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

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 predetermined 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, Sebastian TARCZ, and Maria RAUTIAN

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*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 & IAMAMURA 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.
The systemic 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 Microactinum genus are morphologically similar to those of Chlorella, however cells of Microactinum 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 (Lin et al. 1999; Kvitko et al. 2001), as well as the content of GC pairs in DNA (Kessler & Huss 1990). Fott and Novakova (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 18°C (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: Microactinum reisseri (symbiotic strains: SW1-ZK, EdL_C11_MAF and standard strain: Pbi), Microactinum (free-living strains: KNUA032, MCWWW4, MCWWW5, MCWWW10, MCWWW11, MCWWW15), Chlorella variabilis (EdL_C12_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 (ThermoScien
tific) 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 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 54°C for 2 min, extension at 72°C for 3 min and a final extension at 72°C 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.bi
formatics.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
electrophoresed in 1% agarose gel for 1 hour at 95V. After that, they were purified from the gel using Nu-

cleoSpin Extract II (Macherey-Nagel, Düren, Ger-

many). 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. Se-

quencing products were precipitated using Ex Termi-

nator (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 diver-

sity (π) was done using DnaSP v5.10.01 (LIBRADO &

ROZAS 2009). The identification of the best nucleo-

tide substitution models for Maximum Likelihood

tree reconstruction (T92+G model ) was done using

Table 1

| No. | Paramecium bursaria (host) strain | Taxonomic designation of the host | Origin of the host | Algal (endosymbiont) species | Algal (endosymbiont) strain | GenBank Accession 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                           | LUC et al. 2010 |
| 4.  | host: Euplotes daidaeleos        | unknown                          | unknown            | Chlorella variabilis       | EdL_C12_3NB                 | KF887350.1                           | Unpublished data |
| 5.  | host: Paramecium bursaria        | unknown                          | USA                | Chlorella variabilis       | NC64A                       | AB206549                             | HOSHINA et al. 2010 |
| 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                          | 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-231-1                 | KX639537                     | This study                           |            |
| 12. | SW1                              | unknown                          | Black Forest, Germany | Micractinium reisseri     | SW1-ZK, (SW1)               | AB437244.1                           | HOSHINA & IMAMURA 2009b |
| 13. | host: Euplotes daidaeleos        | unknown                          | unknown            | Micractinium reisseri     | EdL_C11_MAF                 | KF887345.1                           | Unpublished data |
| 14. | host: Paramecium bursaria        | unknown                          | Germany             | Micractinium reisseri     | Pbi                         | FM205851.1                           | HOSHINA et al. 2010; LUC 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 hantzchii     | SAG 2015                     | FM205841.1                           | LUC et al. 2010 |
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).

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   |

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).
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).

**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 unsampled 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.

**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* were 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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