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IDENTIFICATION OF *PARAMECIUM BURSARIA* SYNGENS THROUGH MOLECULAR MARKERS – COMPARATIVE ANALYSIS OF MITOCHONDRIAL CYTOCHROME C OXIDASE SUBUNIT I (*COI*)

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ABSTRACT

The aim of this study is an identification of *Paramecium bursaria* syngens originating from different geographical locations and proving the correlation between distributions and belonging to any of five syngens. Ten strains of *Paramecium bursaria* belonging to five different syngens and strain of *Paramecium multimicronucleatum* were investigated using molecular marker — mitochondrial cytochrome c oxidase subunit I (*COI*). According to results, obtained in this study, using phylogenetic methods like Neighbor Joining (*NJ*) and Maximum Likelihood (*ML*), relationship between analyzing strains through their clustering in clusters and correlation between strains belonging to any syngen and syngen's distribution was confirmed. Phylograms constructed using NJ and ML methods revealed strains' grouping in five clusters. Results which were obtained revealed usefulness of *COI* as a biomarker, which is important in identification of *Paramecium bursaria* syngens. This reports to a great potential of *COI* as a molecular marker and obtaining dependable results through combination of molecular methods with classical ones.

Keywords: *Paramecium bursaria*, cytochrome c oxidase subunit, phylogenetic methods, syngen

INTRODUCTION

Ciliates to which *Paramecium bursaria* belongs are single-celled organisms. They play a very important role in freshwater and marine environments as major trophic links in food chain (**Lynn, 2008).** *Paramecium bursaria* has been used many times as a model organism in biochemical, ecological and genetic investigations. *Paramecium* genus is morphologically divided into "aurelia" group, when a cell has a shape of cigar and the second group, foot-shaped "bursaria". The latest concept based on morphological, biological and molecular differences divides this genus into four subgenera: *"Chloroparamecium", "Helianter", "Cypriostomum"* and *"Paramecium"* composed of only one species *Paramecium bursaria* **(Fokin, *et al*., 2004).** *P. bursaria* species is divided into six syngens including four to eight mating types **(Bomford, 1966).** Syngens are sexually isolated groups among which conjugation does not occur. This process takes place only within the same syngen **(Chen 1956).** Researchers have been interested in concepts of syngen and mating types for a long time. Mating types were first described by **Jennings (1938, 1944)** and by **Siegel and Larison** (**1960**).
The cell of *P. bursaria* posseses inside hundreds of green algae symbionts which provide the photosynthetic products to their host and receive in return carbon dioxide and nitrogen compounds **(Kodama** and **Fujishima, 2009).** Products of photosynthesis, mostly maltose and oxygen play role in regulating the mating reactivity rhythms **(Tanaka** and **Miwa, 1996).**

Mating types are determined genetically and regulated in dominant-recessive mode by two or three loci **(Bomford, 1965; Siegel, 1963).** These ways of determinations are characteristic of outbreeders, to which *P. bursaria* belongs and demonstrates an archetypical outbreeding with a very long period of immaturity, low ratio of cell divisions and very rare autogamy (**Sonneborn, 1957).** There are two models of geographical distribution of protists. **Finlay *et al*., (2006)** states that the majority of them are cosmopolitan and ubiquitous, the opposite statement of Foissner (**2006**) tends toward endemic distribution of most of protists. Both points of view find support in species *Paramecium bursaria.* Some syngens are widely distributed in the world, whereas others have restricted distribution.
The aim of this study was to resolve the phylogenetic relationship among *P. bursaria* strains originating from different localities and to investigate at a molecular level the intraspecific differentiation of this species.

MATERIAL AND METHODS

The strains of *Paramecium bursaria* were culture on a lettuce medium (**Sonneborn, 1970**), fed on *Enterobacter aerogenes* and stored at the temperature of 18^0C, in the light/dark conditions (12L/12D). Strains of *P. bursaria* originating from different locations were used and strain of *Paramecium multimicrinucleatum* as outgroup (Table 1).

Table 1 Strains of *Paramecium bursaria*and strain of *Paramecium multimicronucleatum*with their geographical localizations

Species	Strainindex	Syngen	Location
Paramecium bursaria	UV2-2	1	Vinnitsa, Ukraine
Paramecium bursaria	RV82-3	1	Rostov Veliky, Russia
Paramecium bursaria	BBR49-8	2	Lake Baikal, Russia
Paramecium bursaria	BL15-12	2	Lake Baikal, Russia
Paramecium bursaria	CB11-1	3	Beijing, China
Paramecium bursaria	APS	3	PiburgerSee Lake, Austria
Paramecium bursaria	Ard 7	4	Ardmoore, Oklahoma, USA
Paramecium bursaria	AB2-32	4	Boston, USA
Paramecium bursaria	BS-3	5	Saint Petersburg, Russia
Paramecium bursaria	AZ20-1	5	Astrakhan Nature Reserve, Russia
Paramecium multimicronucleatum	BR	-	Baton Rouge, USA

Paramecium bursaria DNA was isolated from vegetative cell using the NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany). Mitochondrial DNA fragments of COI gene was sequenced and analyzed. The fragment was amplified with F388dt and R118dt primers set (Table 2), using the protocol according to **Strüder-Kypke** and **Lynn (2010)**.

