The Evolutionary Relationships between Endosymbiotic Green Algae of *Paramecium bursaria* Syngens Originating from Different Geographical Locations

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Paramecium bursaria (EHRENBERG 1831), a freshwater ciliate, typically harbors hundreds of green algal symbionts inside the cell. The aim of present study was the molecular identification of newly analyzed *P. bursaria* symbionts. The second aspect of the present survey was testing a hypothesis whether endosymbionts prefer the specified syngen of the host, and the specified geographical distribution. Ten strains of endosymbionts isolated from strains of *P. bursaria* originating from different geographical locations were studied. We analyzed for the first time, both the fragment of plastid genome containing 3'*rpl36-5'infA* genes and a fragment of a nuclear gene encoding large subunit ribosomal RNA (LSU rDNA). The analysis of the LSU rDNA sequences showed the existence of 3 haplotypes and the haplotype diversity of 0.733, and 8 haplotypes for the 3'*rpl36-5'infA* gene fragment and haplotype diversity of 0.956. The endosymbionts isolated from *P. bursaria* strains were identified as *Chlorella vulgaris, Ch. variabilis* and *Micractinium conductrix.* There was no correlation between the syngen of *P. bursaria* and the species of endosymbiont.

Key words: *Paramecium bursaria*, chloroplast 3'rpl36-5'infA gene fragment, LSU rDNA, *Chlorella vulgaris*, *Chlorella variabilis*, *Micractinium conductrix*.

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Paramecium bursaria is a species of ciliate that maintains symbiotic relationships with algae (KODAMA & FUJISHIMA 2009). Mutualistic symbiosis is an important ecological relationship, generally defined as two or more species coexisting and providing benefits to each other (MARGULIS & FESTER 1992). A single cell of P. bursaria possesses about 700 symbiotic algal cells in the cytoplasm (KODAMA & FUJISHIMA 2009). Each alga 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 & RUDZIŃSKA 1981; GU et al. 2002). Irrespective of the mutual relations between P. bursaria and symbiotic algae, the symbiont-free cells and the symbiotic algae retain the ability to grow without a partner (BROWN & NIELSEN 1974). On the other hand, recent studies

(KODAMA *et al.* 2014) have proven that endosymbiont (*Chlorella variabilis*) infections may change the metabolism (gene expression) of the host (*Paramecium bursaria*).

In the literature the term "Zoochlorellae" encompasses almost all *Chlorella*-like algae living in association with various organisms such as ciliates, heliozoans or even invertebrates. Most of these relationships have been described. KAWAIDA *et al.* (2013) has studied the association between *Hydra* (*viridissima* and *vulgaris* group) and *Chlorella sp.* HUSS (1999) has compiled a wide range of organisms including *Amoeba*, *Ciliata*, *Porifera*, *Coelenterata*, *Mollusca*, *Rotatoria* and *Turbellaria* living in symbiosis with green algae. About 25 species of ciliates such as *Climacostomum virens*, *Frontonia leucas*, *Paramecium* *bursaria* or *Vorticella sp.* contain *Chlorella*-like symbionts (REISSER 1994).

KANG et al. (2005) described a virus belonging to the family Phycodnaviridae. This virus is specific, which means that viruses isolated from P. bursaria's algae originating from North America and far East Asia can only infect "American" endosymbiotic algae, but can't infect "European" algae and the viruses isolated from endosymbionts of P. bursaria strains originating from Europe and Warakashiann Asia, can infect only "European" but not "American" algae (REISSER et al. 1988b; REISSER et al. 1990). GAPANOVA et al. (2007) described two groups of symbiotic Chlorella-like species: "American", also referred to as "Southern" and "European" or "Northern" based on differences in introns in the first part of the 18S rRNA gene. HOSHINA et al. (2010) designated these groups as different species: Chlorella variabilis belonging to "American" group and "European" Micractinium conductrix.

P. bursaria is divided into six syngens, reproductively isolated groups, each of which consists of four to eight mating types (BOMFORD 1966). Currently, a representative collection of *P. bursaria* strains belonging to five syngens is maintained at the Culture Collection of Ciliates and their Symbionts (CCCS) of St. Petersburg University, and a new notation of syngens was introduced by GRECZEK-STACHURA *et al.* (2012).

