Symbiosis, 36 (2004) 127–151
Balaban, Philadelphia/Rehovot

Choricystis minor as a New Symbiont of Simultaneous Two-Species Association with *Paramecium bursaria* and Implications for its Phylogeny

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Received August 17, 2003; Accepted November 2, 2003

Abstract

In living specimens of *Paramecium bursaria* collected in Florida in 1992, we found a symbiotic association involving two coccoid algae. One organism had larger cell sizes and pyrenoids, and was identified as *Chlorella* aff. *vulgaris*, which could not be maintained in cultures. The other alga grew slowly in artificial conditions, had smaller cell sizes and lacked pyrenoids. This organism was identified as *Choricystis minor*, and its accommodation in *P. bursaria* is newly recorded. In cells of *P. bursaria*, cells of *Chlorella* aff. *vulgaris* were widely distributed within the cytoplasm while those of *Choricystis minor* were enclosed within visible perialgal vacuoles. The phylogenetic analyses using 18S rRNA gene sequences of the symbiotic strain of *C. minor* resolved that this organism is closely related to the

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0334-5114/2004/$05.50

free-living strain of *C. minor* (SAG 251-1), and forms a monophyletic clade with *Nannochloris atomus* (SAG 14.87) and *Nannochloris* sp. (SAG 251-2) in the Trebouxiophyceae. We discuss the exceptional simultaneous symbiotic mode in *P. bursaria*, and the phylogenetic relationships of *C. minor* and allied organisms.

Keywords: Symbiotic algae, Chlorophyta, *Choricystis minor*, *Nannochloris*, *Paramecium bursaria*, molecular phylogeny, maximum likelihood, 18S rRNA gene

1. Introduction

Symbiotic associations of metazoans and algae have been well known, and *Paramecium*-algal intracellular associations have been described since the 19th century (e.g., Brandt, 1882; Entz, 1882a and b cited in Reisser, 1984). *Paramecium bursaria* Ehrenberg is a common ciliate inhabiting freshwater ponds and small pools, and has been known to include green coccoid algae of the genus *Chlorella* as symbionts that have been generally called "zoochlorellae" in common terms. Many researchers made detailed descriptions of the symbiotic associations between *P. bursaria* and *Chlorella* spp. by light and electron microscopy (e.g., Beijerinck, 1890; Loefer, 1936; Siegel and Karakashian, 1959; Siegel, 1960; Reisser, 1975, 1976, 1984; Reisser et al., 1988; Ikeda and Takeda, 1995). Douglas and Huss (1986) demonstrated sugar releases from symbiotic algal cells under culture conditions, a characteristic which is one of remarkable differences from free-living cells. Reisser (1984) reported an auxotrophy in endosymbiotic *Chlorella vulgaris* and *C. vulgaris*-like algae, in which vitamins B_1 and B_{12} were required for growth. These features clearly indicate the presence of physiological interactions between hosts and symbionts. Kessler and Huss (1989) studied differences in rates of DNA-hybridization and GC contents of the symbiotic green algal cells, and Takeda (1995) researched the extent of variation of cell wall chemical compositions. Recently, Nakahara et al. (2003) reported that symbiotic algae assigned to a species of *Chlorella* from cells of one clone of *P. bursaria* had three physiological types of dependency on the host as revealed by appearances of pyrenoids and survival periods in artificial cultural conditions. In these previous investigations, it has been deemed that the relationship of host to symbiont in *Paramecium*-algal associations is restricted to one-to-one, that is, a single species exclusively accommodates in a single cell of *Paramecium*.

In the present research we found an exceptional case, where two species of symbiotic algae of different sizes are associated with *P. bursaria*. The larger organisms were identified as *Chlorella* aff. *vulgaris*, which unfortunately could not be brought into culture of artificial media. The smaller alga grew in culture for prolonged periods, and we made light and transmission electron

microscopy on this isolate. In recent phylogenetic works using 18S rRNA gene sequences, free-living coccoid green algae allied to these symbionts are recognized as members of the two classes: Chlorophyceae and Trebouxiophyceae (Friedl, 1995; Huss et al., 1999; Katana et al., 2001). But the phylogenetic positions of intracellular symbiotic algae in unicellular hosts have not been studied in large-scale analyses to date. It is expected that the molecular phylogeny is efficacious to determine the evolutionary origin of symbiotic algae.

The objectives of this study are: 1) to demonstrate the two-species association within *P. bursaria*, 2) to describe the detailed morphological features for identification of the smaller alga, and 3) to investigate the phylogenetic status of this symbiotic coccoid alga among the Chlorophyta.

2. Materials and Methods

Isolation, culture and light microscopy

Living specimens of *Paramecium bursaria* were collected from a ditch of several meters in width at Walt Disney World in Orlando, Florida, USA, Dec. 1992 by Dr. T. Kosaka (Hiroshima University, Japan). He isolated the individuals into test tubes as stock cultures at the University of Maryland, USA, which were sent to Japan in April, 1993 by airmail. The stock cultures were kindly donated to MN in April, 1998, from which symbiotic algae were isolated. The cultures of ciliates were maintained in Petri dishes containing the medium used by Sonneborn (1950) under conditions of ca 20°C, 36 $\mu E/m^2/s$, 12:12 h LD cycle.

In order to isolate the algal symbionts, a single cell of *P. bursaria* was ruptured without giving damages to the algae after rinsing ten times in sterile water, and transferred with symbiotic cells to CA medium (Ichimura and Watanabe, 1974) for pre-culture for several weeks. The algal cells were maintained on agar slants in test tubes of the same medium for prolonged culture. Light microscopy was conducted on the cells living in *P. bursaria* and those cultured in artificial conditions. All strains used in this study were deposited in the Department of Biological Science, Graduate School of Science, Hiroshima University, Japan.

Transmission electron microscopy (TEM)

For transmission electron microscopy of the smaller-sized algal strain OL2-1, cells in logarithmic phase culture were pre-fixed using 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 3 hours at 4°C and rinsed for 2 hours with

0.1 M cacodylate buffer three times. Cells were post-fixed in 2% OsO_4 for 1 hour at 4°C. The fixed material was dehydrated in an ethanol series and embedded in Spurr's resin (Spurr, 1969). Ultrathin sections were made with a diamond knife on a Leica Ultracut R (LKB, Bromma). The sections were mounted on grids coated with formvar, and were stained with 2% uranyl acetate and Reynold's lead citrate (Reynolds, 1963). Preparations were observed using a JEM-1010 transmission electron microscope (JEOL, Tokyo) operating at 80 kV.

