PL-ISSN 0015-5497 (print), ISSN 1734-9168 (online)
Folia Biologica (Kraków), vol. 63 (2015), No 1
doi: 10.3409/fb63_1.77

Molecular Identification of *Paramecium bursaria* Syngens and Studies on Geographic Distribution using Mitochondrial Cytochrome C Oxidase Subunit I (*COI*)

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Accepted December 15, 2014

ZAGATA P., GRECZEK-STACHURA M., TARCZ S., RAUTIAN M. 2015. Molecular identification of Paramecium bursaria Syngens and studies on geographic distribution using mitochondrial cytochrome C oxidase subunit I (*COI*). Folia Biologica (Kraków) **63**: 77-83.

Paramecium bursaria is composed of five syngens that are morphologically indistinguishable but sexually isolated. The aim of the present study was to confirm by molecular methods (analyses of mitochondrial *COI*) the identification of *P. bursaria* syngens originating from different geographical locations. Phylograms constructed using both the neighbor-joining and maximum-likelihood methods based on a comparison of 34 sequences of *P. bursaria* strains and *P. multimicronucleatum*, *P. caudatum* and *P.calkinsi* strains used as outgroups revealed five clusters which correspond to results obtained previously by mating reaction. Our analysis shows the existence of 24 haplotypes for the *COI* gene sequence in the studied strains. The interspecies haplotype diversity was Hd = 0.967. We confirmed genetic differentiation between strains of *P. bursaria* and the occurrence of a correlation between geographical distribution and the correspondent syngen.

Key words: *Paramecium bursaria*, *COI*, syngens, geographical distribution, phylogenetic methods.

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Paramecium bursaria (Focke 1836) is a foot-shaped *Paramecium* which contains endosymbiotic algae in individual perialgal vacuoles (KARAKASHIAN & RUDZIŃSKA 1981). Phylogenetic analyses revealed that *P. bursaria* harbors endosymbionts representing different species (PRÖSCHOLD *et al*. 2011; REISSER 1976, 1980) and the establishment of symbiosis is algal species specific (KODAMA & FUJISHIMA 2007, 2009).

P. bursaria was divided into six syngens, i.e. reproductively isolated groups (the term syngen has remained in use for *P. bursaria)* with four to eight mating types for each syngen BOMFORD (1966). Unfortunately Bomford's collection was lost and only a few strains remain available in laboratories in Japan. Currently, a representative collection of *P. bursaria* strains is maintained at St. Petersburg State University, so based on some correspondence between the syngens, a new notation of syngens was introduced by GRECZEK-STACHURA *et al*. (2012). The symbol "R" is used for the "Russian" collection and the symbol "B" for the "British" collection (Table 1).

A fragment of the cytochrome c oxidase 1 (*COI*) gene of mitochondrial DNA can be used to identify protists as well as many other species. Initial studies on ciliates were done on *Tetrahymena* (LYNN & STRÜDER-KYPKE 2006; CHANTANGSI *et al.* 2007). BARTH *et al*. (2006), based on sequences *COI* from *P. caudatum* and *P. multimicronucleatum,* supported the use of this gene as a barcoding marker. PRZYBOŚ *et al*. (2012) used this fragment to analyze phylogenetic diversity in *Paramecium calkinsi,* and TARCZ *et al*. (2013) using mitochondrial loci, studied genetic relationships within *P. novaurelia* originating from distant geographical localities. GRECZEK-STACHURA *et al*. (2012) used mitochondrial *COI* to analyze the degree of speciation within *P. bursaria* belonging to five different syngens.