Molecular analyses have been crucial for inferring evolutionary lineages, especially when organisms, such as the green algal endosymbionts, are difficult to distinguish on the basis of morphological features. Analyses of 18S rDNA have revealed that the cells of P. bursaria possess endosymbiotic algae belonging to two lineages. Most of them represent Chlorellaceae, for example C. vulgaris, and the second group includes representatives of Coccomyxa, for example Paradoxia multiseta (HOSHINA & IMAMURA 2009). PRÖSCHOLD et al. (2011) have also found four species of endosymbionts that were present in P. bursaria cells such as Micractinium conductrix, Chlorella vulgaris, Chlorella variabilis and even Scenedesmus sp. previously described by REISSER & WIDOWSKI (1992). In the present study we investigated 10 strains of Chlorella-like species isolated from P. bursaria, belonging to five syngens collected in remote geographical sites and a strain of Parachlorella kessleri used as outgroup (Table 1). Two loci – a nuclear gene encoding the large subunit of ribosomal RNA (LSU rDNA), and the chloroplast genes encoding ribosomal protein L36 (rpl36) and a translation initiation factor IF-1 (*infA*) were applied to study the relationships of symbiotic algae. Our objective was to test the genetic distinctiveness of the "American" and "European" groups and to examine whether different species of endosymbionts prefer the specific syngen of P. bursaria.

Table 1

 geographical sampling sites.

 No.
 Algal (endosymbiont) strain
 Paramecium (host) strain
 Taxonomic designation of the host
 Origin of the host
 GenBank Accession number
 References

 1
 CVA-KD64
 KD64
 R2
 Kamchatka Ruscia
 KM203668
 KM203657
 This study

Strains of endosymbiotic algae isolated from Paramecium bursaria syngens originating from different

No	(endosymbiont)	1 urumeetum	decignation	Origin of the host			References
110.	strain	(host) strain	of the host	origin of the host	LSU rDNA	3'rpl36-5'infA	iterenetes
1.	CVA-KD64	KD64	R2	Kamchatka, Russia	KM203668	KM203657	This study
2.	CVA-Ard10-3	Ard10-3	R4	Ardmoore, Oklahoma, USA	KM203667	KM203658	This study
3.	CVA-BS-7	BS-7	R5	St. Petersburg, Russia	KM203669	KM203659	This study
4.	CVG-KZ-126	KZ-126	R2	Kaliningrad Region, Russia	KM203672	KM203660	This study
5.	CVG-HKV19-12	HKV19-12	R3	Khabarovsk Region, Russia	KM203671	KM203663	This study
6.	CVG-AB2-51	AB2-51	R4	Boston, USA	KM203673	KM203661	This study
7.	CVG-AZ10-1	AZ10-1	R5	Astrakhan Nature Reserve, Russia	KM203670	KM203662	This study
8.	MC-MS-1	MS-1	R1	St. Petersburg, Russia	KM203675	KM203666	This study
9.	MC-TOS1-7	TOS1-7	R2	Tolyatti Region, Russia	KM203676	KM203665	This study
10.	MC-VM-14	VM-14	R2	Valaam, Karelia, Russia	KM203674	KM203664	This study
11.	CCAP211/83	_	_	Göttingen, Germany	AB506070	_	(HOSHINA et al. 2010)

Abbreviations of algal species names: CVA - Chlorella variabilis, CVG - Chlorella vulgaris, MC - Micractinium conductrix.

Material and Methods

Strain cultivation and strain crosses

The strains of *P. bursaria* were cultivated on a lettuce medium according to Sonneborn (1970), fed on *Klebsiella pneumoniae* (SMC) and stored at a temperature of 18° C, in 12L/12D light/dark conditions. We investigated ten *Chlorella*-like species isolated from cells of *P. bursaria* strains originating from different geographical locations and a strain of *P. kessleri* used as an outgroup (Table 1).

P. bursaria syngens were identified by mating reactions of the studied strain with standard strains representing all the mating types of each syngen. The studied strain was assigned to a certain syngen based on the occurrence of strong clumping at the beginning of the mating reaction, the mating couples observed and the survival of F_1 progeny.

Molecular methods

Endosymbiont DNA was extracted using Gene-JET Plant Genomic DNA Purification Kit (ThermoScientific) according to the protocol. For DNA extraction, 1.5 ml about 2500 clones of dense *P. bursaria* culture was harvested from liquid culture by centrifugation. Then, the pellet was frozen in liquid nitrogen and the mixture was sonicated on ice for 10 s at 40 W to ensure the rupture of the algal cell walls. After this step, we followed the standard extraction protocol.

