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Molecular Investigation of *Paramecium bursaria* Endosymbiotic Algae: the First Records of Symbiotic *Micractinium reisseri* from Kamchatka

Patrycja ZAGATA LEŚNICKA, Magdalena GRECZEK-STACHURA, Sebastian TARCZ, and Maria RAUTIAN

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Original article

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Paramecium bursaria is a symbiotic ciliate species which cells contain hundreds of algae enclosed in perialgal vacuoles. The aim of the present study was to identify endosymbiotic algal strains of *P. bursaria* and to define the geographical distribution of the identified species. We analyzed symbiotic strains of *P. bursaria* originating from distant geographical locations and housed at the Culture Collection of Ciliates and their Symbionts (CCCS) at St. Petersburg University. Based on the obtained results, we identified these strains as *Micractinium reisseri*, *Chlorella vulgaris,* and *Chlorella variabilis.* We did not confirm the occurrence of a division into American and European groups and we guess that this division is only contractual and corresponds to the amount of introns in the 18S rDNA, and that there is no strong correlation with the geographical location. We have demonstrated that the range of *M. reisseri* is greater than previously supposed. We identified algae strains originating from Southern Europe (Serbia), Western Asia, and from the Far East (Kamchatka) as *M. reisseri*. Moreover, we identified two strains originating from Europe as *C. variabilis*, which also contradicts the predetermes about a division into American and European groups.

Key words: Ciliates; *Paramecium bursaria*; ITS1-5.8S rDNA-ITS2 fragment; endosymbionts of *Paramecium bursaria*.

Patrycja ZAGATA LEŚNICKA, Magdalena GRECZEK-STACHURA✉, Institute of Biology, Pedagogical University of Cracow, 31-084 Kraków, Podchorążych 2, Poland.
E-mails: patrycjazagata@gmail.com, magresta@wp.pl,
Sebastian TARCZ, Institute of Systematics and Evolution of Animals, Polish Academy of Sciences, 31-016 Kraków, Sławkowska 17, Poland.
E-mail: starcz@isez.pan.krakow.pl
Maria RAUTIAN, Faculty of Biology and Soil Science, St. Petersburg State, University, Universitetskaya nab. 7-9, 199034 St. Petersburg, Russia.
E-mail: mrautian@mail.ru

Paramecium bursaria is a *ciliate* species that maintains symbiotic relationships with algae. A single cell of *P. bursaria* possesses about 700 symbiotic algal cells in the cytoplasm (KODAMA & FUJISHIMA 2009). Each endosymbiont's cell is enclosed in a perialgal vacuole membrane derived from the host digestive vacuole membrane, which protects the alga from the host's lysosomal fusion (KARAKASHIAN & RUDZINSKA 1981; GU *et al.* 2002). The algae cells provide the photosynthetic products to their host and in return they receive carbon dioxide and nitrogen compounds (KODAMA & FUJISHIMA 2009). Furthermore, symbiotic algae are protected from viral infection and are chauffeured to brightly lit areas for optimum photosynthesis (HOSHINA & IMAMURA 2009a). Meanwhile, the presence of algal symbionts minimizes the level of photooxidative stress that *P. bursaria* is exposed to (HÖRTNAGL & SOMMARUGA 2007).

The symbiotic algae of *Paramecium bursaria* belong to two classes: Trebouxiophyceae and Chlorophyceae (HOSHINA *et al.* 2010a; LUO *et al.* 2010; PRÖSCHOLD *et al.* 2011), and are characterized by a close morphological similarity. PRÖSCHOLD *et al.* (2011) have identified four species of symbionts living in a symbiotic relationship with *P. bursaria: M. reisseri, C. vulgaris, C. variabilis,* and *Scenedesmus sp.* The *Chlorella*-clade includes endosymbiotic algae belonging to two genera: *Chlorella* (HOSHINA *et al.* 2010a) and *Micractinium* (PRÖSCHOLD *et al.*

2011). The systematics of *Chlorella* are constantly being modified since the authors reported from 4 (HUSS 1999; KRIENITZ *et al.* 2004) to 14 species (BOCK *et al.* 2011).