Table 1

Correspondence between *P. bursaria* syngens from the present collection (GRECZEK-STACHURA *et al*. 2012) and the Bomford collection (BOMFORD 1966)

Syngen numbers		Number of mating types	
Present system	Bomford system	Present system	Bomford system
R1	B6	8	8
R2	B4	8	8
R3	B1	8	4
R4	B2	6	8
R5	B3	4	4
Absent in collection	B5	–	8

P. bursaria syngens represent extreme outbreeders. They are characterized by the occurrence of a synclonal system C type of mating inheritance, very long periods of immaturity and maturity, many mating types, low ratio of cell divisions and global geographical distribution (SONNEBORN 1957). BOMFORD (1966) and GRECZEK-STACHURA *et al*. (2012) postulated that most of the sibling species are restricted to certain geographical locations and thus, they are adapted to specific environmental conditions. For example syngens 1, 2 and 3 of the "B" collection were found in the USA (JENNINGS 1938) and later syngen B1 was found in China (CHEN 1956) and Japan (HOSHINA *et al*. 2006). Syngens B4, B5 and B6 were detected in Europe (JENNINGS & OPITZ 1944; BOMFORD 1966). Syngens R1 and R2 of the new collection introduced by GRECZEK-STACHURA *et al*. (2012) are Eurasian. Strains of syngen R3 were reported in the Russian Far East, China, Japan and USA and strains belonging to R4 are restricted to the USA, whereas strains of syngen R5 were found in the Volga River delta.

The aim of the present study was to confirm syngen identification performed by mating reactions by analysing DNA sequences of the mitochondrial *COI* gene for 22 new strains of *P. bursaria*. Another goal of the research was to investigate the level of intraspecific molecular differentiation of 22 strains of *P. bursaria* originating from distant geographical locations and to confirm the correlation between geographical distribution and the correspondent syngen.

Material and Methods

Strain cultivation and strain crosses

A total of 22 strains of *P. bursaria* originating from different geographical regions were used as well as *P. multimicronucleatum, P. caudatum* and *P. calkinsi* included as outgroups (Table 2). The strains were deposited in the CCCS (Culture Collection of Ciliates and their Symbionts) in St. Petersburg State University.

Strains of *P. bursaria* were cultivated on a lettuce medium inoculated with *Enterobacter aerogenes* (SONNEBORN 1970) at a temperature of 18°C, in light/dark conditions (12L/12D).

Syngen identification was performed by mating reaction of a studied strain with standard strains representing all the mating types of each syngen. The studied strain was assigned to a particular 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

P. bursaria genomic DNA was isolated from vegetative cells using the NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany) according to protocol. Mitochondrial DNA fragments of the *COI* gene (651 bp) were amplified, sequenced and analyzed. The fragment was amplified with F388dt and R118dt primers using a protocol previously described by STRÜDER-KYPKE & LYNN (2010) (Table 3). After amplification, the PCR products were electrophoresed in 1% agarose gels for 1 hour at 95V. NucleoSpin Extract II (Macherey-Nagel, Düren, Germany) was used for purifying DNA from gels. The sequencing reaction was done in both directions with primers M13F and M13R (STRÜDER-KYPKE & LYNN 2010; Table 3) using BigDye Terminator v3.1 (Applied Biosystems, Foster City, USA) according to protocol. Sequencing products were precipitated using Ex Terminator (A&A Biotechnology, Poland) and separated on an ABI PRISM 377 DNA Sequencer (Applied-Biosystems, USA). The sequences are available in the NCBI GenBank database (Table 2).

Data analyses

Sequences were examined using Chromas Lite software (Technelysium, Australia) to evaluate and correct chromatograms. Alignments of the studied sequences were done using BioEdit software (HALL 1999). Phylograms were constructed with Mega 5.2 (TAMURA *et al*. 2011), using the neighbor-joining (NJ) (SAITOU & NEI 1987) and maximum likelihood (ML) (FELSENSTEIN 1981) methods. The NJ analysis was performed using the Kimura 2-parameter correction model (KIMURA 1980) by bootstrapping with 1000 replicates (FELSENSTEIN 1985). Analysis of haplotype diversity (Hd), nucleotide diversity (π) and polymorphic sites (NEI 1987) was done with DnaSP v5.10.01 (LIBRADO & ROZAS 2009). Analysis of nucleotide frequencies and identification of sub-