The large subunit rDNA was amplified by polymerase chain reaction (PCR) using the Chlorella-like specific primer pair HLR0F/HLR4R (HOSHINA & IMAMURA 2008) according to the protocol described by HOSHINA et al. (2004). The fragment of the 3'rpl36-5' infA genes was amplified using primer set UCP2F and UCP2R according to PROVAN et al. (2004). After amplification, the PCR products were electrophoresed in 1% agarose gel for 1 hour at 95V and after that purified from the gel using NucleoSpin Extract II (Macherey-Nagel, Düren, Germany). Sequencing reactions were done in both directions using BigDye Terminator v3.1 (Applied Biosystems, Foster City, USA). Sequencing products were precipitated using Ex Terminator (A&A Biotechnology, Gdynia, Poland).

Sequences were examined and corrected using Chromas Lite (Technylesium), aligned using Bio-Edit (HALL 1999). Trees were constructed in Mega 5.1 (TAMURA *et al.* 2011), using the Neighbor-Joining (NJ) (SAITOU & NEI 1987) and Maximum Likelihood (ML) (FELSENSTEIN 1981) methods by bootstrapping with 1000 replicates (FELSENSTEIN 1985). Analysis of haplotype diversity (Hd), nucleotide diversity (π) and polymorphic sites (NEI & KUMAR 2000) was done with DnaSP v5.10.01 (LIBRADO & ROZAS 2009). Analysis of nucleotide frequencies, and identification of best nucleotide substitution models for maximum likelihood tree reconstruction (T92+G for both the LSU rDNA and 3'*rpl36-5*'*infA* genes) were done using Mega v5.1 (TAMURA *et al.* 2011). Haplotype networks of the 3'*rpl36-5*'*infA* genes, which presented the distribution and relationships among haplotypes of the studied algae strains, were done by using the median-joining methods as implemented in the program Network 4.6.1.3 (http://www.fluxus-engineering.com/, BANDELT *et al.* 1999).

Results

We selected the LSU rDNA and 3'*rpl36-5'infA* genes as markers because we wanted to analyze two independent genomes, nuclear and chloroplast. In addition, the selected rDNA primers are specific for *Chlorella*-like algae. We analyzed the large subunit ribosomal RNA (LSU rDNA), which is characterized by higher variability than the small subunit ribosomal RNA (SSU rDNA). Additionally, the 3'*rpl36-5'infA* gene fragment was selected due to the presence of intergenic region which was suspected to have more potential substitution sites than coding gene regions.

Analysis of the LSU rDNA fragment

We obtained ten LSU rDNA fragments (505bp) of *Chlorella*-like species isolated from different syngens of *P. bursaria* and identified 3 haplo-types. The interspecific haplotype diversity value was Hd = 0.733 and the nucleotide diversity was $\pi = 0.02033$. The nucleotide frequencies were A = 25.3%, T = 18.6%, C = 24.3% and G = 31.8%. There were 22 variable positions (22 parsimony informative) in the analyzed rDNA fragment.

The phylogenetic tree (NJ, ML) constructed on the basis of the sequenced LSU rDNA fragments and a sequence of outgroup revealed that strains are grouped into three clusters. Endosymbionts isolated from *P. bursaria* strains: VM-14 (Valaam, Karelia, Russia), MS-1 (St. Petersburg, Russia) and TOS1-7 (Tolyatii Region, Russia) are grouped into the first cluster. We assigned these three strains to *Micractinium conductrix* after comparing the obtained sequences with sequences published in GenBank using the Basic Local Alignment Search Tool (BLAST, available from http://blast.ncbi.nlm.nih.gov/Blast.cgi). The second cluster is composed of green algae isolated from *P. bursaria* strains KD64 (Kamchatka, Rus-



Fig. 1. Distribution of the "American" and "European" *P. bursaria* symbionts with their strain index. Abbreviations: CVA – *Chlorella variabilis*, CVG – *Chlorella vulgaris*, MC – *Micractinium conductrix*.