The genus *Chlorella* includes non-motile spherical cells 2-10 μm in diameter, a single nucleus, vacuoles, mitochondria, a few peroxisomes, and a single chloroplast with a pyrenoid surrounded by starch grains (KESSLER & HUSS 1992). Algae from the *Micractinium* genus, are morphologically similar to those of *Chlorella*, however cells of *Micractinium* are equipped with bristles and they are usually organized into colonies (LUO *et al.* 2005). A surprising fact is that when *M. reisseri* is isolated from *P. bursaria*, it does not form bristles and lives as a single cell (PRÖSCHOLD *et al.* 2011). Therefore, *P. bursaria* endosymbionts which belong to two classes are very hard to distinguish through microscopic observations (morphological analysis).

Up-to-date attempts at symbiotic algae identification have been carried out through the microscopic observation and physiological parameters measurement (REISSER 1984; DOUGLAS & HUSS 1986; KESSLER & HUSS 1990), analyzing cell wall structure (TAKEDA 1995), isoenzymes and sensitivity to viruses (LINZ *et al.* 1999; KVITKO *et al.* 2001), as well as the content of GC pairs in DNA (KESSLER & HUSS 1990). FOTT and NOVÁKOVÁ (1969) suggest that the morphological and biochemical features which are used as identification tools, as well as the size and the shape of the cell are highly variable parameters and depend on the age of the culture as well as nutrition and environmental conditions. Therefore, the application of molecular markers seems to be a promising tool for algae taxonomy (TAYLOR & HARRIS 2012).

Our objective was to identify symbiont species of *P. bursaria* strains from the CCCS collection and to define the geographical distribution of the identified species. Taking into account the fact that endosymbionts of *P. bursaria* are indistinguishable when comparing morphological features, identification based on molecular analyses seemed to be the only way to classify them into a particular taxon.

Material and Methods

Strains cultivation

The strains of *P. bursaria* were cultivated on a lettuce medium according to SONNEBORN (1970), fed on *Klebsiella pneumoniae* (SMC), and stored at 18ºC (12L/12D). We investigated 7 symbiont strains isolated from cells of *P. bursaria* originating from different geographical locations and maintained at the CCCS of St. Petersburg University. Furthermore, we analyzed 14 sequences available in GenBank: *Micractinium reisseri* (symbiotic strains: SW1-ZK, EdL_Cl1_MAF and standard strain: Pbi), *Micractinium* sp. (free-living strains: KNUA032, MCWWW4, MCWWW5, MCWWW10, MCWWW11, MCWWW15), *Chlorella variabilis* (EdL_Cl2_3NB and standard strains: SAG 211-6 and NC64A), *Chlorella vulgaris:* strain DRL3, and a strain of *Actinastrum hantzschii* as an outgroup (Table 1). The range of symbiotic algae strains isolated from *P. bursaria* cells is presented in Figure 1.

Molecular methods

Symbiont's DNA was extracted using a GeneJET Plant Genomic DNA Purification Kit (ThermoScientific) according to protocol. Before isolation, culture of *P. bursaria* was carefully purified using special filters which allowed us to obtain pure culture of *P. bursaria* cells. 1.5 ml of dense *P. bursaria* culture was harvested from liquid culture by centrifugation. The pellet was frozen in liquid nitrogen and the mixture was sonicated on ice for 10 s at 40 W. After that, we followed the standard extraction protocol.

For molecular analysis we applied a fragment of the ITS1-5.8S rDNA-ITS2, as the most widely used, marker for *Paramecium* algal endosymbiont identification (for example BOCK *et al.* 2011; PRÖSCHOLD *et al.* 2011 and the other literature cited herein), due to its high degree of nucleotide substitutions, which allows for the comparison of closely related taxa, and which is highly variable among different species, whilst it is conserved within the same species (HOSHINA *et al.* 2010a). The fragment of ITS1-5.8S rDNA-ITS2 was amplified using primer pairs: ITS1 (WHITE *et al.* 1990)/ITS2R (primer designed in the present study, Table 2) or ITS1F/ITS2R (primers designed in the present study, Table 2) according to protocol with the following parameters: initial denaturation at 95ºC for 5 min, followed by 30 cycles of denaturation at 95ºC for 1 min, annealing at 54ºC for 2 min, extension at 72ºC for 3 min and a final extension at 72ºC for 5 min. The primers, which would amplify the DNA fragment we were studying, are specific to algae, and were designed according to the following scheme: (I) comparison of several algae sequences available in GenBank and identification of homologous, conservative fragments, (II) application of Reverse Complement software (http://www.bioinformatics.org/sms/rev_comp.html) in order to obtain sequences of reverse primers, (III) determining the Tm for the Forward (sequence 5'-3') and Reverse primer (sequence 3'-5') (the temperature of both primers should be similar) using the Primer Blast program (https://www.ncbi.nlm.nih.gov/tools/primer-blast). PCR amplification for all analyzed DNA fragments was carried out in a final volume of 40 μl containing 4 μl of DNA, 1.5 U Taq-Polymerase (EURx, Poland), 0.8 μl of 20 μM of each primer, 10 × PCR buffer, and 0.8 μl of 10 mM dNTPs in a Thermal Cycler PCR (G-storm). After amplification, the PCR products were

