PL-ISSN 0015-5497 (print), ISSN 1734-9168 (online)
Folia biologica (Kraków), vol. **58** (2010), No 1-2

doi:10.3409/fb58_1-2.35-45

Intra-Specific Differentiation of *Paramecium bursaria* Strains by Molecular Methods – Preliminary Studies

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Accepted September 15, 2009

GRECZEK-STACHURA M., TARCZ S., PRZYBOŚ E. 2010. Intra-specific differentiation of *Paramecium bursaria* strains by molecular methods – preliminary studies. Folia biol. (Kraków) **58**: 35-45.

Ten strains of *Paramecium bursaria* and also *P. caudatum, P. multimicronucleatum*, *P. tetraurelia* strains (as outgroups) were characterized by using Random Amplified Polymorphic DNA (RAPD), Amplified Ribosomal DNA Restriction Analysis (ARDRA) and sequencing of the non-coding ribosomal internal transcribed spacer (ITS) regions. RAPD analysis revealed that all *Paramecium bursaria* strains possessed characteristic band patterns; there was a correlation between the degree of differentiation of DNA revealed by RAPD-fingerprinting and the geographic origin of a particular strain. ARDRA riboprinting (using a fragment of SSU-LSU rDNA, about 3085bp) with restriction enzymes *Dra*I, *Eco*RV, *Hha*I, *Hind*III, *Msp*I, *Pst*I distinguished groups of *P. bursaria* strains with characteristic band patterns originating from different sites. Comparison of the 550bp ITS1-5.8S-ITS2 fragment showed differentiation (0.9%) of the *P. bursaria* strains as three main groups of strains connected by site of origin in the constructed tree.

Key words: *Paramecium bursaria* strains, biodiversity, RAPD, ARDRA, ITS1-5.8S-ITS2.

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An understanding of the process of speciation in protozoans is very important and worthy of study as they are exceptionally differentiated at many levels. They are thus valuable research subjects. Species belonging to the genus *Paramecium* spp. (Ciliophora) are the most studied in Protozoa in this regard. Among these, an interesting species is *Paramecium bursaria* EHRENBERG 1831 which maintains intracellularly several hundred symbiotic algae. The character of the association of the *Paramecium bursaria* with the *Chlorella* spp. is mutual symbiosis (KODAMA & FUJISHIMA 2009). The molecular mechanism responsible for this phenomenon is still under investigation.

The phylogeny of *P. bursaria* and its intraspecific differentiation seem equally interesting. The problem was previously investigated by HOSHINA *et al*. (2006) who compared 18S and ITS2 sequences in 10 strains of *P. bursaria*. Four types of 18S rDNA sequences and six different ITS2 sequences were revealed. The unrooted tree revealed large intraspecific distances of *P. bursaria* in comparison to the other *Paramecium* species (*P. multimironucleatum, P. aurelia* complex and *P. caudatum)*. Generally, the subgeneric classification of *Paramecium* by FOKIN *et al*. (2004) into four subgenera: *Chloroparamecium, Helianter, Cyprostomum and Paramecium*, was supported. *Chloroparamecium* is composed of only one species, *P. bursaria,* and on the basis of biological and molecular differences it was suggested that this species diverged first in the genus (STRŰDER-KYPKE *et al*. 2000; FOKIN *et al.* 2004).

Paramecium bursaria is divided into six syngens, sexually isolated groups (BOMFORD 1966), each of which consists of four to eight mating types. Successful conjugation can take place only between clones of different mating types within the same syngen (CHEN 1956).

According to SONNEBORN (1975) the species of the *Paramecium aurelia* complex show inbreeding in general, but to a varying degree. *Paramecium bursaria* was the archetypical outbreeder

(SONNEBORN 1957), and as a result shows intraspecific genetic differentiation. The high degree of outbreeding within the same species could play an important role in its life history and evolutionary strategies.