Fig. 2. Phylogram constructed for 11 endosymbionts of *P. bursaria* strains and a strain of *P. kessleri* used as an outgroup based on a comparison of LSU rDNA sequences using the Neighbor Joining and Maximum Likelihood (T92+G model) methods. Bootstrap values for Neighbor Joining and Maximum Likelihood are presented. Bootstrap values less than 50% are not shown.

sia), BS-7 (St. Petersburg, Russia) and Ard10-3 (Ardmoore, USA). These strains were assigned to *Chlorella variabilis*. Finally, the third group is the *Chlorella vulgaris* clade composed of endosymbionts isolated from strains AB2-51 (Boston, USA), KZ-126 (Kaliningrad Region, Russia), HKV19-12 (Khabarovsk, Russia) and AZ10-1 (Astrakhan Nature Reserve, Russia). Clades composed of *C. vulgaris* and *C. variabilis* are closely related to each other, whereas *M. conductrix* is more distant (Fig. 2).

Analysis of the 3'*rpl36-5*'*infA* gene fragment of the plastid genome

Sequences of the 3'*rpl36-5*'*infA* gene fragment (256bp) were obtained from 10 strains of *Chlorella*-like species isolated from different syngens of *P. bursaria*. In the studied dataset 8 haplo-types were indentified. The interspecific haplotype

diversity value was Hd = 0.956 and the nucleotide diversity was $\pi = 0.07595$. The nucleotide frequencies were A = 29.0%, T = 36.8%, C = 18.6% and G = 15.6%. There were 42 variable positions (36 parsimony informative) in the analyzed 3'*rpl36-5*'*infA* gene fragment. Similarly as in the case of the ribosomal locus, the phylogenetic tree (NJ, ML) constructed on the basis of the sequenced 3'*rpl36-5*'*infA* fragments and outgroup sequence revealed that the strains of endosymbionts were grouped into three clades representing three different algae species (*C. vulgaris, C. variabilis, M. conductrix*) (Fig. 3).

The haplotype network of the 3'*rpl36-5*'*infA* gene fragment divided the strains into three clades, but included more than one haplotype. The clade *Chlorella variabilis* is composed of three unique haplotypes (Cva-h01, Cva-h02 and Cva-h03), each corresponds to a different strain, which sug-



Fig. 3. Phylogram constructed for 10 endosymbionts of *P. bursaria* strains and a strain of *P. kessleri* used as an outgroup based on a comparison of 3'*rpl36-5*'*infA* sequences using the Neighbor Joining and Maximum Likelihood (T92+G model) methods. Bootstrap values for Neighbor Joining and Maximum Likelihood are presented. Bootstrap values less than 50% are not shown.



Fig. 4. Haplotype network constructed for 10 endosymbionts of *P. bursaria* strains based on a comparison of 3'*rpl36-5*'*infA* (b) sequences. The size of the circles is proportional to the haplotype frequency (empty circles represent sizes for 1 and 2 strains for a particular haplotype). The median vectors that represent hypothetical intermediates or un-sampled haplotypes are shown as black circles. Black dots on particular branches represent nucleotide substitutions between particular haplotypes (if over 10 then the corresponding number was given).

gests a high level of genetic diversity within this species. The clade Chlorella vulgaris consisted of three unique haplotypes. Haplotype Cvg-h01 represented the strain CVG-KZ-126, haplotype Cvgh02 is composed of two strains: CVG-HKV19-12 and CVG-AZ10-1 and haplotype Cvg-h03 represented the strain CVG-AB2-51, which corresponds to results presented in the phylogram (Fig. 3). The Micractinium conductrix clade consisted of two unique haplotypes. Haplotype Mc-h01 is represented by two strains: MC-MS-1 and MC-TOS1-7. Haplotype Mc-h02 included the strain MC-VM-14. This haplotype network also revealed that C. variabilis and C. vulgaris are more closely related to each other (14 nucleotide substitutions), than M. conductrix to C. variabilis (41 nucleotide substitutions) and C. vulgaris (20 nucleotide substitutions), which is in accordance to results in the phylogram (Figs 2, 3, & 4).

Discussion

Division of *Paramecium bursaria* symbionts into "American" and "European" groups

Recent molecular analyses have revealed that nearly all *P. bursaria* symbionts belong to either "American" or "European" groups (HOSHINA *et al.* 2004; HOSHINA *et al.* 2005). KESSLER & HUSS (1990) revealed that the "American" and "European" algal strains demonstrated different physiological and biochemical characters including formation of secondary carotenoids or utilization of inorganic nitrogen. GAPANOVA *et al.* (2007) compared these two groups of symbionts, named respectively "Southern" and "Northern", and free-living *Chlorella sp.* All strains of "Northern" and "Southern" ecotypes were different from free-living *Chlorella vulgaris* in having introns in the first part of the 18S rRNA gene. In the present study, we confirmed the genetic diversity of the "American" and "European" groups. There are three clades on the phylograms and each is represented by a distinct species of algae: *C. vulgaris, C. variabilis* and *M. conductrix* (Figs 2 & 3).