Table 1

Strains of algae used in the present study

No.	*Paramecium bursaria* (host) strain	Taxonomic designation of the host	Origin of the host	Algal (endosymbiont) species	Algal (endosymbiont) strain	GenBank Accesion number (ITS1-5.8S-ITS2)	References
1.	BS-7	R5	Botanical Garden in St. Petersburg, Russia	*Chlorella variabilis*	CVA-BS-7	KX639522	This study
2.	AZ20-4	R2	Astrakhan Nature Reserve, Russia	*Chlorella variabilis*	CVA-AZ20-4	KX639521	This study
3.	host: *Paramecium bursaria*	unknown	unknown	*Chlorella variabilis*	SAG 211-6	FM205849.1	LUO *et al.* 2010
4.	host: *Euplotes daidaleos*	unknown	unknown	*Chlorella variabilis*	EdL_Cl2_3NB	KF887350.1	Unpublished data
5.	host: *Paramecium bursaria*	unknown	USA	*Chlorella variabilis*	NC64A	AB206549	HOSHINA *et al.* 2010a
6.	GB15-2	R2	Lake Loch Linnhe, Scotland	*Chlorella vulgaris*	CVG-GB15-2	KX639525	This study
7.	KZ-126	R2	Kaliningrad, Russia	*Chlorella vulgaris*	CVG-KZ-126	KX639533	This study
8.	unknown	unknown	unknown	*Chlorella vulgaris*	DRL3	JX139000.1	BAILUNG *et al.* 2012
9.	SRB9-1	R2	River Danube, Serbia	*Micractinium reisseri*	MC-SRB9-1	KX639539	This study
10.	MS-1	R1	St. Petersburg, Russia	*Micractinium reisseri*	MC-MS-1	KX639538	This study
11.	KAM231-1	R2	Kamchatka, Russia	*Micractinium reisseri*	MC-4 231-1	KX639537	This study
12.	SW1	unknown	Black Forest, Germany	*Micractinium reisseri*	SW1-ZK, (SW1)	AB437244.1	HOSHINA & IMAMURA 2009b
13.	host: *Euplotes daidaleos*	unknown	unknown	*Micractinium reisseri*	EdL_Cl1_MAF	KF887345.1	Unpublished data
14.	host: *Paramecium bursaria*	unknown	Germany	*Micractinium reisseri*	Pbi	FM205851.1	HOSHINA *et al.* 2010a; LUO *et al.* 2010
15.	free-living	unknown	Mill Cove, Canada	*Micractinium* sp.	MCWWW15	KP204593.1	PARK *et al.* 2015
16.	free-living	unknown	Mill Cove, Canada	*Micractinium* sp.	MCWWW4	KP204582.1	PARK *et al.* 2015
17.	free-living	unknown	Mill Cove, Canada	*Micractinium* sp.	MCWWW5	KP204583.1	PARK *et al.* 2015
18.	free-living	unknown	Mill Cove, Canada	*Micractinium* sp.	MCWWW10	KP204588.1	PARK *et al.* 2015
19.	free-living	unknown	Mill Cove, Canada	*Micractinium* sp.	MCWWW11	KP204589.1	PARK *et al.* 2015
20.	free-living	unknown	West Antarctica	*Micractinium* sp.	KNUA032	KM243324.1	HONG *et al.* 2015
21.	unknown	unknown	unknown	*Actinastrum hantzschii*	SAG 2015	FM205841.1	LUO *et al.* 2010

electrophoresed in 1% agarose gel for 1 hour at 95V. After that, they were purified from the gel using NucleoSpin Extract II (Macherey-Nagel, Düren, Germany). Cycle sequencing was done in both directions with the application of BigDye Terminator v3.1 chemistry (Applied Biosystems, USA). The primers that were used for amplification were also applied for sequencing. Each sequencing reaction was carried out in a final volume of 10 µl containing 3 µl of template, 1 µl of BigDye Master Mix (1/4 of standard reaction), 1 µl of sequencing buffer, and 1 µl of 5 µM primer. Sequencing products were precipitated using Ex Terminator (A&A Biotechnology, Poland) and separated using the Genomed Company (Poland). Sequences are available in the GenBank database (for accession numbers see Table 1).