Paramecium bursaria is regarded as an old species and we suspected that genetic divergence will be larger than in the other *Paramecium* species. The aim of the present study was an investigation of strain relationships by a comparison of ten different strain genotypes of *Paramecium bursaria* and some strains belonging to other species of *Paramecium*: *P. tetraurelia, P. caudatum,* and *P. multimicronucleatum*. We applied RAPD-fingerprinting (Random Amplified Polymorphic DNA) to discern the polymorphism of genomic DNA, ARDRA riboprinting (Amplified Ribosomal DNA Restriction Analysis) to analyze a fragment of 3085bp rDNA and sequencing of fragment 550bp rDNA (SSUrDNA ITS1-5,8S-ITS2, LSU rDNA). This preliminary work on intraspecific differentiation of *P. bursaria* using these three methods resulted in the selection of a suitable molecular marker and a comparison of the obtained results.

Material and Methods

Material

The strains of *Paramecium* spp. were cultivated on a lettuce medium inoculated with *Enterobacter aerogenes* (SONNEBORN 1970) at a temperature of 24°C with cool white light illumination (24L). Strains of *P. bursaria* originating from different geographical regions were used (Table 1), as well as *P. tetraurelia, P. multimicronucleatum* and *P. caudatum* as outgroups.

Methods used in molecular analyses

Paramecium genomic DNA was isolated (500μl of cell culture was used for DNA extraction) from vegetative cells at the end of the exponential phase using the NucleoSpin Tissue Kit (Macherey-Nagel, Germany).

RAPD analysis

Random Amplified Polymorphic DNA - PCR (RAPD-PCR) analysis of *Paramecium* strains, carried out in general as in STOECK & SCHMIDT (1998), was previously described in PRZYBOŚ *et al.* 2003b. RAPD-PCR was performed with primers Ro360-04, Ro 460-01, Ro 460-02, Ro 460-03, Ro 460-04, Ro 460-05, Ro 460-06, Ro 460-07, Ro 460-08, Ro 460-10 (Oligo, Poland) (Table 2) using Taq polymerase (Qiagen). The RAPD-PCR was done in a Biometra thermocycler using the PCR conditions as described in STOECK and SCHMIDT (1998). The products of the PCR reactions were separated by electrophoresis on 1.5% agarose gels for 1.5h at 85V together with a molecular weight marker XIV (Roche, France), stained with ethidium bromide, and visualized in UV light using the program Scionimage (Scion Corporation, USA). Analysis of similarity was carried out by comparing the molecular mass of DNA band patterns obtained by the RAPD method (the Bio1D++ program, Vilbert Lourmat, France) according to the NEI and LI (1979) similarity coefficient. Dendrograms were produced using the UPGMA (unweighted pair group match average) algorithm.

Table 1

Strains of *Paramecium bursaria* and other *Paramecium* spp. strains used in molecular studies

	Strain designation	Species	Geographical origin	GenBank
1	IP	*P. bursaria*	Italy, Pisa	GQ869825
2	JT	*P. bursaria*	Japan, origin unknown	GQ869826
3	UK	*P. bursaria*	Ukraine, Krzemieniec	GQ869827
4	PB1	*P. bursaria*	Poland, Biebrza National Park	GQ869828
5	PB2	*P. bursaria*	Poland, Biebrza National Park	GQ869829
6	PB3	*P. bursaria*	Poland, Biebrza National Park	GQ869830
7	FP	*P. bursaria*	France, Paris	GQ869831
8	GG	*P. bursaria*	Germany, Göttingen	GQ869832
9	AW	*P. bursaria*	Austria, Wieldbiche	GQ869833
10	APS	*P. bursaria*	Austria, Pitburger See	GQ869834
11	P. t	*P. tetraurelia*	Australia, Sydney	GQ869835
12	P. m	*P. multimicronucleatum*	USA, Louisiana	GQ869837
13	P. c	*P. caudatum*	Cyprus, Akamas	GQ869836