Geographical distribution of the 'American' and 'European' *P. bursaria* symbionts

Figure 1 shows the geographical distribution of both of these groups. HOSHINA & IMAMURA (2009) as well as HOSHINA et al. (2010) assigned strains from eastern part of USA, Japan, China and Australia to the "American" group, whereas strains from western and northern Europe and Karelia Region were assigned to the "European" group. PRÖSCHOLD et al. (2011) revealed that "European" strains of green algae belong to C. vulgaris or to Micractinium. HOSHINA et al. (2010) assigned a strain originating from North Carolina, USA to *C. variabilis* and a strain originating from Göttingen, Germany to M. conductrix. Our strains of M. conductrix: MC-MS-1, MC-TOS1-7 and MC-VM-14 were collected in St. Petersburg (Russia), Tolyatti Region (Russia) and Valaam Karelia (Russia), respectively. Strains of C. variabilis used in the present study were collected in the USA (CVA-Ard10-3) and Kamchatka (CVA-KD64). The strain of C. variabilis, CVA-BS-7 was collected in the Botanical Garden of St. Petersburg State University. According to the literature (HOSHINA et al. 2010), this part of Europe is an unusual occurrence record of this species. This outlying geographical record may be explained by the observation that botanical gardens contain species imported from all over the world and therefore this strain could have been transported from elsewhere with other plant or animal organisms (RICHARDSON 1994). The strains of C. vulgaris used in the present study originated from the Kaliningrad Region (Russia) (CVG-KZ-126), Boston (USA) (CVG-AB2-51), Khabarovsk Region (Russia) (CVG-HKV19-12) and Astrakhan Nature Reserve (Russia) (CVG-AZ10-1). DOUGLAS & HUSS (1986) suggested that strains from the USA can be assigned to C. vulgaris or C. sorokiniana. REISSER et al. (1988a) stated that representatives of C. vulgaris can be both "American" and "European" green algae. Furthermore, he assigned symbionts from Japan to C. vulgaris as well. KODAMA et al. (2007) as well as KODAMA & FUJISHIMA (2008) assigned strains collected in Japan to C. vulgaris.

Correlation between the syngen and geographical distribution of *Paramecium bursaria* and the species of endosymbiont

The issue of the occurrence of a correlation between the symbiotic algae species living inside the cell of host and the syngen and the geographical distribution of P. bursaria has not been investigated yet. P. bursaria is divided into six syngens, which are sexually isolated groups (BOMFORD 1966), each of which has a specific geographical distribution (JENNINGS 1938; JENNINGS & OPITZ 1944; CHEN 1956; BOMFORD 1966; HOSHINA et al. 2006). We can put forward two hypotheses on the establishment of endosymbiosis between green algae and P. bursaria. The first suggests that the symbiosis took place before the speciation of P. bursaria species into syngens. In result, each species of endosymbiotic algae may form a symbiosis with any syngen. The second hypothesis suggests the aposymbiotic P. bursaria after speciation to the syngens had a symbiotic relationship with green algae. When considering speciesspecificity in symbioses, it is important to distinguish between host and symbiont specificities. In general the host seems to be more specific (BAKER 2003). HOSHINA et al. (2005) concluded that the type of Chlorella symbiont depends on the distribution of Paramecium. Our analyses indicated the existence of a correlation between geographical distribution of syngens and species of endosymbiotic algae. P. bursaria syngens R1 and R2 are Eurasian (GRECZEK-STACHURA et al. 2012). The strain MS-1 of syngen R1 from St. Petersburg harbors M. conductrix (Table 1, Fig. 1). Strains of syngen R2 from the Kaliningrad Region (KZ-126) and Tolyatti Region (TOS1-7), harbor C. vulgaris. The strain from Karelia (VM-14) possesses M. conductrix and the strain from Kamchatka (KD64) lives in symbiosis with C. variabilis (Table 1, Fig. 1). Strains of syngen R3 were reported in the Russian Far East, China, Japan and USA (GRECZEK-STACHURA et al. 2012) and the strain HKV-19-12 was collected in Khabarovsk. The endosymbiotic algae isolated from this strain was assigned to C. vulgaris (Table 1, Fig. 1). The strains belonging to syngen R4 are restricted to USA, and the strains AB2-51 and Ard10-3 were from Boston and Ardmoore. Interestingly, that these two strains from America have different species of endosymbiotic algae, C. vulgaris and C. variabilis, respectively (Table 1, Fig. 1). This case supports the hypothesis that endosymbionts of P. bursaria do not have a preference for the syngen of the host. Syngen R5 of *P. bursaria* was found in the Volga Delta (GRECZEK-STACHURA et al. 2012) and in this paper is represented by strains from Astrakhan (AZ10-1) and St. Petersburg (BS-7). This syngen also can establish endosymbiosis with C. vulgaris (AZ10-1) and C. variabilis (BS-7). Although the latter species wasn't reported in Europe, the host strain of P. bursaria originated from a botanical garden, where we speculate that species may have been imported from all over the world (Table 1, Fig. 1). REISSER et al. (1988a) found that two algal-free P. bursaria strains, both originating