Table 2 Primers used in study

DNA fragment	Primer	Sequence 5' - 3'	References
COI mtDNA	F388dt	TGTAAAACGACGGCCAGTGGCbAAAGATGTGC	Strüder-Kypke and Lynn (2010)
COI mtDNA	R118dt	CAGGAAACAGCTATGACTAACTCAGGGTGACCAAATCA	Strüder-Kypke and Lynn (2010)

After amplification, the PCR products were electrophoresed in 1% agarose gel for 1 hour at 95V and after that purified from gel using NucleoSpin Extract II (Macherey-Nagel, Düren, Germany).Sequencing reaction was done in both directions using the BigDye Terminator v3.1 (Applied Biosystems, Foster City, USA) according to protocol shown in table (Table 3).

Table 3 Sequencing reaction profile

Reaction	Temperature	Time of reaction	Number of cycles
Initialdenaturation	94°C	3 min	1
Denaturation	96°C	10 s	25
Annealing	55°C	5 s	
Extension	60°C	2 min	

Sequencing products were precipitated using Ex Terminator (A&A Biotechnology, Gdynia, Poland).Sequences were examined and corrected using Chromas Lite (Technylesium), aligned using BioEdit **(Hall 1999)**. Trees were constructed in Mega 5.1 **(Tamura *et al*. 2007)**, using the Neighbor-Joining (NJ) and Maximum Likelihood (ML) methods by bootstrapping with 1000 replicates.

RESULTS AND DISCUSSION

Analysis of mitochondrial cytochrome oxidase gene (*COI*mtDNA) fragments by construction the tree using Maximum Likelihood method revealed strains' grouping into five clusters. Strains of syngen 2, originating from Russia are grouped into cluster A, American strains of syngen 4 are grouped into cluster B. Cluster C groups strains of syngen 1, which originate from Russia and Ukraine. Cluster D is composed of 5th syngen's strains and the last one, cluster E groups strains from China and Austria, belonging to syngen 3 (Fig. 1).

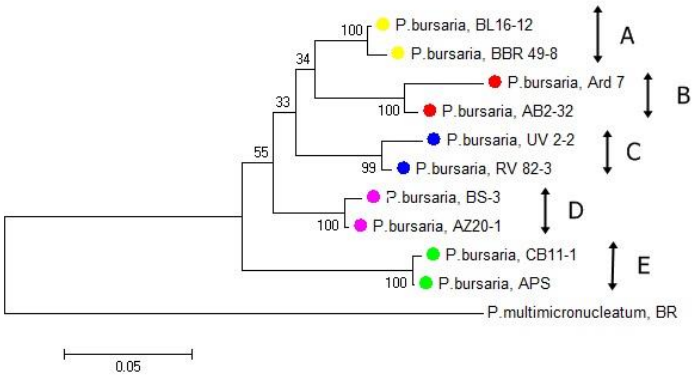

Figure 1 Phylogram constructed for 10*P. bursaria* strains (*P. multimicronucleatum* as an outgroup), based on a comparison of sequences from *COI* gene fragment using the Maximum Likelihood method

The second tree, constructed using Neighbor Joining method demonstrates *P. bursaria* strains grouping also into 5 clusters. The first of them, cluster A groups strains of syngen 2, cluster B groups American strains of syngen 4. Russian strains of syngen 5 are grouped into cluster C. Cluster B is composed of Eurasian strains of syngen 1 and cluster E groups strains of syngen 3 originating from Austria and China (Fig. 2).

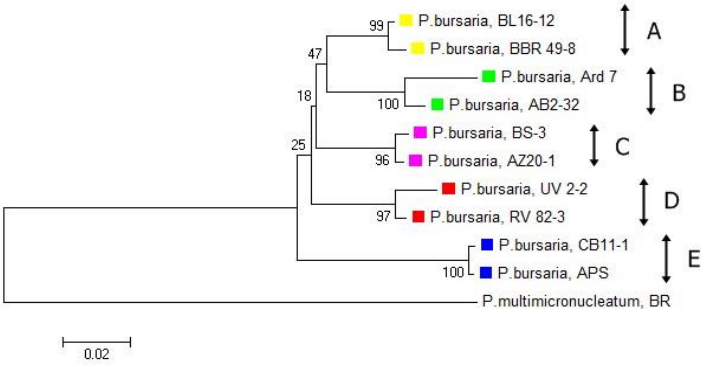

Figure 2 Phylogram constructed for 10*P. bursaria* strains (*P. multimicronucleatum* as an outgroup), based on a comparison of sequences from *COI* gene fragment using the Neighbor Joining method