Table 2

Primers used in this study

Analysis	Primer	Sequence 5'-3'	References
RAPD	Ro-360-04	5'-CCCTCATCAC-3'	FOISSNER *et al.* 2001
RAPD	Ro-460-01	5'-TGCGCGATCG-3'	STOECK & SCHMIDT 1998
RAPD	Ro-460-02	5'-GCAGGATACG-3'	STOECK & SCHMIDT 1998
RAPD	Ro-460-03	5'-CTGCGATACC-3'	STOECK & SCHMIDT 1998
RAPD	Ro-460-04	5'-GCAGAGAAGG-3'	STOECK & SCHMIDT 1998
RAPD	Ro-460-05	5'-CTAGCTCTGG-3'	STOECK & SCHMIDT 1998
RAPD	Ro-460-06	5'-GTAGCCATGG-3'	STOECK & SCHMIDT 1998
RAPD	Ro-460-07	5'-AACGTACGCG-3'	STOECK & SCHMIDT 1998
RAPD	Ro-460-08	5'-CGATGAGCCC-3'	STOECK & SCHMIDT 1998
RAPD	Ro-460-10	5'-CTAGGTCTGC-3'	STOECK & SCHMIDT 1998
ARDRA	82F	5'-GAAACTGCGAATGGCTC-3'	ELWOOD *et al.* 1985
ARDRA	LR6R	5'-CGCCAGTTCTGCTTACC-3'	universal eukaryotic primer*
Sequencing	ITS1	5'-TCCGTAGGTGAACCTGCGG-3'	universal eukaryotic primer*
Sequencing	ITS4	5'-TCCTCCGCTTATTGATATGC-3'	universal eukaryotic primer*

*http://www.biology.duke.edu/fungi/mycolab/primers.htm

ARDRA analysis

For ARDRA analysis, PCR amplification of genomic DNA was performed using the forward primer, an internal ciliate specific sequencing primer (82F) from the 5' end of the SSU rRNA gene (ELWOOD *et al*. 1985) and the reverse primer (LR6R). Each amplification reaction was carried out in 100μl of reaction mixture containing 2μl DNA template, 1xQiagen PCR buffer, 1xQ solution, 200μM dNTP mix, 0.4 μM of each primer, and 2.5U of Taq DNA polymerase (Qiagen). The PCR reaction was carried out as in PRZYBOŚ *et al.* (2007a). The PCR product was purified using NucleoSpin Extract II (Macherey-Nagel, Germany) (Table 2). Restriction digestion was performed directly on the purified PCR products. The following restriction enzymes were used: *Dra*I, *Eco*RV, *Hha*I, *Hind*III, *Msp*I, *Pst*I (Promega).

Digestion reactions were carried out separately for each enzyme at 37°C for 1.5h. The final volume of the reaction mixture was 20μl and contained: 10μl of PCR product, 5U of restriction enzyme, 1x reaction buffer and 0.1ug/μl of acetylated BSA. Digested PCR products were run on 1.5% agarose gels for 1.5h at 85V.

Analysis of the ribosomal DNA fragment

The primers used for PCR amplification of the 3' end of SSU rDNA gene ITS1, 5.8S gene and ITS2 (550bp), are listed in Table 2. Both are universal eukaryotic primers for amplification of rDNA fragments. PCR amplification was carried out in a final volume of 40 μl containing: 4μl of DNA, 1.5 U Taq-Polymerase (Qiagen, Germany), 0.6μl 10mM of each primer, 10x PCR buffer, 0.6μl of 10mM dNTPs in a T-personal thermocycler ™ (Biometra GmbH, Germany). The amplification protocol consisted of initial denaturation at 94°C, followed by 34 cycles of denaturation at 94°C for 45s, annealing at 50°C for 60s, and extension at 72°C for 60s, with final extension at 72° for 5 min. After amplification, the PCR products were electrophoresed in 1% agarose gels for 45 min at 85V with a DNA molecular weight marker (XIV Roche, France). For purification, 30 μl of each PCR product was separated on a 1.8 % agarose gel (100V/60min). Then, the band representing the examined fragment was cut out and purified using the NucleoSpin Extract II (Macherey-Nagel, Germany). Cycle sequencing was done in both directions using the BigDye Terminator v3.1™ chemistry (Applied Biosystems, USA). Sequencing products were precipitated using Ex Terminator (A&A Biotechnology, Poland) and separated on ABI PRISM 377 DNA Sequencer (Applied Biosystems, USA).