Phylograms were constructed in Mega v5.1 (TAMURA *et al.* 2007), using the Neighbor Joining (NJ) (SAITOU & NEI 1987) and Maximum Likelihood (ML) (FELSENSTEIN 1981) methods by bootstrapping with 1000 replicates (FELSENSTEIN 1985). The analysis of haplotype diversity (Hd) and nucleotide diversity (π) was done using DnaSP v5.10.01 (LIBRADO & ROZAS 2009). The identification of the best nucleotide substitution models for Maximum Likelihood tree reconstruction (T92+G model) was done using

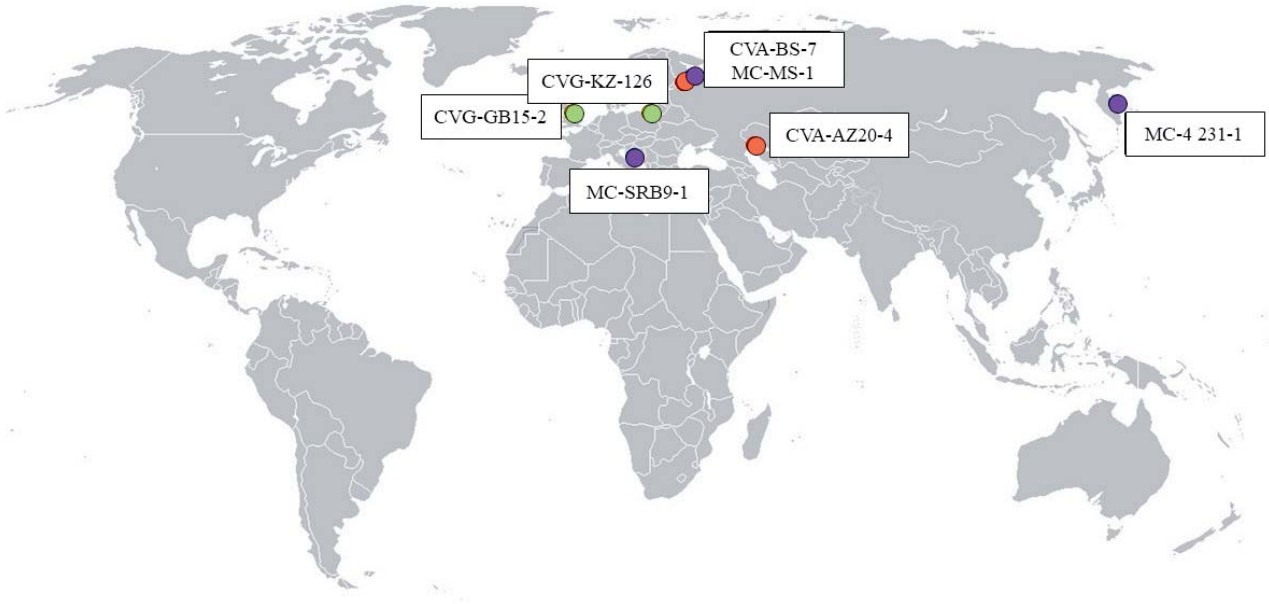

Fig. 1. Distribution of *P. bursaria* symbionts: *Micractinium reisseri* (violet circles), *Chlorella vulgaris* (green circles), and *Chlorella variabilis* (red circles). Abbreviations: MC – *Micractinium reisseri*, CVG – *Chlorella vulgaris*, CVA – *Chlorella variabilis.*

Table 2

Primers used in the present study

DNA fragment	Primer	Sequence 5'-3'	References
ITS1-5.8S rDNA-ITS2	ITS1	TCCGTAGGTGAACCTGCGG	WHITE *et al.* (1990)
	ITS1F	AATCTATCGAATCCACTTTGGTAAC	This study
	ITS2R	CTGCTAGGTCTCCAGCAAAG	This study