Phylogenetic analyses

Total DNA was extracted from actively growing cells of strain OL2-1, using the modified CTAB method (Murray and Thompson, 1980). Several segments of 18S rRNA gene were amplified by standard polymerase chain reaction (PCR) or nested PCR with Ex Taq polymerase PCR amplification kit (Takara, Kyoto, Japan) on a DNA thermal cycler (ABI Thermal Cycler-9600, Tokyo, Japan) with synthetic primers (Table 1). The amplification products were checked on agarose gels and purified with concentrating filters (Takara). Direct DNA sequence analyses of the PCR products were performed by dideoxy chain termination method using the ABI kits with additional internal primers (Table 1). The sequences were electrophoresed on automated sequencers (ABI PRISM 310, ABI).

The 18S rRNA gene sequence from OL2-1 (accession number AB109544) was aligned with 130 species/strains registered in the DNA database using the program Clustal W (Thompson et al., 1994), and refined by comparison with 18S rRNA secondary structures proposed by Neefs et al. (1993). The dataset includes 35 OTUs of Chlorophyceae, 64 OTUs of Trebouxiophyceae, 8 OTUs of Ulvophyceae, 7 OTUs of Prasinophyceae, 15 OTUs of Streptophyta, and *Cyanophora padoxa* as the most distant outgroup (Table 2). Undetermined sites, gaps, and regions not clearly alignable were excluded from the data set, and thus, 1,412 bases were used for phylogenetic analyses.

Phylogenetic analyses were performed by the maximum likelihood criteria with the HKY85 model (Hasegawa et al., 1985) using NucML in MOLPHY version 2.3b3 (Adachi and Hasegawa, 1996). Tree topologies for NucML were obtained by the neighbor-joining (NJ) method (Saitou and Nei, 1987) with the local rearrangement search by NucML and maximum parsimony (MP) method (Fitch, 1971) by PAUP* 4.0b10 (Swofford, 2002) with PAUPRat (Sikes and Lewis, 2001) to implement the Parsimony Ratchet searches (Nixon, 1999). Tree comparison to evaluate the resulting trees was carried out with standard errors (SEs) of the difference in log-likelihood (Kishino and Hasegawa, 1989). For the best ML topology, we used MEGA2 software (Kumar et al., 2001) with

Table 1. Primers used for PCR amplification and sequencing of the 18S rRNA gene of *Choricystis minor* (OL2-1). The positions correspond to ones in the 18S rRNA gene of *Chlorella vulgaris* (X13688).

Primers	Sequence (5'–3')	Position	References
Forward			
18S1Fh	AACCTGGTTG ATCCTGCC	1–18	Present study
18S2Fh	GTTGATCCTG CCAGTAGTCA	7–26	Present study
18SNS1	GTAGTCATAT GCTTGTCTC	20–38	Kocher and White (1992)
18S410	CCACATCCAA GGAAGGCAGC	409–428	Handa et al. (2003)
18S575	CGGTAATTCC AGCTCCA	576–592	Handa et al. (2003)
18S921	GAAAGACGAA CTACTGCGA	923–941	Handa et al. (2003)
18S1208	TACCAGGTCC AGACATAGTG AGG	1209–1231	Handa et al. (2003)
18S1288	TGGGTTGCCT TGTCA	1295–1309	Present study
18S1307	ACGAGACCTC AGCCTGCT	1328–1345	Present study
18S1421	CAGGTCTGTG ATGCCCTTAG A	1428–1448	Present study
Reverse			
18SNS2	GGCTGCTGGC ACCAGACTTG C	574–554	Kocher and White (1992)
18S909R	CCAAGAATTT CACCTCTGAC	916–897	Handa et al. (2003)
18S1125R	CAGCCTTGCG ACCATACTCC	1132–1113	Handa et al. (2003)
18S1442R	TCTAAGGGCA TCACAGAC	1448–1431	Handa et al. (2003)
18S1617R	CAGGGACGTA ATCAACGC	1623–1606	Handa et al. (2003)
18S4Rh	CTGCAGGTTC ACCTACGGA	1789–1771	Present study
18S3Rh	TGATCCTTCT GCAGGTTC	1797–1780	Present study

Table 2. List of organisms used in the analyses, with origins and accession numbers of 18S rRNA gene sequences.

Species	Strain[a]	Accession No.
Chlorophyta		
Chlorophyceae		
Ankistrodesmus stipitatus (as *A. falcatus* var. *stipitatus*)	SAG 202-5	X56100
Bracteacoccus minor	UTEX 66	U63097
Chaetophora incrassata	UTEX LB1289	D86499
Characium hindakii	UTEX2098	M63000
Chlamydomonas asymmetrica	SAG 70.72	U70788
Chlamydomonas humicola	UTEX 225	U13984
Chlamydomonas moewusii	CC-1419	U41174
Chlamydomonas reinhardtii	CC-400	M32703
Chlamydopodium vacuolatum (as *Characium vacuolatum*)	UTEX2111	M63001
Chlorella zofingiensis	SAG 211-14a	X74004
Chlorococcum hypnosporum	UTEX 119	U41173
Desmodesmus pirkollei	Hegewald et al. unpubl.	AF348496
Dunaliella parva	UTEX LB 1983	M62998
Dunaliella salina	Wilcox et al. (1992a)	M84320
Ettlia minuta (as *Chlorococcopsis minuta*)	UTEX776	M62996
Hydrodictyon reticulatum	Wilcox et al. (1992b)	M74497
Muriella aurantiaca	SAG 249-1	X91268
Mychonastes homosphaera	Hanagata et al. (1999)	AB025423
Mychonastes homosphaera (as *Chlorella homosphaera*)	CCAP 211/8e	X73996
Neochloris aquatica	UTEX138	M62861
Neochloris vigensis	UTEX 1981	M74496
Pediastrum duplex	UTEX LB 1364	M62997
Pleurastrum insigne	SAG 30.93	Z28972
Scenedesmus abundans (as *Chlorella fusca* var. *fusca*)	UTEX 343	X73995
Scenedesmus communis	UTEX 76	X73994
Scenedesmus costato-granulatus	SAG 18.81	X91265
Scenedesmus obliquus	SAG 276-3a	X56103
Scenedesmus ovalternus	SAG 52.80	X81966
Scenedesmus producto-capitatus	SAG 21.81	X91266
Scenedesmus pupukensis	UTEX 2219	X91267