Table 2

Strains of *P. bursaria*, *and P. multimicronucleatum*, *P. caudatum*, *P. calkinsi* used in the present study

Species	Strain index	Syngen	Location	GenBank accession numbers COI mtDNA	References
Paramecium bursaria	BOB130-6	R1	Lake Baikal, Russia	KJ701556	this study
Paramecium bursaria	AZ 12-9	R1	Astrakhan Nature Reserve, Russia	KJ701557	this study
Paramecium bursaria	AS 62-9	R1	Lake Sewan, Armenia	KJ701558	this study
Paramecium bursaria	T 24-5	R1	Tajikistan	KJ701559	this study
Paramecium bursaria	PB1	R1	Biebrza National Park, Poland	JF708920	GRECZEK-STACHURA *et al*. 2012
Paramecium bursaria	PB2	R1	Biebrza National Park, Poland	JF708921	GRECZEK-STACHURA *et al*. 2012
Paramecium bursaria	OLI	R1	Unknown	FJ905152	STRÜDER-KYPKE & LYNN 2012
Paramecium bursaria	GG	R1	Göttingen, Germany	JF708919	GRECZEK-STACHURA *et al*. 2012
Paramecium bursaria	BBR51-1	R2	Lake Baikal, Russia	KJ701560	this study
Paramecium bursaria	BBR178-9	R2	Lake Baikal, Russia	KJ701561	this study
Paramecium bursaria	NRB217-1	R2	Novosibirsk, Russia	KJ701562	this study
Paramecium bursaria	BBK197-2-2	R2	Lake Baikal, Russia	KJ701563	this study
Paramecium bursaria	RA 2-1	R2	Altai Forelands, Russia	KJ701564	this study
Paramecium bursaria	KZ-126	R2	Kaliningrad region, Russia	KJ701565	this study
Paramecium bursaria	96 Bi-2	R2	St. Petersburg, Russia	KJ701566	this study
Paramecium bursaria	V 6-1	R2	Volgograd, Russia	KJ701567	this study
Paramecium bursaria	AZ 21-3	R2	Akstrahan Nature Reserve, Russia	KJ701568	this study
Paramecium bursaria	AZ 20-4	R2	Akstrahan Nature Reserve, Russia	KJ701569	this study
Paramecium bursaria	KT 1-1	R2	Krasnoyarsk, Russia	KJ701570	this study
Paramecium bursaria	Obv	R2	St.Petersburg, Russia	JF708937	GRECZEK-STACHURA *et al*. 2012
Paramecium bursaria	Ek	R2	St.Petersburg, Russia	JF708936	GRECZEK-STACHURA *et al*. 2012
Paramecium bursaria	BOB-1	R2	Vyborg, Russia	JF708938	GRECZEK-STACHURA *et al*. 2012
Paramecium bursaria	AZ17-5	R2	Akstrahan Nature Reserve, Russia	JF708929	GRECZEK-STACHURA *et al*. 2012
Paramecium bursaria	BP-28	R3	Morskoy Nature Reserve, Russia	KJ701571	this study
Paramecium bursaria	Cs2	R3	Shanghai, China	KJ701572	this study
Paramecium bursaria	SKS4-5	R3	Fukushima, Japan	KJ701573	this study
Paramecium bursaria	Pb1C10	R3	China	JX082021	ZHAO *et al*. 2013
Paramecium bursaria	Pb1C17	R3	China	JX082018	ZHAO *et al*. 2013
Paramecium bursaria	Pb1C19	R3	China	JX082020	ZHAO *et al*. 2013
Paramecium bursaria	Ard 7	R4	Ardmore, Oklahoma, USA	KJ701574	this study
Paramecium bursaria	Ard 9	R4	Ardmore, Oklahoma, USA	KJ701575	this study
Paramecium bursaria	AB2-32	R4	Boston, USA	JF708916	GRECZEK-STACHURA *et al*. 2012
Paramecium bursaria	BS-3	R5	St. Petersburg, Russia	KJ701576	this study
Paramecium bursaria	AZ20-1	R5	Astrakhan Nature Reserve, Russia	KJ701577	this study
Paramecium multimicronucleatum	BR	–	Baton Rouge, USA	JF304189	PRZYBOŚ *et al*. 2012
Paramecium caudatum	PcC40	–	Australia	JX082103	ZHAO *et al*. 2013
Paramecium calkinsi	PRO165-7	–	Vladivostok, Russia	JF304181	PRZYBOŚ *et al*. 2012