Data analysis

Sequences were examined using Chromas Lite (Technelysium, Australia) to evaluate and correct chromatograms. Alignment and consensus of the studied sequences were performed using Clustal W (THOMPSON *et al*. 1994) in the Bio Edit program (HALL 1999). Trees were constructed for the studied fragments in Mega version 4.1 (TAMURA *et al*. 2007), using the Neighbor-Joining method (NJ) (SAITOU & NEI 1987) and Maximum Parsimony (MP) (NEI & KUMAR 2000) and Bayesian analysis. The NJ analysis was performed using a

KIMURA 2-parameter correction model (KIMURA 1980) and Jukes-Cantor method (JUKES & CANTOR 1969) by bootstrapping with 1000 replicates (FELSENSTEIN 1985). The MP analysis was evaluated with the Min-mini heuristic parameter (level =2) and bootstrapping with 1000 replicates. Bayesian analyses were performed in MrBayes 3.1.2 (RONQUIST & HUELSENBECK 2003). The analysis was run with 5,000,000 generations and trees were sampled every 100 generations. All trees were displayed with Tree View 1.6.6 (PAGE 1996).

Results

RAPD analysis

The band patterns characteristic for the *Paramecium bursaria* strains were dependent on the primer used. Ten random primers were preliminarily used to amplify DNA samples. On the basis of the RAPD pattern analysis (on several gels) four primers (Ro360-04, Ro460-04, Ro460-07, Ro460-08) were used in further analysis. All results of RAPD analysis are shown in Figure 1, and dendrograms are presented in Figure 2.

Amplification with the primer Ro360-04 (Fig.1A) produced characteristic band patterns and an overall comparison distinguished three genotypes among the *P. bursaria* strains. The first one appeared in the strains from Italy (IP) and Japan (JT) (the similarity was about 62%), the second genotype was represented by strains from Poland (PB_1, PB_2, PB_3) and Ukraine (UK) (the similarity was about 45%) and the third genotype was composed by both strains from Austria (AW, APS) (the similarity inside this group was 90%) and the strain from Germany (GG), homology was about 25%. The strain from France (FP) is characterized by different band patterns. On the dendrogram it matches with the first and the second genotype with similarity of about 15% (Fig. 2A).