Table 3

Variability of the ITS1-5.8S-ITS2 rDNA fragments of the studied algae species

Species of algae	Number of sequences N	Number of sites	Number of polymorphic (segregating) sites S	Number of haplotypes h	Haplotype diversity Hd (SD)	Nucleotide Diversity π
ITS1-5.8S-ITS2 rDNA						
Chlorella variabilis	5	570	19	3	0.7	0.02095
Chlorella vulgaris	3	570	47	3	1.0	0.07407
Micractinium reisseri	12	570	35	8	0.894	0.01972
All species	20	570	154	14	0.937	0.07407

Mega v5.1. The haplotype network, which presented the relationships between and within studied algae species, was reconstructed by means of the Median Joining method (BANDELT *et al.* 1999), as implemented in the PopART software v. 1.7 (LEIGH & BRYANT 2015). Identification of isolated algae species was based on the comparison of obtained sequences with the standard sequences available in GenBank using Basic Local Alignment Search Tool (BLAST, available from http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results

Analysis of ITS1-5.8S-ITS2 rDNA fragments variation

We analyzed 20 ITS1-5.8S-ITS2 rDNA fragments (570 bp) of algae including 12 sequences of *Micractinium*, 3 sequences of *C. vulgaris,* 5 sequences of *C. variabilis*, and identified 14 haplotypes. The interspecific haplotype diversity value (Hd) was 0.937 and the nucleotide diversity (π) was 0.07407 (Table 3).

Taxonomic classification, and reciprocal relationship of the currently studied algal species

The phylogram (ML/NJ) constructed on the basis of fragments of ITS1-5.8S rDNA-ITS2 (Fig. 2), isolated from 20 algae strains, revealed strains grouping into three clusters (A, B, and C). The first of them – A is composed of the symbiotic algae of *P. bursaria* originating from the Danube River in Serbia (MC-SRB9-1) and St. Petersburg in Russia (MC-MS-1). Furthermore, there is also a strain, MC-4 231-1, isolated from *P. bursaria* collected from Kamchatka (Russia) (Tab. 1). Additionally, the cluster included 6 sequences of *Micractinium* sp. and 3 sequences of *M. reisseri* obtained from GenBank. Our strains of this cluster were assigned to *M. reisseri* after comparing the analyzed sequences with records published in GenBank (97% similarity to the closest match) as well as based on the constructed tree: they form a monophyletic clade together with *Micractinium* strains with a rather high bootstrap support (ML/NJ: 81/78). The second cluster – B includes symbiotic strains originating from Lake Loch Linnhe, Scotland (CVG-GB15-2) and Kaliningrad, Russia (CVG-KZ-126) (Tab. 1, Fig. 2) and a sequence of *C. vulgaris* from GenBank. These strains have been assigned to *C. vulgaris* based on the grouping with the strain DRL3 (92% similarity and bootstrap values for ML/NJ: 100/100). The third clade – C is composed of strains originating from St. Petersburg, Russia (CVA-BS-7) and the Astrakhan Nature Reserve, Russia (CVA-AZ20-4) and 3 sequences of *C. variabilis* obtained from GenBank (97% similarity to the closest match, bootstrap values for ML/NJ: 99/93). Our strains of this cluster have been identified as *C. variabilis*, because of a monophyly with 3 sequences of *C. variabilis* obtained from GenBank (Fig. 2).

The haplotype network of the fragment of ITS1-5.8S rDNA-ITS2 (Fig. 3) divided the strains into 3 haplogroups. The first one – *Micractinium* includes 7 haplotypes. Five of them correspond to single strains: 2 of the *Micractinium* sp. from GenBank and 3 of *M. reisseri* (newly analyzed strains marked in a darker violet). One of the remaining 2 haplotypes represents 4 strains of *Micractinium* sp. (GenBank) and the last haplotype represents 3 strains of *M. reisseri* (*conductrix*) obtained from GenBank. Molecular variability between particular haplotypes of that haplogroup oscillates from 1 to 10 nucleotide substitutions (Fig. 3).