Table 3

Primers used in this study

DNA fragment	Primer	Sequence 5'-3'	References
COI mtDNA	F388dt	TGTAAAACGACGGCCAGTGGCAAAGATGTGC	STRÜDER-KYPKE & LYNN 2010
COI mtDNA	R118dt	CAGGAAACAGCTATGACTAACTCAGGGTGACCAAATCA	STRÜDER-KYPKE & LYNN 2010
Sequencing primer	M13F	TGTAAAACGACGGCCAGT	STRÜDER-KYPKE & LYNN 2010
Sequencing primer	M13R	CAGGAAACAGCTATGAC	STRÜDER-KYPKE & LYNN 2010

stitution models for maximum likelihood tree reconstruction (T92+G) were done using Mega v5.2 (TAMURA *et al*. 2011).

Results

The amplified fragment of the mitochondrial *COI* gene was used to identify *P. bursaria* syngens. The number of haplotypes was 24 and the interspecific haplotype diversity value was Hd = 0.967 and nucleotide diversity was $\pi = 0.09709$. The nucleotide frequencies were A = 35.3, T = 42.5, C = 11.1 and G = 11.1. There were 310 variable positions (209 parsimony informative) in the analyzed *COI* fragment.

A neighbor-joining phylogram based on mitochondrial *COI* fragments revealed five clusters denoted A, B, C, D, and E (Fig. 1), respectively. Strains of syngen R2 (RA2-1, 96Bi-2, NRB217-1, V6-1, BBR178-9, KT1-1, AZ21-3, AZ20-4, BBR51-1,