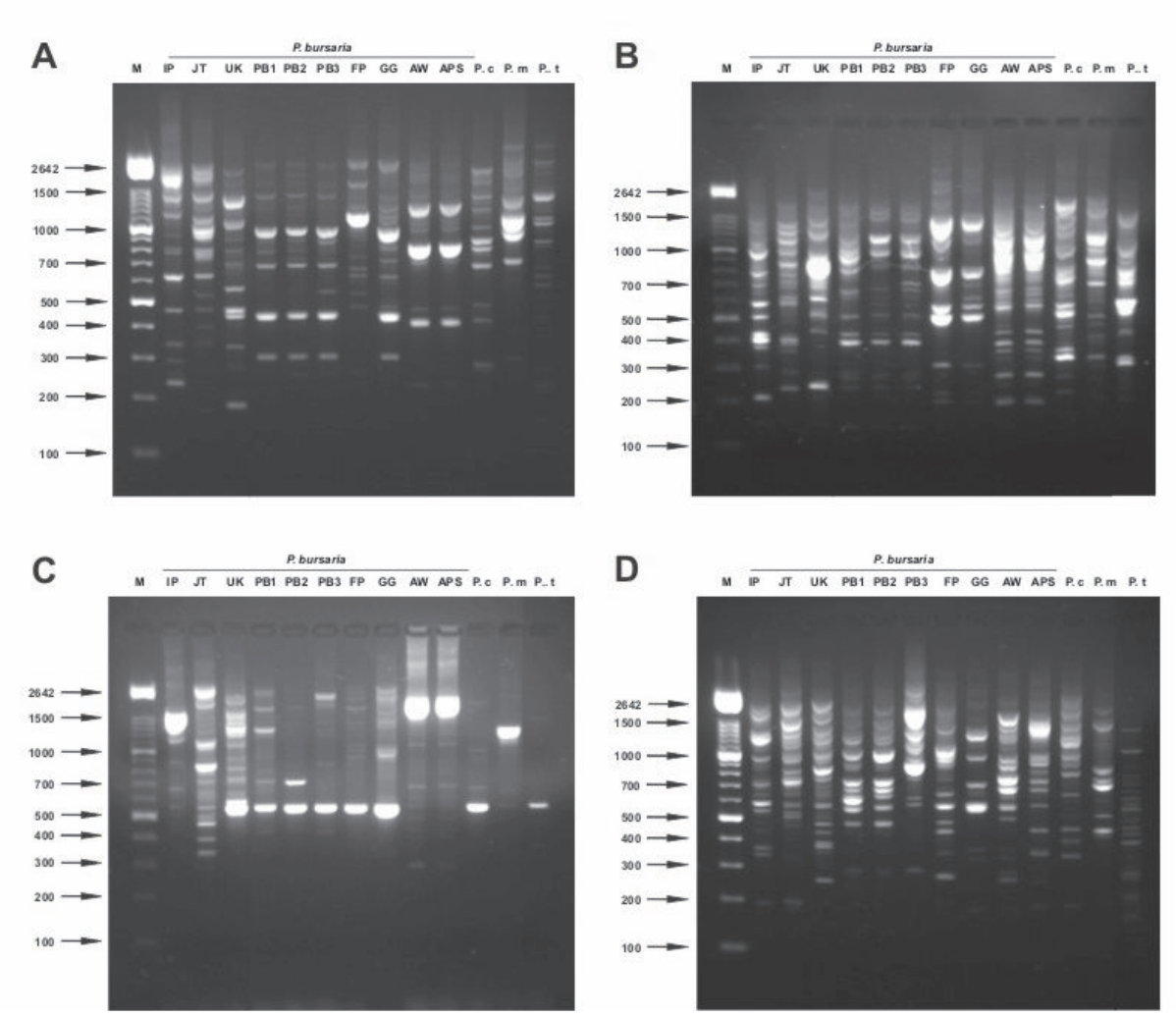

Fig. 1. RAPD fingerprints of species of the *P. bursaria* strains and *P. tetraurelia, P. caudatum, P. multimicronucleatum* strains as outgroups with primers: Ro360-04 (A), Ro460-04 (B), Ro460-07 (C), Ro460-08 (D). M-molecular pGEM marker, molecular weight of the marker DNA bands are given in bp.