Table 2. Continued

Species	Strain[a]	Accession No.
Scenedesmus rubescens (as *Chlorella fusca* var. *rubescens*)	CCAP 232/1	X74002
Scenedesmus vacuolatus (as *Chlorella fusca* var. *vacuolata*)	SAG 211-8b	X56104
Schroederiella apiculata	SAG 47.86	AB037098
Spermatozopsis similis	SAG B 1.85	X65557
Volvox carteri	UTEX 1885	X53904
Trebouxiophyceae		
Auxenochlorella protothecoides (as *Chlorella protothecoides*)	SAG 211-7a	X56101
Chlorella ellipsoidea	SAG 211-1a	X63520
Chlorella kessleri	IAM C-531	AB080309
Chlorella kessleri	SAG 211-11g	X56105
Chlorella lobophora	Andreyeva 750-I	X63504
Chlorella luteoviridis	SAG 211-2a	X73997
Chlorella minutissima	Bethesda C-1.1.9	X56102
Chlorella minutissima	SAG 1.80	AB006046
Chlorella mirabilis	Andreyeva 748-I	X74000
Chlorella saccharophila	SAG 211-9a	X63505
Chlorella saccharophila	SAG 211-9b	X73991
Chlorella sorokiniana	Baslerová Prag A14	X74001
Chlorella sorokiniana	IAM C-212	AB080307
Chlorella sorokiniana	SAG 211-8k	X62441
Chlorella sorokiniana	SAG 211-40a	X73993
Chlorella sp.	SAG 211-18	X73992
Chlorella sphaerica	SAG 11.88	AJ416105
Chlorella vulgaris	IAM C-536	AB080308
Chlorella vulgaris	SAG 211-11b	X13688
Choricystis minor	SAG 251-1	X89012
Choricystis minor	OL2-1	AB109544
Coenocystis inconstans	K4-3	AB017435
Dicloster acuatus	SAG 41.98	AB037085
Dictyochloropsis reticulata	CCHU 5616	Z47207
Friedmannia israeliensis	UTEX1181	M62995

Table 2. Continued

Species	Strain[a]	Accession No.
Fusochloris perforatum	UTEX2104	M62999
Leptosira obovata	SAG 445-1	Z68695
Leptosira terrestris	SAG 463-3	Z28973
Lobosphaera tirolensis	ASIB S234	AB006051
Micractinium pusillum	Hegewald 1983-3	AF237662
Microthamnion kuetzingianum	UTEX 1914	Z28974
Muriella terrestris	ASIB V38	AB012845
Myrmecia astigmatica	IB T76	Z47208
Myrmecia biatorellae	UTEX 907	Z28971
Myrmecia bisecta	IB T74	Z47209
Nannochloris atomus	CCAP 251/7	AB080303
Nannochloris atomus	SAG 14.87	AB080305
Nannochloris bacillaris	Ogawa et al. (1995)	AB080300
Nannochloris coccoides	CCAP 251/1b	AB080301
Nannochloris eucaryotum	KSW 0203	AB080304
Nannochloris maculata	CCAP 251/3	AB080302
Nannochloris sp.	SAG 251-2	AB080306
Nanochlorum eucaryotum	Mainz 1	X06425
Pabia signensis	SAG 7.90	AJ416108
Parietochloris pseudoalveolaris	UTEX975	M63002
Prasiola crispa	SAG 43.96	AJ416106
Prasiola fluviatilis	Sherwood et al. (2000)	AF189072
Prasiola meridionalis	Sherwood et al. (2000)	AF189074
Prasiola mexicana	MEX12	AF189075
Prasiola mexicana	CR24	AF189076
Prototheca wickerhamii	SAG 263-11	X74003
Prototheca wickerhamii	Pore 1283	X56099
Prototheca zopfii	SAG 263-1a	X63519
Pseudochlorella subsphaerica	CCAP 264-3	AB006050
Raphidonema nivale	CCAP 470/4	AF448477
Stichococcus bacillaris	CCAP 379/7	AB055864
Stichococcus bacillaris	D10-1	AB055865

Table 2. Continued

Species	Strain[a]	Accession No.
Stichococcus bacillaris	K4-4	AB055866
Stichococcus bacillaris	SAG 397-1b	AJ416107
Stichococcus chodatii	UTEX1177	AB055867
Trebouxia asymmetrica	SAG 48.88	Z21553
Trebouxia erici	IAM C-593	AB080310
Trebouxia impressa	UTEX 892	Z21551
Trebouxia magna	UTEX 902	Z21552
Ulvophyceae		
Acrosiphonia sp.	SAG 127.80	U03757
Gloeotilopsis planctonica	SAG 29.93	Z28970
Gloeotilopsis sarinoidea (as *Protoderma sarinoidea*)	UTEX 1710	Z47998
Pseudendoclonium basiliense	UTEX 2593	Z47996
Pseudoneochloris marina (as *Neochloris* sp.)	UTEX 1445	U41102
Trentepohlia aurea	Handa-840(a)	AB110783
Ulothrix zonata	SAG 38.86	Z47999
Ulva rigida	EL0102	AJ005414
Outgroups: Prasinophyceae		
Nephroselmis olivacea	SAG 40.89	X74754
Nephroselmis pyriformis (as *Pseudoscourfieldia marina*)	CCMP 717	X75565
Mamiella sp.	Shizugawa	AB017129
Mantoniella squamata	CCAP 1965/1	X73999
Ostreococcus tauri	Courties et al. (1998)	Y15814
Scherffelia dubia	SAG 17.89	X68484
Tetraselmis striata	CCMP 443	X70802
Streptophyta		
Chara australis	Ragan et al. (1994)	U05260
Chara foetida	Steinkötter et al. (1994)	X70704
Chlorokybus atmophyticus	UTEX LB 2591	M95612
Coleochaete orbicularis	Wilcox et al. (1993)	M95611
Coleochaete scutata	SAG 110.80	X68825

Table 2. Continued

Species	Strain[a]	Accession No.
Genicularia spirotaenia	SCK 329	X74753
Klebsormidium flaccidum	Wilcox et al. (1993)	M95613
Klebsormidium flaccidum	SAG 335-2b	X75520
Klebsormidium nitens	SAG 335-1a	AJ250112
Halosphaera sp.	Shizugawa	AB017125
Nitella capillaries	Marin and Melkonian (1999)	AJ250111
Nitella flexilis	Ragan et al. (1994)	U05261
Nitella sp.	Wilcox et al. (1993)	M95615
Pyramimonas parkeae	Hachijo	AB017124
Staurastrum sp.	M 752	X74752
Cyanophyta		
Cyanophora paradoxa	Kies	X68483