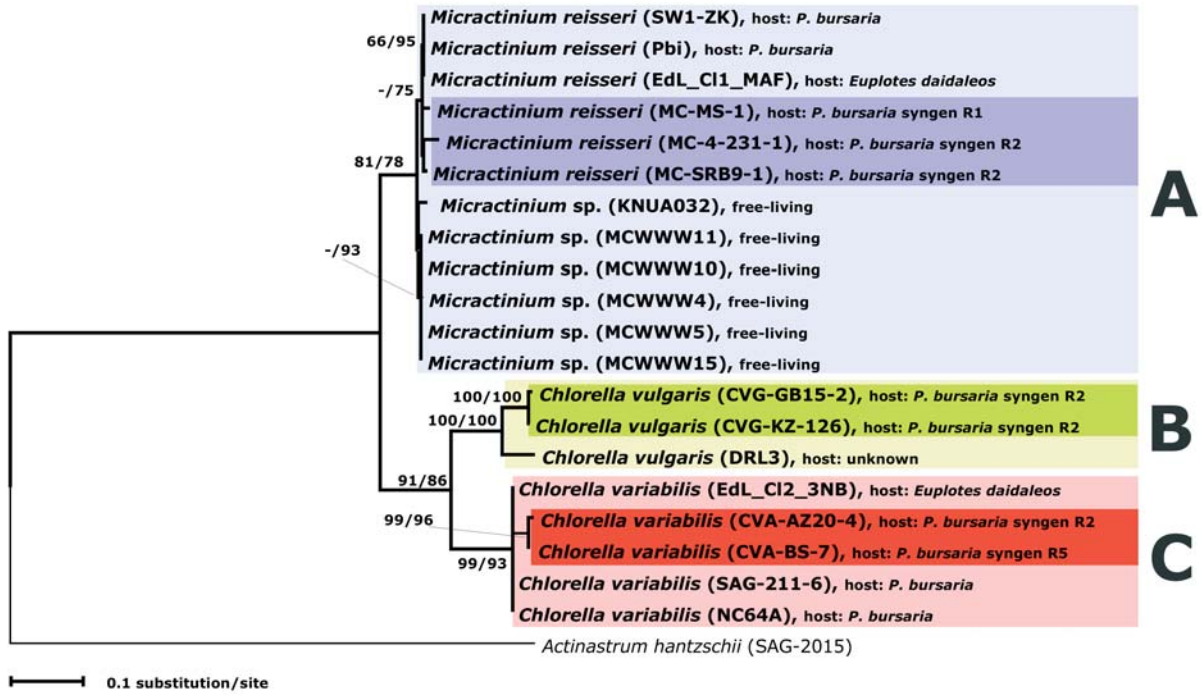

Fig. 2. Phylogram constructed for 20 symbiotic algae strains and a strain of *Actinastrum hantzschii* used as an outgroup based on a comparison of the ITS1-5.8S rDNA-ITS2 sequences using the Maximum Likelihood (T92+G model) and Neighbor Joining methods. The strains highlighted by lighter colours represent data from GenBank, whereas those highlighted by darker colours are currently studied strains. Bootstrap values for Maximum Likelihood and Neighbor Joining are presented. Bootstrap values less than 50% are not shown.