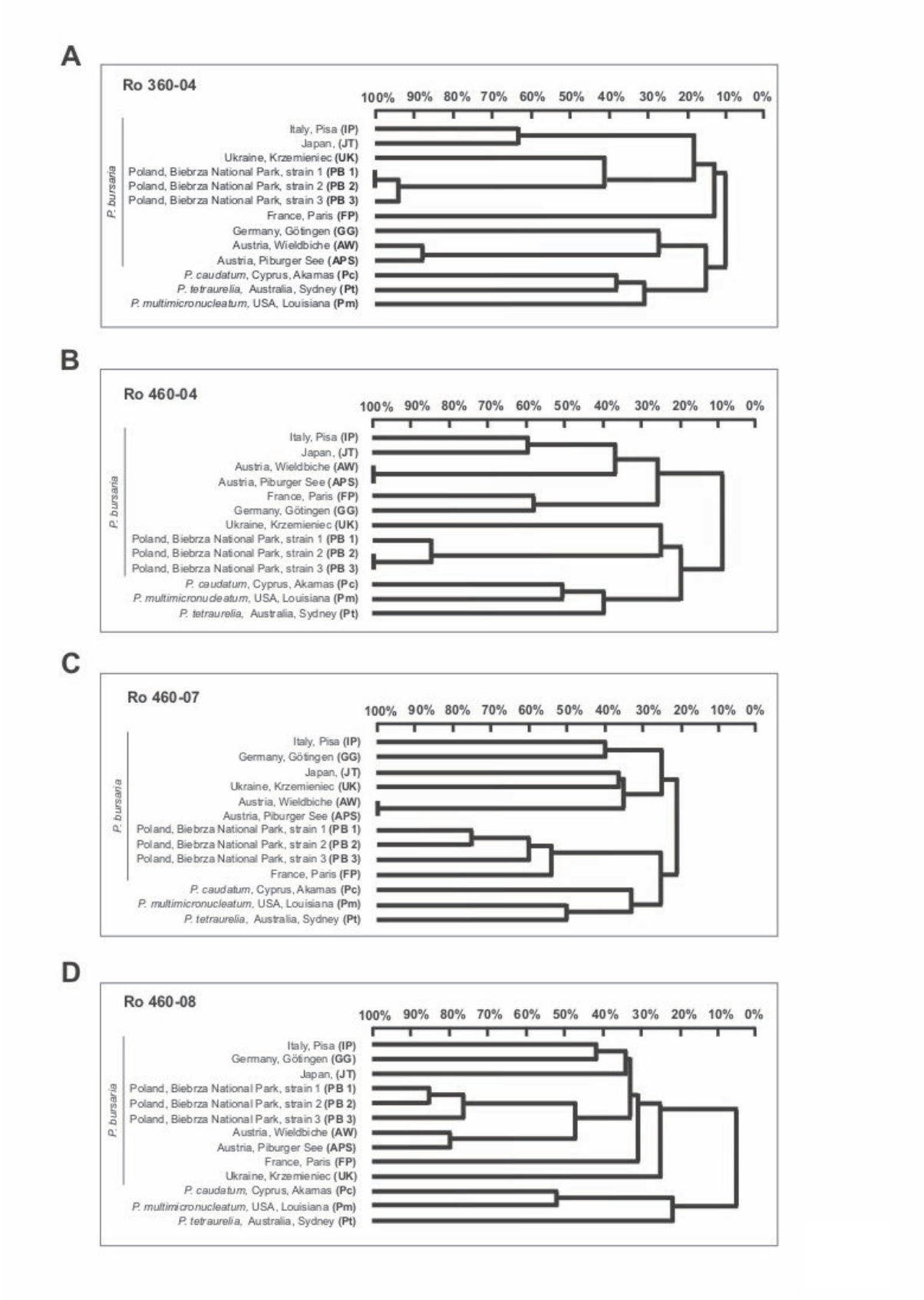

Fig. 2. Intraspecies dendrograms of the *P. bursaria* strains and P. *tetraurelia, P. caudatum*, *P. multimicronucleatum* strains as outgroups, based on RAPD fingerprinting with primers: Ro360-04 (A), Ro460-04 (B), Ro460-07 (C), Ro460-08 (D).

The band patterns revealed by the random primer Ro460-04 (Fig. 1B) divided the strains of *P. bursaria* into three genotypes. The first genotype is similar to the one revealed by primer Ro360-04 and it is constructed by strains that originated from Italy and Japan. Their band patterns showed 60% similarity and were related with strains from Austria with 40% of homology. The second genotype appeared in strains from France and Germany (about 60% similarity). The strains from Poland represented the third genotype, which had a relationship of about 25% with the genotype of the strain from Ukraine. The strains from Germany and France represented specific band patterns which differ from other patterns of *P. bursaria* strains (Fig. 2B).

Therefore two primers, Ro360-04 and Ro460-04, revealed a very low similarity of strains from France, Germany and Ukraine to the remaining studied strains of *P. bursaria*.

The relationship of strains constructed on the basis of the band patterns revealed by the primer Ro460-07 (Fig. 1C) showed three main groups of *P. bursaria* strains. The strains from Italy (IP) and Germany (GG) constituted the first group (with about 40% similarity). The second group of genotypes was composed by strains from Japan (JT) and Ukraine (UK) together with strains from Austria (AW, APS) with about 38% similarity. The strains from Poland (PB_1, PB_2, PB_3) and France (FP) composed the third genotype (similarity 55% to 80%) (Fig. 2C).

The diagram constructed on the basis of the cluster analysis of the fingerprints with the primer Ro460-08 (Fig. 1D) established three groups of strains. The strains from Italy (IP), Germany (GG) and Japan (JT) appeared in the first genotype and showed 42% to 34% similarity. The next genotype was constructed on the basis of band patterns of the strains from Poland (PB_1, PB_2, PB_3) and Austria (AW, APS). Similarity between the two groups of genotypes was 48%. Different genotypes were presented by fingerprints of strains from France (FP) and Ukraine (UK) (Fig. 2D).

Based on band patterns revealed by two primers, Ro460-07 and Ro460-08, very low homology of strains from Ukraine and from Japan to other *P. bursaria* strains was shown.