[a]: Names of culture collections where the strains are deposited: Andreyava; V. M. Andreyava, St. Petersburg, Russia. Baslerová; M. Baslerová, Praha, Czech Republic. Bethesda; Culture Collection at Bethesda, MD, USA. CC; The Chlamydomonas Genetics Center at Duke University. CCAP; Culture Collection of Algae and Protozoa, Ambleside, UK. CCMP; Provasoli-Guillard National Center for Culture of Marine Phytoplankton, Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, ME, USA. IAM; Culture Collection at the Institute of Molecular and Cellular Biosciences, the University of Tokyo, Japan. IB; Culture Collection of the Botanical Institute at Innsbruck, Austria. Pore; R. S. Pore, Morgantown, WV, USA. KSW; The Laboratory of Plant Life System, Department of Integrated Biosciences, Graduate School of Frontier Sciences, University of Tokyo, Kashiwa, Japan. M; Culture Collection Melkonian, Koeln, FRG. SAG; Sammlung von Algenkulturen der Universität Göttingen, Germany. UTEX; Culture Collection of Algae at the University of Texas at Austin, TX, USA. For algal strains whose cultural sources are unknown, references are cited.

10,000 replications to apply a standard bootstrapping test (Felsenstein, 1985), using the Kimura (1980) 2-parameter distances, based on NJ. The program package CONSEL 0.1e (Shimodaira and Hasegawa, 2001) was used to calculate *p*-values of confidence of candidate topologies using the approximately unbiased (AU) test (Shimodaira, 2000, 2002), and a 50% majority-rule consensus tree for the topologies with high ranking log-likelihood values that passed the AU test was also computed by PAUP*. DNA-homologies among different strains of *Choricystis* and *Nannochloris* were calculated manually.

3. Results

Symbiotic conditions in P. bursaria and morphological features of the algae

Symbiotic conditions of algae in P. bursaria. In the cytoplasm of individual cells of *P. bursaria* (OL-2), two kinds of coccoid green algae were accommodated with numerous non-living small granules (Fig. 1). These algae were easily distinguished from each other by the size of vegetative cells and the presence or absence of pyrenoids. All algal cells and non-living small granules moved along with cyclosis occurring within the cytoplasm of *P. bursaria*. Two to dozens of the smaller cells were aggregated in perialgal vacuoles (Karakashian et al., 1968) which randomly distributed in the cytoplasm of hosts.

Features of the larger alga. The larger alga has the following features in symbiotic conditions (Fig. 1). Cells are ellipsoidal and 3.0 × 4.0 μm in size when young, and become broadly ellipsoidal to spherical to attain 7.0 μm in diameter at maturity. The chloroplast is single and assumes saucer-, cup-, and girdle-shapes with a single pyrenoid. The pyrenoid is spherical to broadly ellipsoidal in shape, and surrounded by two starch sheaths. The cell wall is smooth and thin. Reproduction occurs by formation of 2 or 4 autospores of nearly equal size.

These features of this symbiotic alga are identical to characteristics of *Chlorella vulgaris* described from free-living specimens. However, unlike in the culture of free-living strains of *Chlorella vulgaris*, it was very difficult to bring this symbiotic alga into culture, and we were unable to obtain isolated cells regardless of many trials.

Features of the smaller alga. The smaller alga has the following features in symbiotic conditions (Fig. 1). Cells are ellipsoidal, ellipsoidal to spherical, or slightly kidney-shaped with rounded ends, and 1.2–2.0 × 1.9–3.0 μm in size. The chloroplast is single, saucer- and cup-shaped, and lacks pyrenoids. The cell wall is smooth and thin. Reproduction occurs by formation of 2 or 4

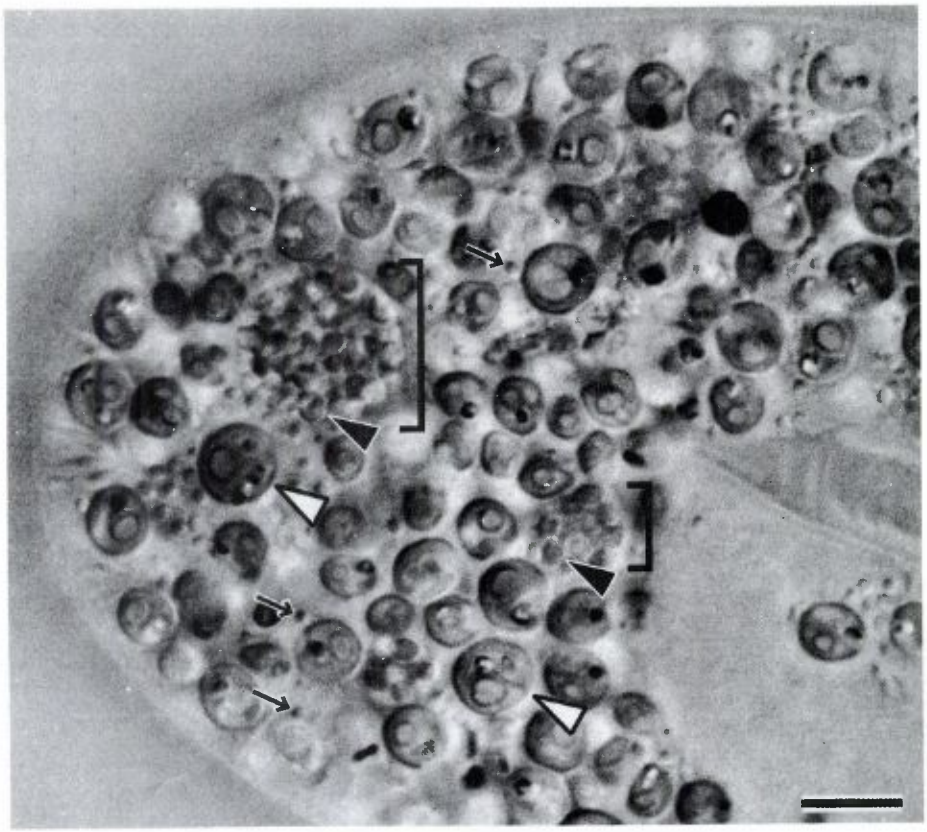

Figure 1. Symbiotic association of *Chlorella* aff. *vulgaris* and *Choricystis minor* in their host *Paramecium bursaria*. Black arrowheads indicate cells of smaller species, *C. minor*, within a spherical cluster in perialgal vacuoles of various sizes (brackets). White arrowheads indicate cells of larger species, *Chl.* aff. *vulgaris* with a pyrenoid. Many small granules (arrows) are mixed with algae. Scale bar: 10 μm.

autospores of nearly equal size; however, the breakdown of the mother cell wall was not observed after accomplishment of autosporulation in cells of *P. bursaria*.