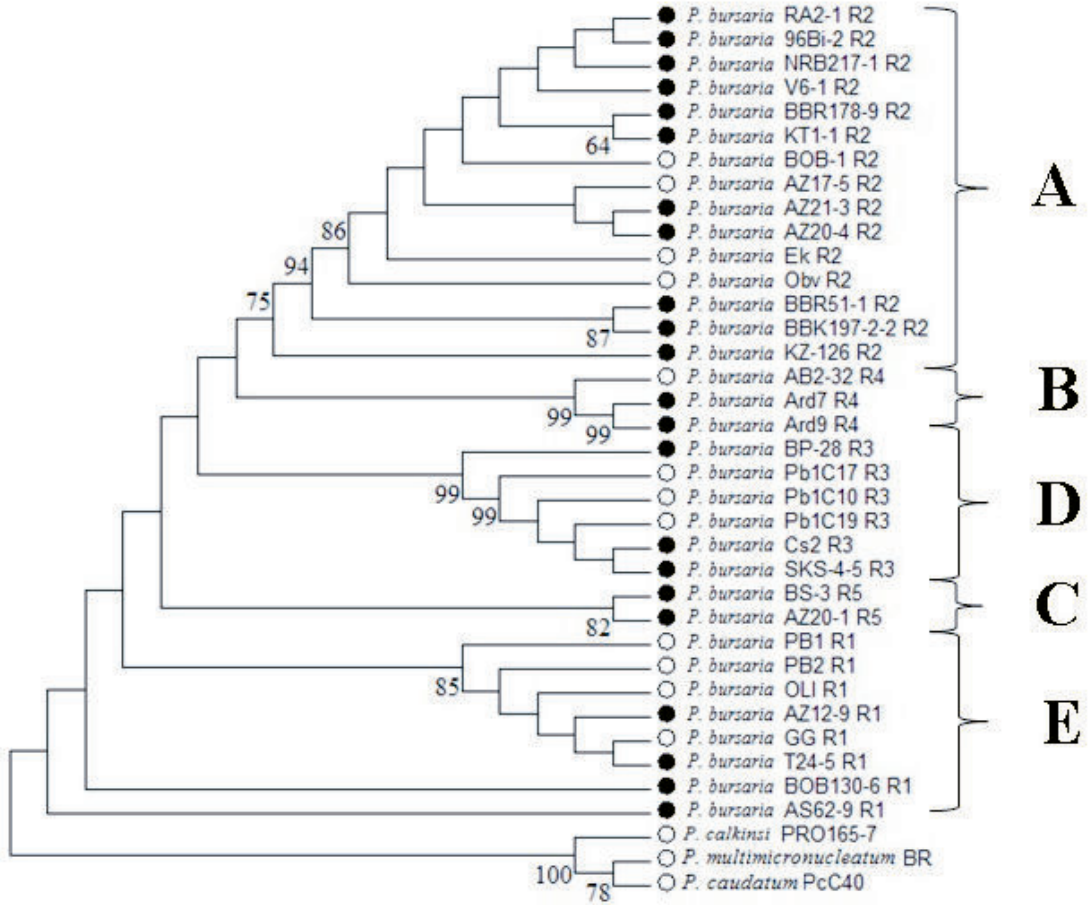

Fig. 1. Phylogram constructed for 34 *Paramecium bursaria* strains and strains of *Paramecium multimicronucleatum, Paramecium caudatum* and *Paramecium calkinsi* used as outgroups, based on a comparison of sequences from the mitochondrial *COI* gene fragment using the neighbor-joining method. Bootstrap values for neighbor-joining are presented. Bootstrap values less than 50% are not shown. Black circles present newly used strains and white circles indicate strains previously published.