ARDRA analysis

A fragment of about 3085bp of the SSU-LSU ribosomal RNA gene with internal transcribed spacers (ITS) was amplified and restriction enzymes were used for cleaving DNA of the studied *P. bursaria* strains as well as *P. tetraurelia, P. caudatum, P. multimicronucleatum* used as outgroups. Results of the ARDRA analysis are shown in Figure 3. *Eco*RV produced different restriction patterns in the *P. bursaria* strain from Japan (JT) and in *P. caudatum* (P.c). *Msp*I cleaved DNA at a different site in the strain from Ukraine (UK). The *P. caudatum* strain also differed from other strains. *Dra*I produced a different restriction pattern in the strain of *P. bursaria* from Japan (JT) and differentiated the outgroups with characteristic band patterns for each species. A similar effect was produced by the enzyme *Pst*I, which differentiated the Japanese strain of *P. bursaria* from the other studied strains. *Hha*I did not produce different restriction patterns except for the outgroups, which were different from *P. bursaria. Hind*III revealed polymorphism in the studied strains from Japan (JT) (one additional band of 2600bp) and from Ukraine (UK) (one additional band of 380bp).

The result of the ARDRA analysis suggests that the strain from Japan is the most polymorphic and also that the strain from Ukraine presents different restrictions patterns in comparison to other the studied strains of *P. bursaria*.

Analysis of the ITS1-5.8S-ITS2 fragment

The fragment of rDNA (550bp) containing the 3' end of the SSU rDNA gene, ITS1, 5.8S gene and ITS2 was obtained from ten *P. bursaria* strains and from *P. tetraurelia*, *P. caudatum* and *P. multimicronucleatum* used outgroups.

Among the studied sequences we found 6 genotypes of *P. bursaria* strains and 17 polymorphic sites. Seven of the polymorphic sites were parsimony informative (#63, #91, #290, #293, #461, #472, #549), four of them had deletions of nucleotides (#101, #102, #290, #475) and one had an insertion (#295) in one strain. The same genotype was observed in three strains from Poland and three other strains, two from Austria and one from Japan. The most divergent sequence was obtained from the strain from France, which had seven characteristic substitutions (#102, #104, #108, #288, #294, #295, #493). Polymorphic positions are presented in Table 3.

The distance matrix shows (Table 4) no differences among strains of *P. bursaria* from Poland (PB_1, PB_2, PB_3), and among strains from Austria (AW and APS). The most divergent strain from France (FP) differed from the other strains by 1.6-1.8%. Mean divergence among studied strains of *P. bursaria* was 0.9%. Differentiation between studied strains of *P. bursaria* and other investigated *Paramecium* representatives was 19.9% - 22%. Mean divergence between all studied strains was 11%. Nucleotide frequencies within the studied sequences from *P. bursaria* were: A=34.4, C=16.5, G=16.2, T=32.9.