We isolated 24 strains of the smaller alga but their growth was generally very slow, and from them only 8 were maintained in culture long enough for observations. They varied in number of autospores in a mother cell, that is, 2, 4, 8 or 16 (Fig. 2). Numbers of autospores formed in a cultured mother cell were generally more than those living in *P. bursaria*. In the logarithmic phase of growth, cells were ellipsoidal (Fig. 2A) when 2 autospores were formed in a

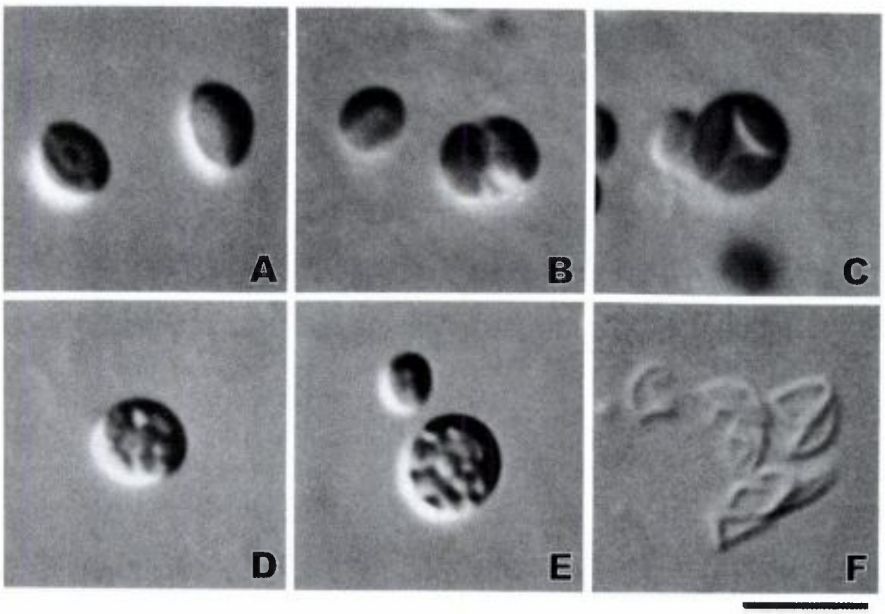

Figure 2. Light micrographs of *Choricystis minor* OL2-1 in culture isolated from *P. bursaria*. A. Ellipsoidal cell. B–E. Mother cells containing various numbers of autospores. B. Two autospores. C. Four autospores. D. Eight autospores. E. Sixteen autospores. F. Persistent sporangial walls after release of autospores. Scale bar: 5 μm for all figures.

mother cell (Fig. 2B), or suborbicular to spherical when more than 4 autospores were formed (Fig. 2C). In old cultures most cells became spherical, to attain 4.0 × 4.0 μm at maximum. Autospores were released after the mother cell wall deeply split (Fig. 2F). Although some variations were observed in cultural strains of the smaller symbiotic alga, all of these isolates could be identified as *Choricystis minor* (Skuja) Fott.

By transmission electron microscopy on OL2-1, the next ultrastructural features were observed. The chloroplast covers more than half of the peripheral region. Thylakoid bundles comprised of three to four lamellae extend into the chloroplast, and a few starch segments are located in the stroma (Figs. 3A, 3E). Absence of pyrenoids was ascertained from many sections of cells. In the cytoplasm of vegetative cells a single nucleus is situated beside the chloroplast, and a mitochondrion is positioned in the space between the nucleus and chloroplast (Fig. 3A). In the cytoplasm typical dictyosomes were not observed. The cell wall is composed of three layers (Fig. 3B), that is, an outer and inner electron-dense layer, and between them an electron-sparse