Ciliates are a comprehensive group including various organisms. Some of them have become models for range of fields in biological researches in recent years, which is among other things due to discovery of mating types and possibilities to control their sexual process. Many new methods of investigation appeared and enabled to study their complex structure. Incompleteness of knowledge of *P. bursaria* complex structure is a reason to requiring further investigations. To study this issue more precisely, molecular methods have been used and their development has made it possible to establish phylogenetic relationships between strains belonging to different syngens of *P. bursaria*. The most complete description of *Paramecium bursaria* syngens was done by **Bomford (1966)**. The occurrence of six syngens within this species, containing from four to eight mating types was documented. Furthermore, the list of syngens' typical geographical distribution was introduced (Table 4). Unfortunately, the great number of collection was lost and a few strains remained available in laboratories. That was a significant obstruction of conducting researches on *Paramecium bursaria*for a long time. Therefore the new notation of *Paramecium bursaria*s syngens and a new reference to Bomford's syngens using "R" symbol was done by **Greczek-Stachura** and colleagues (**2011**). Table 4 shows that new syngen R1 corresponds to Bomford's syngen 6. Bomford's syngens: B2 and B4 correspond to introduced R4 and R2 respectively. And the last one which revealed correspondence to new data is B1 which refers to new R3. In our collection strains of Bomford's syngen 5 are probably absent. There are two models of geographical distribution of protists proposed by Finlay and Foissner. First of them states that the majority of protists are cosmopolitan and ubiquitous **(Finlay *et al*., 2006)** whereas the opposite statement of **Foissner (2006)** defines protists' distribution to be endemic. Both points of view find support in distribution of syngens of *Paramecium bursaria*, which tend to be found in certain geographical localities. Bomford's syngens: B1, B2 and B3 were found in the USA **(Jennings, 1938)** and after almost two decades B1 was also found in China **(Chen, 1956). Hoshina *et al*., (2006)** identified strains from Japan also as belonging to syngen 1. European syngens B4, B5 and B6 were found in Russia **(Jennings and Opitz, 1944).** According to notes included in table 4, "old" and

"new" syngens corresponding to each other occupy the same regions (Table 4). Syngens R1 and R2 are Eurasian and they were obtained in Ukraine and Russia. Strains of syngen 3 have been found in China and Austria, the second localizations in Austria is probably due to migration of tropical plants **(Greczek** and **Stachura *et al*., 2011).** Syngen 4 is restricted to US territory. Strains of syngen 5 were obtained on Russia (Table 1).Geographical factors seem to play very important role in *Paramecium bursaria* distribution, but it still requires further investigations because most of them remain a mystery. Some of them, for example syngen 1 and 2 are sympatric and occupy common territory, whereas strains of syngen 3 have a worldwide distribution, though it is rarely found in Europe. It is hard to find a good and unequivocal explanation for this fact. Distribution of syngens 1 and 2 may be due to some unknown genetic factors. On the other hand the worldwide distribution can be correlated to migration of birds or anthropogenic transport. The outbreeding strategy, which is characteristic to *Paramecium bursaria* also seem to play a significant role in reaching that sort of distribution and may be a way to maintenance genetic conservation of strains belonging to the same syngen, geographically isolated from each other **(Greczek-stachura *et al*., 2011).** The next possible explanation for restricted occurrence of some syngens is too short period of time to reach worldwide distribution or too specific ecological requirements **(Foissner, 2008).**

Table 4 Possible correlation between syngens introduced by Bomford and new syngens' numbers proposed by **Greczek-Stachura** and colleagues (2011)

Syngennumber		**Geographicaldistribution**	
Greczek *et al*., system (R)	Bomford's system (B)	Greczek *et al*., data	Bomford's data
R1	B6	All over Europe, to the east up to Siberia	Europe
R2	B4	All over Europe, to the east up to Siberia, Australia	Europe
R3	B1	Far East of Russia, Japan, China, USA	Asia
R4	B2	USA	USA
-	B3	-	USA
R5	-	Fewlocalities in Europe	-
Absent in collection	B5	-	Western Europe

Mitochondrial cytochrome c oxidase subunit I, which was a molecular marker in this study is a protein, located in inner membrane of mitochondria and it is a crucial enzyme of electrons transport in oxidative chain **(Strüder-Kypke** and **Lynn, 2010).** Mitochondrial genes change by nucleotide substitution at a much faster rate than nuclear genes. The *COI* gene sequences revealed significant genetic divergence (21-26%) within Paramecium Aurelia complex and made possible to define them as different species. *Paramecium bursaria* divergence was lower (0-10.4%) but it was similar to *Paramecium multimicrinucleatum* (10.3%) and higher that was observed in *Paramecium caudatum* (7.6%) **(Strüder-Kypke** and **Lynn, 2010).** Therefore, this gene has been extensively used in evolutionary studies and has proved its usefulness as a molecular marker.

CONCLUSION

In this experiment the occurrence of five syngens of *Paramecium bursaria* has been revealed. Furthermore genetic polymorphism between strains originating from different geographical locations has been demonstrated as well as occurrence of correlation between belonging to syngen and geographical distribution has been revealed. It can be said that mitochondrial cytochrome c oxidase subunit I is an useful molecular tool.

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