from America and Europe, can form a stable symbiosis with two *Chlorella*-like strains of American and European origin. SUMMERER *et al.* (2007) investigated the endosymbiotic ciliate *Askenasia chlorelligera* from two lakes and found different species of endosymbiotic *Chlorella* because of different environmental conditions. MEIER & WIESSNER (1989) and TAKEDA *et al.* (1998) found that symbionts of *P. bursaria* can be occasionally lost and symbiont-free cells can be reinfected with different species of symbiotic algae (REISSER *et al.* 1988a).

Usefulness of DNA markers for *Paramecium bursaria* symbiont identification

Molecular analyses have been crucial for resolving phylogenetic relationships, especially if organisms, such as the green algal endosymbionts, are difficult to distinguish on the basis of morphological features. The present survey, based on molecular data, revealed the existence of three algae species (C. vulgaris, C. variabilis and M. conductrix) in ten studied strains of P. bursaria. We analyzed the LSU rDNA and a fragment of 3'rpl36-5' infA gene. They were found to be appropriate molecular markers for distinguishing P. bursaria symbionts due to their variability. The LSU rDNA region of nuclear DNA has been found to vary in sequence and evolves eight times faster than the complete SSU rDNA sequence. HOSHINA & IMAMURA (2008) found 59 nucleotide substitutions in the LSU rDNA fragment between C. vulgaris and symbiotic algae of *P. bursaria* – *Chlorella* sp. PROVAN et al. (2004) amplified the various regions of 3'rpl36-5' infA gene of Nephroselmis olivacea, Chlorella vulgaris, Mesostigma viride and Chaetosphaeridium globosum and showed that pairwise identities between C. vulgaris and Chaetosphaeridium globosum ranged even to 69% (using pair UCP2). SUMMERER et al. (2008) revealed that the chloroplast gene segment in all P. bursaria symbionts they examined was identical. Analysis of the 3'rpl36-5' infA gene fragment revealed the division of the 10 strains studied herein into three clades, each included more than one haplotype. LUO et al. (2006) made the important finding that Micractinium pusillum (characterized by cells with bristles) loses bristles and becomes similar to Chlorella under monoculture conditions, which suggests that molecular methods are a suitable complement for conventional methods. WU et al. (2001) tested *Chlorella* spp. isolates using ribosomal DNA sequences and revealed that phylogenetic analysis was in line with results obtained by conventional methods (morphological and biochemical data). According to the literature quoted above, we suggest that the application of LSU

rDNA and the fragment of the 3'*rpl36-5*'*infA* gene allows the identification of *P. bursaria* symbionts.

Conclusions

Based on the obtained results we assigned endosymbiotic algae of *Paramecium bursaria* to three species: Chlorella. vulgaris, Ch. variabilis and Micractinium conductrix. We also confirmed the genetic diversity between strains originating from different geographical locations and included them to the "American" and "European" groups. Furthermore, we also demonstrated that there is no congruence between the species of endosymbiont and the syngen of P. bursaria. We can conclude that both the LSU rDNA fragment and 3'rpl36-5' infA genes are useful molecular tools for distinguishing closely related taxa of *P. bursaria* endosymbionts, the identification of which is very difficult based on morphological features. Moreover, the application of two independent fragments of the genome makes the results more reliable.

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