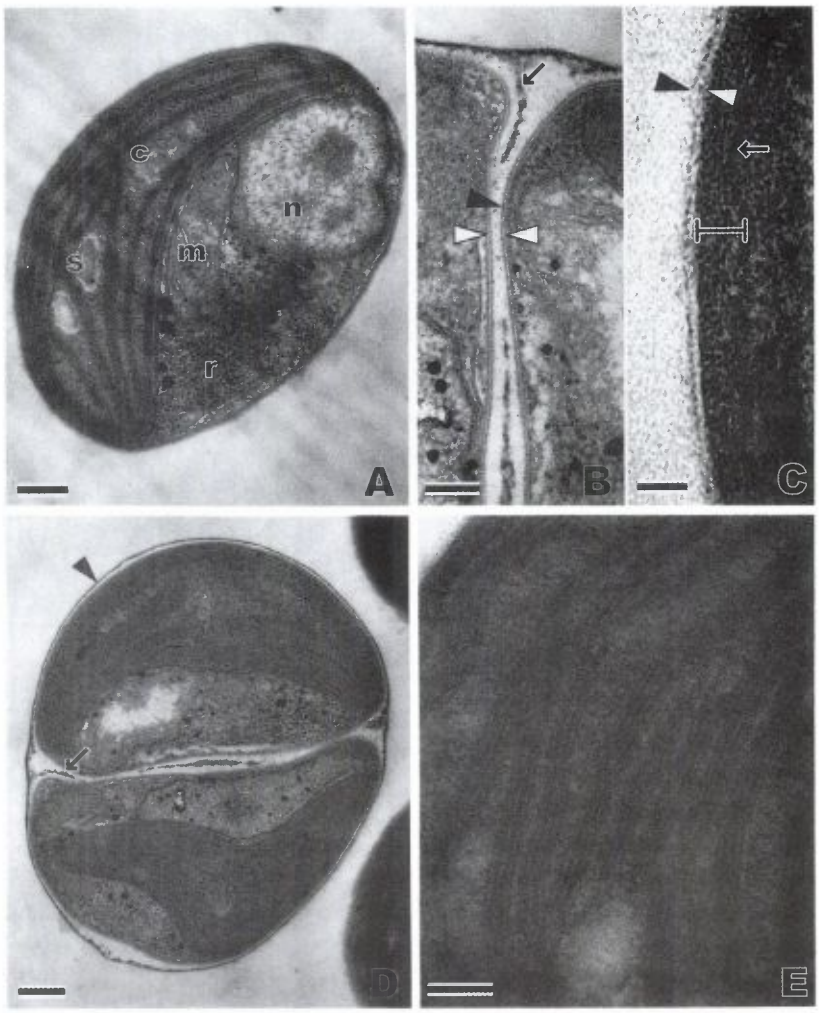

Figure 3. Transmission electron micrographs of *Choricystis minor* OL2-1. A. Ellipsoidal vegetative cell, showing chloroplast (c), nucleus (n), mitochondrion (m), numerous ribosomes (r), and starch grain (s). B. Part of mother cell with two autospores. TL-layer is comprised of outer (black arrowhead) and inner (white arrowheads) electron-dense layers, and an electron-sparse layer between them. Note intracellular materials (arrow) continuous to mother cell wall. C. Close-up image of part of cell wall including granulo-fibrillar layer (bar) between inner electron-dense layer (white arrowhead) and plasmalemma (arrow). Black arrowhead indicates the outer TL-layer. D. Mother cells containing two autospores. Structure of TL-layer (arrowhead) of mother cell wall is not clear. Note intracellular materials (arrow) continuous to mother cell wall. E. Close up image of part of vegetative cell, showing the thylakoid bundles comprised of three to four lamellae that extend into the chloroplast. Scale bars: 200 nm (A, D), 50 nm (B), 100 nm (C, E).

middle layer, which is called a triple-layered structure (TL-layer, Krienitz et al., 1996). The TL-layer is clear in autospores (Fig. 3B), but in mature vegetative cells differences between the outer and inner layers become unclear. Between the inner layer and the plasmalemma lies the granulo-fibrillar layer (Krienitz et al., 1996) (Fig. 3C), which is smooth or irregularly lined, and 9–40 nm thick in vegetative and autospore-forming cells, attaining 120 nm thickness in mature cells. Processes of autosporulation, confirmed by transmission electron microscopy (Fig. 3D), show that 2 autospores are formed in a single mother cell. Cell wall materials that apparently connect to the inner TL-layer of the mother cell wall are present between autospores.

In a rare occasion a mitochondrion is situated at the lateral periphery outside of the chloroplast in autospores (Fig. 3D). In autospores within a mother cell, the chloroplasts are placed in peripheral regions, while nuclei are settled in inner regions. These ultrastructural features were generally coincident with the previous report of *Choricystis minor* (Skuja) Fott by Krienitz et al. (1996).

Phylogenetic analyses of OL2-1

A total of 557 topologies were constructed by the two methods, and analyzed by the maximum likelihood criteria with preparation of an AU test. A single ML tree was obtained and shown in Fig. 4. In the AU test, a total of 527 topologies for the 557 topologies were passed. The ML tree resolved a united clade of the Chlorophyceae plus *Chaetophora incrassata* and Ulvophyceae, which is supported by 96% by the AU test. The Trebouxiophyceae appeared to be paraphyletic, forming five major clades: Clade I comprised of taxa from *Chlorella kessleri* (IAM C-531) to *Chlorella minutissima* (C-1.1.9) [97% AU]; Clade II from *Microthamnion kuetzingianum* to *Nannochloris* sp. (SAG 251-2) [89% AU]; Clade III from *Leptosira obovata* to *Myrmecia biatorellae* [61% BP, 98% AU]; Clade IV from *Dictyochloropsis reticulata* to *Chlorella saccharophila* (SAG 211-9b) [59% BP, 87% AU]; and Clade V from *Stichococcus bacillaris* (CCAP 379/7) to *Coenocystis inconstans* [78% AU], although all clades were not well supported with a low bootstrap probability.

Within Clade I, five species of *Nannochloris* including *N. bacillaris* (Ogawa et al., 1995), *N. coccoides* (CCAP 251/1b), *N. maculata* (CCAP 251/3), *N. atomus* (CCAP 251/7) and *Nanochlorum eucaryotum* (Mainz 1) constitute a weak branch. In this branch, *N. bacillaris* (Ogawa et al., 1995) and *N. coccoides* (CCAP 251/1b) formed the *N. bacillaris* clade [73% BP, 100% AU], which is sister to the *N. maculata* clade comprised of *N. maculata* (CCAP 251/3), *N. atomus* (CCAP 251/7) and *Nanochlorum eucaryotum* (Mainz 1) [96% BP, 100% AU]. Clade I also includes a robust *Chlorella minutissima* clade that