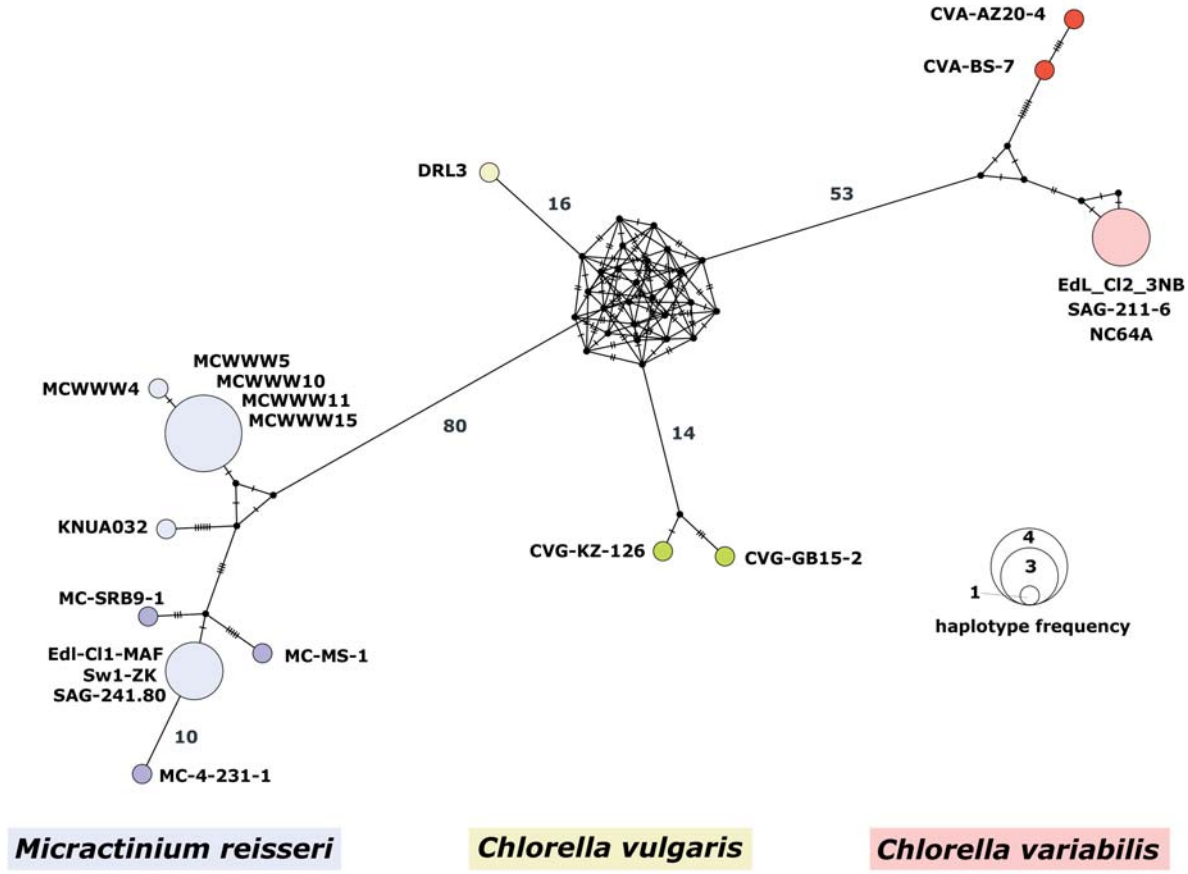

Fig. 3. Haplotype network constructed for 20 symbiotic algae strains based on a comparison of the ITS1-5.8S rDNA-ITS2 sequences. The size of the circles is proportional to the haplotype frequency. The median vectors that represent hypothetical intermediates or un-sampled haplotypes are shown as black circles. Haplotypes highlighted by lighter colours represent data from GenBank, whereas those highlighted by darker colours are currently studied strains. Hatch marks on particular branches represent nucleotide substitutions between particular haplotypes (in the case of 10 or more, a corresponding number was given). Analyses were conducted using the Median Joining method in PopART software v. 1.7.

The second haplogroup is composed of 3 unique haplotypes: 2 of newly analyzed strains (marked in darker green) with 4 differences between them and a haplotype of *C. vulgaris* (strain DRL3) from GenBank which is different from the other two haplotypes mentioned above by over 30 nucleotide substitutions.

And finally, the last haplogroup contains 3 haplotypes. One of them represents 3 strains of *C. variabilis* from GenBank, and the other two haplotypes (marked in darker red) correspond to the currently studied strains. Molecular variability between particular haplotypes of that haplogroup oscillates from 4 to 15 nucleotide substitutions. In turn, distances between different algal species are much greater: there are about 70-80 nucleotide substitutions between *C. vulgaris* and *C. variabilis*, 100-120 nucleotide substitutions between *C. vulgaris* and *Micractinium*, and 140-160 nucleotide substitutions between *C. variabilis* and *Micractinium* (Fig. 3).