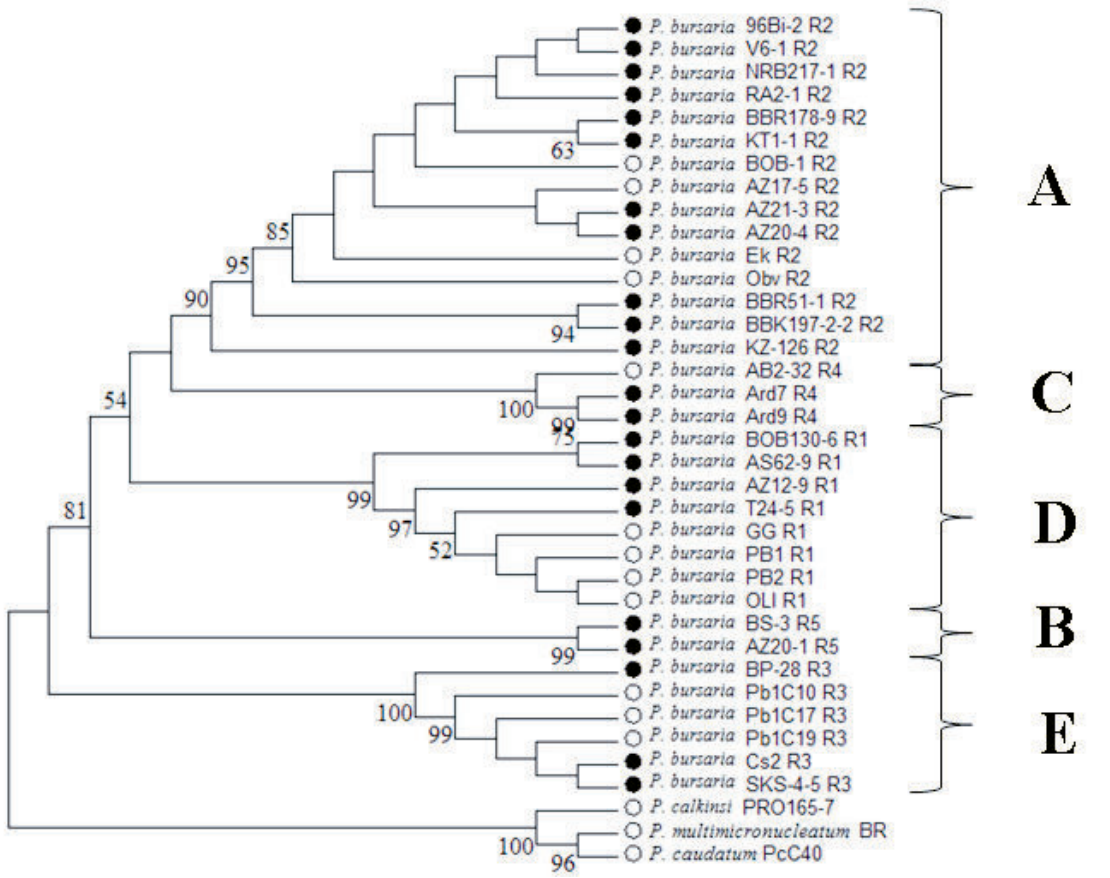

Fig. 2. Phylogram constructed for 34 *Paramecium bursaria* strains and strains of *Paramecium multimicronucleatum, Paramecium caudatum* and *Paramecium calkinsi* used as outgroups, based on a comparison of sequences from the mitochondrial *COI* gene fragment using maximum-likelihood (T92+G model). Bootstrap values for maximum-likelihood are presented. Bootstrap values less than 50% are not shown. Black circles present newly used strains and white circles indicate strains previously published.

BBK197-2-2, KZ-126, Obv, Ek, BOB-1, AZ17-5) originating from regions of Russia (Altai Forelands, St. Petersburg, Novosibirsk, Volgograd, Lake Baikal, Krasnoyarsk, Astrakhan Nature Reserve, Kaliningrad region, Vyborg) are grouped into cluster A, strains of syngen R3 (BP-28, Cs2, SKS4-5, Pb1C10, Pb1C17, Pb1C19) originating from Morskoy Nature Reserve (Russia), Shanghai (China) and Fukushima (Japan) are grouped into cluster D. Cluster B groups American strains of syngen R4 (Ard7, Ard9, AB2-32) originating from Ardmoore and Boston, and cluster C is composed of strains of syngen R5 (BS-3, AZ20-1) originating from Russia (St. Petersburg and Astrakhan Nature Reserve). Strains of syngen R1 (BOB130-6, AZ12-9, AS62-9, T24-5, PB1, PB2, OLI, GG) are grouped into cluster E. These strains originate from Lake Baikal (Russia), Astrakhan Nature Reserve, Lake Sewan (Armenia), Tajikistan, Poland and Germany.

The second phylogram constructed using the maximum-likelihood method reveals a very similar topology as the previous one (NJ) and showed that *P. bursaria* strains are divided into 5 clusters. The first, cluster A, groups Russian strains of syngen R2, cluster B groups two strains of syngen R5. American strains of syngen R4 are grouped into cluster C. Cluster D is composed of strains of syngen R1. Cluster E groups strains of syngen R3 (Fig. 2).