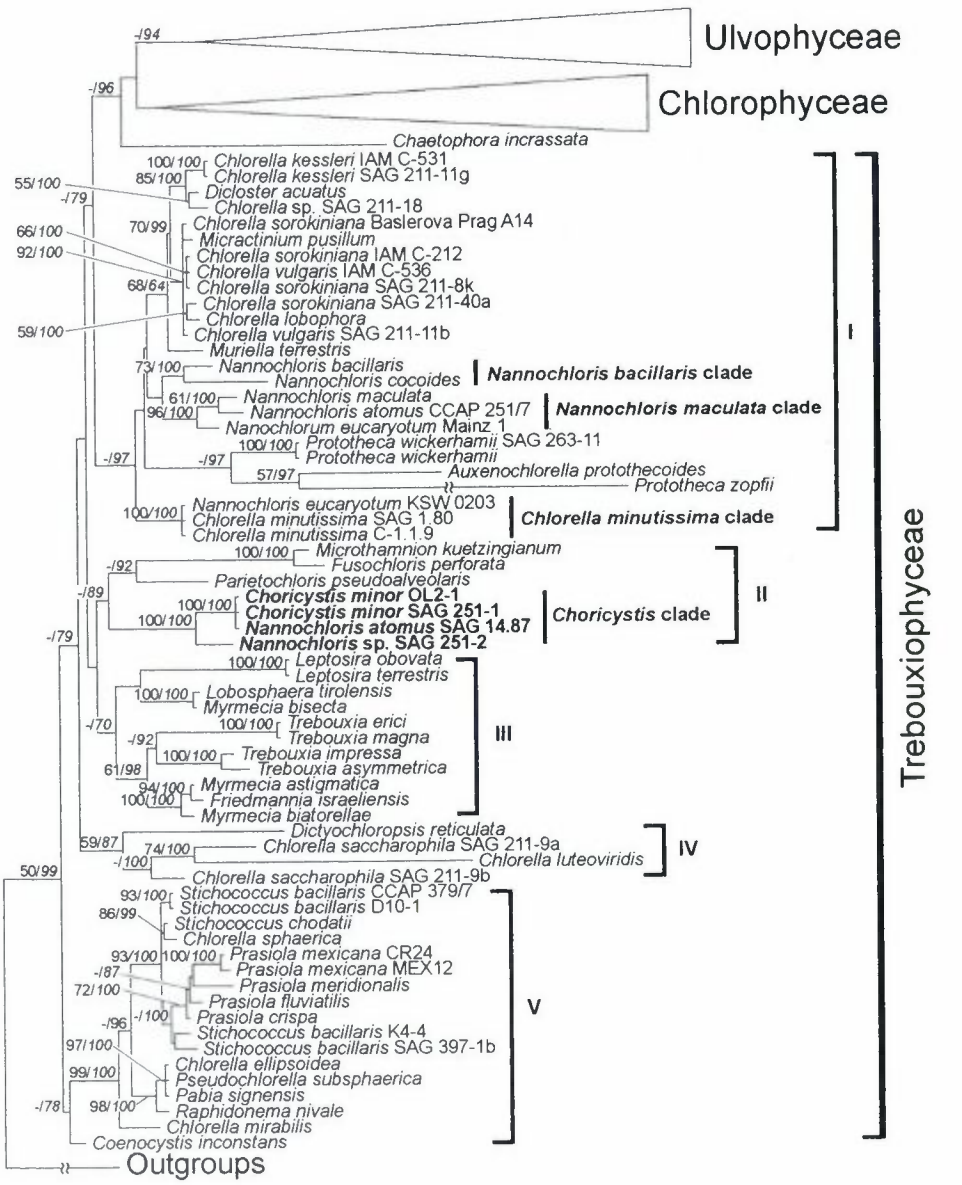

Figure 4. The best-supported NucML tree (HKY85 model; $2\alpha/\beta = 3.83$; ln $L = -17894.7 \pm 755.7$) for 130 algal 18S rRNA gene sequences. The root is arbitrarily placed on the branch leading to the *Cyanophora paradoxa*. Bootstrap probabilities based on 10,000 replications by NJ (BPs; in %) and percentage of number of topologies which passed the AU test (*AU*; in %) more than 50% are shown on branches (BP/*AU*).

Table 3. Comparisons of 18S rRNA gene sequences of two strains of *Choricystis minor*, *Nannochloris atomus*, and *Nannochloris* sp. of the *Choricystis* clade. The numbers of introns, and the homology (%) with ratios (lower-left matrix) of different sites per total ones (in parentheses) and numbers of gaps added to the sequences for alignment (upper-right matrix). Between two strains of *C. minor*, homologies were obtained, including introns, while between *C. minor* and two species of *Nannochloris* introns were excluded.

Species/strains	Number of introns	*C. minor* OL2-1	*C. minor* SAG251-1	*N. atomus* SAG14.87	*N.* sp. SAG 251-2
C. minor OL2-1[1)]	2	–	5	0	2
C. minor SAG251-1[2)]	2	99.97% (7/2649)	–	1	2
N. atomus SAG14.87[3)]	0	99.94% (2/1791)	99.94% (2/1791)	–	1
N. sp. SAG 251-2[3)]	0	98.16% (33/1791)	98.16% (33/1790)	98.60% (25/1791)	–

1)Present study, 2)Krienitz et al. (1996), 3)Yamamoto et al. (2003).

was comprised of *Nannochloris eucaryotum* (KSW0203) and two strains of *Chlorella minutissima* (SAG 1.80 and C-1.1.9) [100% BP, 100% AU].

Within Clade II, both the symbiotic and free-living strains of *Choricystis minor* (OL2-1 and SAG 251-1), *Nannochloris atomus* (SAG 14.87) and *Nannochloris* sp. (SAG 251-2) formed a monophyletic *Choricystis* clade [100% BP, 100% AU]. Numbers of introns and homologies of nucleotide sequences were compared among the algal strains of this clade (Table 3). Both strains of *C. minor* commonly possess two introns, while *N. atomus* and *Nannochloris* sp. lack them. The sequence homology including the intron regions between two strains of *C. minor* was 99.97%. When introns of *C. minor* (OL2-1) were omitted from homology calculations, the value between *C. minor* (OL2-1) and *N. atomus* (SAG 14.87) was 99.94% and that between *C. minor* (OL2-1) and *Nannochloris* sp. (SAG 251-2) was 98.16%.

4. Discussion

Simultaneous symbiotic association in P. bursaria

Simultaneous endosymbiotic associations with two different algal species in seawater have been reported from various host organisms. Muscatine (1971) reported the simultaneous mixed infection of zooxanthellae and marine zoochlorellae within tissues of sea anemones, *Anthopleura*. Lee and McEnery (1983) observed that a single cell of one species of *Amphistegina* has cells of *Chlorella* or diatoms in addition to a usual dinophycean symbiont *Symbiodinium microadriaticum*.

However, simultaneous two-species associations of unicellular organisms in ciliates have not been reported in fresh-water environments. Nakahara et al. (2003) observed that the survival terms of 56 isolated algal clones from *P. bursaria* varied from one month to about 20. In these cultural periods the starch segments around pyrenoids disappeared and pyrenoids could not be detected by light microscopy. From these prolonged observations, Nakahara et al. (2003) emphasized that the symbiotic algae depend on the host for growth and they become different from free-living clones in physiological requirements. As Reisser and Widowski (1992) pointed out, heterotrophic hosts of fresh-water endosymbiotic associations with eukaryotic algae seem to be very fastidious in choosing a potential autotrophic partner.

When we consider that the *Chlorella*-species has been currently known as a representative symbiotic alga of *P. bursaria*, and the association with *C. minor* is the first finding in a heterotrophic host, it could be appreciated that the accommodation of *C. minor* is rare and this species is possibly a secondary candidate of an association partner.

