.77. Longitudinal cell section.

Fig. 3.78. Tangential cell section. Open arrowheads indicate chloroplast membranes.

Fig. 3.79. Transverse cell section.

Figs 3.80–3.82. *Chr. pichinchae* strain UTEX SNO33.

Fig. 3.80. Longitudinal cell section.

Fig. 3.81. Tangential cell section.

Fig. 3.82. Transverse cell section.

Figs 3.83–3.85. *Chr. chenangoensis* strain UTEX SNO143.

Fig. 3.83. Longitudinal cell section.

Fig. 3.84. Tangential cell section.

Fig. 3.85. Transverse cell section.

Figs 3.86–3.88. *Chr. krienitzii* strain GsCl-54.

Fig. 3.86. Longitudinal cell section.

Fig. 3.87. Tangential cell section. Open arrowheads indicate chloroplast membranes.

Fig. 3.88. Transverse cell section.

Chapter 4. General discussion

Correct taxonomic positions of field-collected aplanozygotes of snow **Chloromonas** *species*

Life cycle data encompassing both motile vegetative cells and aplanozygotes have previously been demonstrated in only North American materials of five snow-inhabiting species of *Chloromonas* (Hoham 1975; Hoham & Mullet 1977; Hoham *et al.* 1979, 1983, 2006). Nevertheless, snow *Chloromonas* species collected from various regions are often identified based only on aplanozygote morphology (e.g. Müller *et al.* 1998; Kvíderová *et al.* 2005; Lukavský & Cepák 2010).

In the present study, I obtained long sequences of multiple DNA regions from the field-collected aplanozygotes which were morphologically identical under LM, in order to examine the detailed phylogenetic positions of the aplanozygotes using phylogenetic analyses of multiple DNA regions (chapter 2). Based on the results of multigene phylogeny and comparison of fast-evolving ITS2 of nuclear rDNA cistron, I demonstrated that field-collected aplanozygotes and a culture strain belong to the same species in two lineages (chapter 2). The present LM, TEM and molecular analysis of culture strains in chapter 3 exhibited effectiveness of such polyphasic methods to classify snow-inhabiting species of *Chloromonas* even lacking information of aplanozygotes. Thus, additional worldwide culture strains of snow *Chloromonas* species identified by polyphasic methods are needed to clarify correct taxonomic positions of aplanozygotes of snow *Chloromonas* collected from various regions of the world by analyses of multiple DNA regions of the aplanozygotes.

Unveiling the life cycle of snow-inhabiting **Chloromonas**

Whole life cycle data are important to understand actual biodiversity and activity of organisms especially in algae and fungi (e.g. heterococcolith and holococcolith phase in Coccolithophyceae [Geisen *et al.* 2002]; anamorph and teleomorph in pleomorphic fungi [Hennebert 1971]). The present study demonstrated that polyphasic analyses of cultured materials are useful for delineating snow species of *Chloromonas* (chapter 3). However, induction of aplanozygote formation in culture is difficult in these algae (Hoham *et al.* 1998, 2000, 2006). Thus, combining the polyphasic analyses of

culture strains and the multi-DNA phylogenetic analyses of field-collected aplanozygotes is a possible and practical strategy to unveil the life cycle including vegetative cells and aplanozygotes in each species of snow *Chloromonas*.

Subdivision of the cosmopolitan species of snow-inhabiting **Chloromonas**

The present phylogenetic analyses unambiguously demonstrated that the cosmopolitan species "*Chr. nivalis*" is actually composed of multiple cryptic species. Thus, one of the cryptic species should be identified as *Chr. nivalis sensu stricto* when it includes the holotype, and others should be classified under different species names. However, information of motile vegetative cells of *Pteromonas nivalis* (the basionym of *Chr. nivalis*; Hoham & Mullet 1977) collected from French mountainous areas (Chodat 1892) (Table 1.1) is lacking. Only vegetative cells of "*Chr. nivalis*" originating from North America are known in this cosmopolitan species (Hoham & Mullet 1977). The present multi-DNA analysis exhibited that culture strains of "*Chr. miwae"* NIES-2379 and the specimen Gassan-C (the small type aplanozygotes of "*Chr. nivalis*" from Japan) belong to the same species, but the vegetative cells of "*Chr. miwae*" can be compared with only those of the North American material within "*Chr. nivalis.*" Thus, *Chr. nivalis* should be tentatively circumscribed by having "tear-shaped vegetative cells with a posterior tail" as reported by Hoham & Mullet (1977) in the North American material.

Similarly, the present study showed that "*Chr. brevispina,*" other cosmopolitan species of snow-inhabiting *Chloromonas*, actually contains multiple cryptic species. Therefore, taxonomic re-examination as those described above is also required for this species. However, features of motile vegetative cells of *Chodatella brevispina* (the basionym of *Chr. brevispina*; Hoham *et al.* 1979) collected from South Orkney, Antarctica (Fritsch 1912) (Table 1.1) are unknown; the life cycle data encompassing both motile vegetative cells and aplanozygotes of "*Chr. brevispina*" are reported only from North American materials (Hoham *et al.* 1979). North American "*Chr. brevispina*" produces

two types of aplanozygotes (*Cryocystis*- and *Trochiscia*-type; Hoham *et al.* 1979) (Fig. 1.4), but the present molecular analyses exhibited that the specimens Gassan-A and Hakkoda-2 (*Trochiscia*-type aplanozygotes of "*Chr. brevispina*" from Japan) and *Chr. krienitzii* strains GsCl-54 and GsCl-49 are assigned to the same species. Since the vegetative cells of *Chr. krienitzii* can be compared with only those of North American "*Chr. brevispina*," *Chr. brevispina* should be tentatively demarcated by its vegetative morphology (ovoid or pyriform in shape) which was provided based on light microscopic observation of North American samples (Hoham *et al.* 1979).

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