Discussion

DNA barcoding as a method of species discrimination and establishment of phylogenetic relationships between closely related taxa is a useful molecular tool. Mitochondrial DNA has been used in phylogenetic studies of protists because it evolves much more rapidly than nuclear DNA, resulting in the accumulation of differences between closely related species. The database of *COI* for *Paramecium* is growing and becoming more comprehensive and that is why the assignment of new, unknown species is now possible. Thus, error detection is now easier, for example the sequences

previously identified as *P. multimicronucleatum* seem to be *P. caudatum* (TARCZ *et al*. 2012; TARCZ 2013). ZHAO *et al*. (2013) assessed diversity at the *COI* locus in five species of *Paramecium: P. bursaria*, *P. duboscqui*, *P. nephridiatum*, *P. caudatum* and *P. sp.* They found various *COI* haplotypes in all of them. The level of intraspecific haplotype differentiation was between 0.1% and 10.9%. The inter-specific haplotype divergence was higher than 23%. The *COI* gene sequences revealed significant genetic differentiation (21-26%) within the *P. aurelia* complex and supported their status as different species. *P. bursaria* divergence was lower (10.4%) but similar to *P. multimicronucleatum* (10.3%) and higher than in *P. caudatum* (7.6%) (STRÜDER-KYPKE & LYNN 2010). GRECZEK-STACHURA *et al*. (2012) stated that phylograms constructed for strains of *P. bursaria* based on *COI* sequences had a higher resolution than phylograms inferred from rDNA.

Similarly to the previous results obtained by GRECZEK-STACHURA *et al*. (2012), the sequence analysis of *COI* confirmed the occurrence of five syngens of *P. bursaria*, and genetic polymorphism between strains originating from different geographical locations as well. Furthermore, the analysis confirmed the correlation between syngen type and geographical distribution.

The distribution of strains used in this study corresponds to locations proposed by BOMFORD (1966), JENNINGS & OPITZ (1944) and GRECZEK-STACHURA *et al*. (2012). Strains of syngen R1 originate from Russia, Armenia and Tajikistan. Strains of syngen R1 (PB1, PB2, GG) described by GRECZEK-STACHURA *et al*. (2012) originate from Poland and Germany. Strains of syngen R2 also occur in Russia, ranging from the Kaliningrad region up to the Russian Far East. Strains of the same syngen cited in this paper (Obv, Ek, BOB-1. AZ17-5) also originate from Russia (GRECZEK--STACHURA *et al*. 2012). Strains of syngen R3 used in this study originate from China, Japan and the Russian Far East (Morskoy Nature Reserve). ZHAO *et al*. (2013) restricted the distribution of syngen R3 strains to China (Pb1C10, Pb1C17, Pb1C19). The strains of syngen R4 originate from USA (Ardmore and Boston). JENNINGS (1938) also collected strains of the same syngen on US territory. Strains of syngen R5 used in this study originate from Astrakhan and St. Petersburg (Russia) and correspond to the distribution of strain AZ20-1 proposed by GRECZEK-STACHURA *et al*. (2012).

There are two intensively debated models of the geographical distribution of protists. FINLAY *et al*. (2006) states that the majority of protists are cosmopolitan and ubiquitous –"ubiquity model", whereas FOISSNER (2008) defines some protist distributions as endemic – "the moderate endemicity model". Both points of view find support in the distribution of syngens of *P. bursaria*. The unusual feature of *P. bursaria* syngens is that they tend to be found in certain geographical areas and that geographical factors seem to play a significant role in the species distributions. For example syngen R3 is present mainly in Asia (China, Japan and the Russian Far East), whereas syngens 1 and 2 are present in Europe and also in Australia (strains Hg5g and Hg24g) (GRECZEK-STACHURA *et al*. 2012). The distribution of syngen R4 is restricted to the USA and the distribution of syngen R5 is restricted to the Volga River delta and Astrakhan in Russia. However, there are exceptions to this rule. Syngen R3 was noted in Italy. It was collected in botanical gardens, i.e. places in which species are imported from all over the world, thus this strain could have been transported with exotic plants (GRECZEK STACHURA *et al*. 2012). Strains collected in the Volga River delta, a place known for great waterfowl migrations, are also suspected to be transmitted from other possibly distant territories.

The distribution of *P. bursaria* seems to be moderately endemic. The outbreeding strategy, characteristic of *P. bursaria*, has a significant role in reaching new locations, but the genetic conservation of strains belonging to the same syngen makes them geographically isolated from each other.

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