From the previous observations of endosymbiotic associations with *Chlorella* sp. (e.g., Vivier et al., 1967; Karakashian et al., 1968; Reisser, 1976; Meier and Wiessner, 1987), algal cells are enclosed in a perialgal vacuole of a single-layered membrane. Since symbiotic algae in the perialgal vacuoles reproduce two or four daughter cells and the membranes of vacuoles are newly formed to follow cell divisions, each algal cell is enclosed in a single perialgal vacuole. These phenomena involving the perialgal vacuoles have been reported in various hosts: *P. bursaria* (e.g., Vivier et al., 1967; Karakashian et al., 1968; Reisser, 1976; Meier and Wiessner, 1987; Ikeda and Takeda, 1995), *Vorticella* sp. (Graham and Graham, 1978), *Hydra viridis* (Oschman, 1967), *Spongilla lacustris* (Williamson, 1979; Masuda, 1990), *Radiospongilla cerebellata* (Masuda, 1990), *R. sendai* (Masuda, 1990), *Heteromeyenia stepanowii* (Masuda, 1990), *Anthopleura elegantissima* (Muscatine, 1971), *Stentor polymorphus* (Reisser, 1981), and *Anthopleura xanthogrammica* (Muscatine, 1971; O'Brien, 1978).

However, in light microscopy on the symbiotic associations, we only observed perialgal vacuoles surrounding aggregations of cells of *C. minor*, but failed to find its membranous structure around each cells of *Chlorella* aff. *vulgaris*. In the present study the symbiotic association of *P. bursaria* and symbiotic algae was not observed by electron microscope; however, we consider that cells of *Chlorella* aff. *vulgaris* are surrounded by perialgal vacuoles, since cells of *Chlorella*, a single symbiotic organism of the host, were observed within individual perialgal vacuoles by electron microscopy in previous studies (e.g., Reisser, 1976; Ikeda and Takeda, 1995; Nakahara unpublished data). It is interesting that perialgal vacuoles housing numerous cells of *C. minor* were easily detected by the light microscopy. This may be caused by large aggregations of daughter cells that made the membranes of perialgal vacuoles visible.

Phylogenetic and taxonomic relationships of C. minor and allied organisms

The genus *Choricystis* was established by Fott (1976) with a type species *C. minor* (Skuja) Fott, which was originally described under the name of *Coccomyxa minor* Skuja. Bourrelly (1966) introduced that the genus *Coccomyxa* includes species with or without mucilage around cells, and those reproducing by autosporulation or binary division along the diagonal direction of the mother cell. Bourrelly (1966) also quoted a proposal by Skuja (1948) to subdivide the genus *Coccomyxa* into two sections, *Coccomyxa* with mucilage surrounding cells and *Choricystis* without it. To follow Skuja's idea, Fott (1976) transferred *Choricystis* from a rank of section to an independent genus which was circumscribed as lacking mucilage around cells and propagating by

autosporulation with two daughter cells. This species has been found free-living from freshwater, terrestrial or aerial environments (e.g., Fott, 1976; Handa and Nakano, 1988; Nakano et al., 1991; Krienitz et al., 1996; Belykh et al., 2000).

In the present phylogenetic study using 18S rDNA sequence data, the isolated strain OL2-1 formed a robust clade with the free-living strain of *C. minor* (SAG251-1), and between these strains 99.97% homology was obtained in the sequence of 2649 nucleotides including two introns (Table 3). In our prolonged cultural observations, the symbiotic strains survived in limited periods in artificial conditions, in which the free-living strains were easily maintained. From the morphological features the symbiotic strain was identical to the free-living one as supported by molecular analyses, but they obviously varied in physiological properties. It could be said that the symbiotic association with *P. bursaria* has influenced physiological requirements of *C. minor* to an extent to give difficulties in growing in artificial media. When *C. minor* is successively maintained in culture, we will understand the physiological dependency of this alga on the host.

Our molecular analyses resulted in four clades to which strains of the genus *Nannochloris* belong: *Choricystis* clade, *N. bacillaris* clade, *N. maculata* clade and *Chlorella minutissima* clade. In phylogenetic analyses of seven isolates of *Nannochloris* using sequence data of actin and 18S rRNA genes, Yamamoto et al. (2003) showed that the strains of *Nannochloris* are members of the Trebouxiophyceae, and resolved into similar clades that we obtained here, with some topological differences. From our phylogenetic tree and Yamamoto et al. (2003), it appears that the *Choricystis* clade is distantly related to clades containing other species of *Nannochloris*. *Nannochloris atomus* (SAG14.87) and *Nannochloris* sp. (SAG251-2) are known to reproduce by autosporulation (Yamamoto et al., 2003), and the members of the *Choricystis* clade commonly have the same reproductive feature as circumscribed by Fott (1976) for a generic character.

Symbiotic and free-living strains of *C. minor* share two introns, while *Nannochloris atomus* (SAG14.87) and *Nannochloris* sp. (SAG251-2) have no intron. Krienitz et al. (1996) determined partial sequences of 18S rDNA from two strains of *C. minor*, KR1986/11 and KR1986/27, which contain one group I intron and no intron respectively. Krienitz et al. (1996) considered that *C. minor* of SAG251-1 and two other strains are conspecific, since no discrepancy was detected among partial sequences of these strains. If their incomplete sequences are informative enough to compare, it may be suggested that the presence or absence of introns could not be regarded as features of even specific taxonomy. Given that *C. minor* had diverged to species either having introns or lacking, the symbiotic association of an intron-bearing strain of *C. minor*

with *P. bursaria* is possibly a recent event that has occurred after the acquisition of introns.

The present phylogenetic analyses and those by Yamamoto et al. (2003) resolved that *C. minor* is very close to *N. atomus* (SAG14.87). Differences between these species are found in the presence or absence of introns; however, this feature may be infraspecific as suggested by Krienitz et al. (1996). It will be necessary to compare detailed morphological attributes and molecular data of members of the *Choricystis* clade to obtain a better understandings of taxonomic status of these organisms.

Acknowledgements

We wish to express our special gratitude to Prof. J.M. Glime (Michigan Technological University, USA) for kindly reading the manuscript and making helpful suggestions; Dr. T. Kosaka (Hiroshima University, Japan) for giving us living specimens of *P. bursaria*; Prof. S. Sato (Ehime University, Japan) for guiding the technique of TEM; Dr. N. Hanagata (Tokyo University of Technology, Japan) for his help on an earlier version of the dataset; Dr. H. Tsubota (Hiroshima University, Japan) for help with the phylogenetic analysis and making helpful suggestions. Financial support in the form of a Sasakawa Scientific Research Grant to M. Nakahara (12-218K) is gratefully acknowledged.

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