Discussion

The application of molecular analyses is crucial to resolve phylogenetic relationships, especially when the organisms in question are not distinguishable using conventional methods, like microscopic observation or the analysis of physiological parameters. Based on molecular analyses, almost all symbiotic algae of *P. bursaria* were divided into two groups: American and European (HOSHINA *et al.* 2004; HOSHINA *et al.* 2005). According to the results obtained using gene encoding 18S rRNA as a marker, the symbiotic algae of *P. bursaria* are related to three species: *C. vulgaris*, *C. sorokiniana* and *C. lobophora* (GAPANOVA *et al.* 2007). HOSHINA and IMAMURA (2009a) described the characteristic geographical distribution of the two groups. The symbiotic algae belonging to the European group originate usually from Great Britain, Germany, Austria and Kaliningrad and

the algae of the American group originate from USA, Japan, China and the Southern Australia. HOSHINA *et al.* (2010a) assigned the strains originating from England, Germany, Austria, Karelia (Russia) and Northern Europe to *M. reisseri*. GAPANOVA *et al.* (2007) stated that strains of the European group are closely related to *C. vulgaris* or *C. sorokiniana* while American symbionts are closely related to *C. lobophora*. PRÖSCHOLD *et al.* (2011) revealed that symbiotic algae assigned to the European group belong to *C. vulgaris* and *M. reisseri*. The present results concerning strains of *C. vulgaris* are in concordance with PRÖSCHOLD *et al.* (2011) as they were collected from Great Britain and Kaliningrad region. Similarly, the origin of strains of *M. reisseri* collected in Russia (St. Petersburg,) and the River Danube in Serbia (Fig. 1) are within the boundaries of the occurrence of the European group (HOSHINA *et al.* 2010a).

However, the most significant result of the present study was determining the presence of *M. reisseri* on Kamchatka (Fig. 1) which is contradictory to the previous hypothesis that geographical distribution of *M. reisseri* is restricted to Europe and the presence of *C. variabilis* in Europe, whose distribution was limited to the USA and the Far East (HOSHINA & IMAMURA 2009b; HOSHINA *et al*. 2010a or by PRÖSCHOLD *et al*. (2011).

Before our current findings, there were not any reports of *M. reisseri* occurring in the Far East. Both viruses, CvV (infecting *C. variabilis*) and MrV (infecting *M. reisseri*), have been detected from distant regions of the world, but MrV has never been recorded from East Asia (VAN ETTEN 2003; YAMADA *et al.* 2006; HOSHINA *et al.* 2010b). The results obtained in the present study are supported by values of bootstrap reaching 81/78% for the ML/NJ phylogram constructed based on a comparison of the ITS1-5.8S rDNA-ITS2 sequences (Fig. 2). Furthermore, regions of Kamchatka (Russia) are located close to the boundaries of the American group which includes the Far East. PRÖSCHOLD *et al.* (2011) stated that strains belonging to the American group can be assigned to *C. vulgaris* or *C. variabilis*. However, in the present study, strains of *C. variabilis* were collected from Austria (Wien) and Russia (St. Petersburg and the Astrakhan Nature Reserve).

According to our results, we can conclude that the geographical distribution of *M. reisseri* is not restricted to only Europe and that the division of symbiotic algae into two groups is only contractual and is related to the number of introns in 18S rDNA (GAPANOVA *et al.* 2007). Moreover, these differences do not refer to all species of symbiotic algae and what is even more evident is that they don't have a strong connection with the geographical locations of algae. All of the divergences can be due to the fact that, so far, there has been an analysis of strains collected from a few places located very far from each other (for example Western Europe and the Far East). Analyses carried out on symbiotic algae were usually limited to few samples of a particular region. A persistent problem in many of the molecular phylogenetic investigations thus far might be caused by undersampling, which results in systematic errors in phylogenetic reconstruction. For example, some early 18S phylogenies showed a sister relationship between Chlorophyceae and Trebouxiophyceae (KRIENITZ *et al.* 2001), while more recent studies, that increased taxon sampling, revealed a sister relationship between Chlorophyceae and Ulvophyceae (WATANABE & NAKAYAMA 2007; DE WEVER *et al.* 2009). Therefore, an analysis which involves dense taxon sampling is important in order to avoid systematic errors in phylogenetic analyses. In order to resolve the phylogenetic relationships between symbiotic algae of *P. bursaria* originating from all over the world, the next step should be a research extension to new regions from Europe to the Far East and an increase in the taxon sampling of Palearctic and Nearctic ecozones.

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Author Contributions

Research concept and design: P.Z.L., S.T.; Collection and/or assembly of data: P.Z.L., M.R.; Data analysis and interpretation: P.Z.L., S.T.; Writing the article: P.Z.L., M.G.-S., S.T.; Critical revision of the article: S.T.; Final approval of article: P.Z.L.

Conflict of Interest

The authors declare no conflict of interest.

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