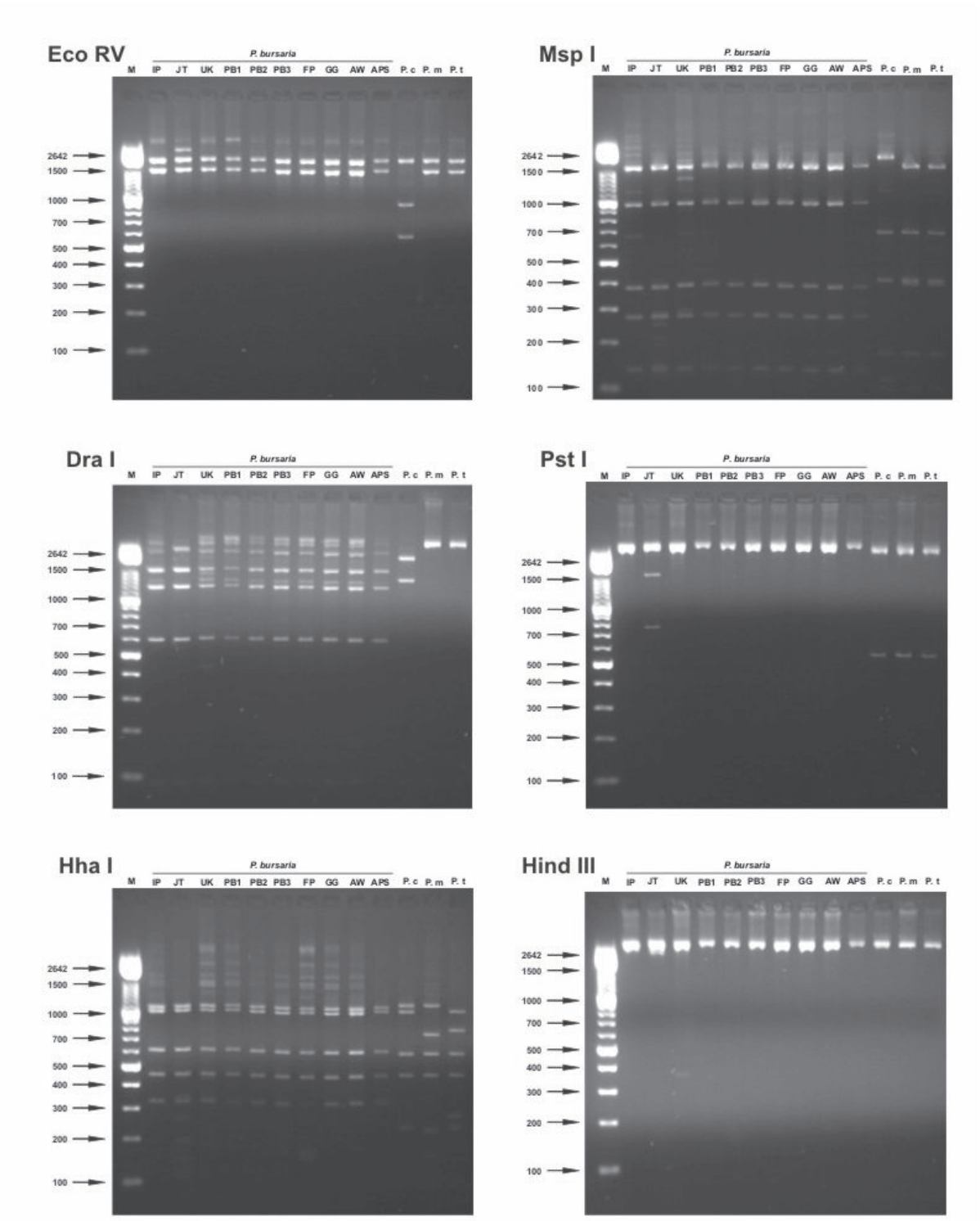

Fig. 3. ARDRA riboprinting patterns (a fragment of about 3085bp of SSU-LSU) after digestion with restriction enzymes *Eco*RV, *Msp*I, *Dra*I, *Pst*I, *Hha*I, *Hind*III of the *P. bursaria strains* and outgroups (*P. tetraurelia, P. caudatum, P. multimicronucleatum*) on an agarose gel. M-molecular pGEM marker, molecular weight of the marker DNA bands are given in bp.

Based on the obtained sequences, trees were constructed using NJ, MP and BI methods (Fig. 4). All three methods gave very similar tree topologies. It was possible to discriminate the studied strains of *P. bursaria* from other species of the genus *Paramecium*. The studied strains were divided into three clades: one group contained strains from Poland, Germany and Ukraine (PB_1, PB_2, PB_3, GG, UK), the second group contained strains from Italy, Austria and Japan (IP, AW, APS, JT) and the

Table 3

Polymorphic sites in the rDNA fragment ITS1-5.8S-ITS2 obtained from ten *P. bursaria* strains

	#63	#91	#101	#102	#104	#108	#288	#290	#293	#294	#295	#361	#461	#472	#475	#493	#549
IP	A	A	A	C	T	T	A	C	G	T	---	C	C	A	---	T	A
JT	.	.	.	.	.	.	.	.	.	.	---	A	.	.	---	.	G
UK	.	.	.	.	.	.	.	---	A	.	---	A	T	T	T	.	.
PB1	T	G	.	.	.	.	.	---	A	.	---	A	T	T	T	.	G
PB2	T	G	.	.	.	.	.	---	A	.	---	A	T	T	T	.	G
PB3	T	G	.	.	.	.	.	---	A	.	---	A	T	T	T	.	G
FP	T	.	.	---	A	C	G	G	A	C	C	A	.	T	T	A	G
GG	T	G	.	.	.	.	.	---	A	.	---	A	T	T	T	.	.
AW	.	.	---	.	.	.	.	.	.	.	---	A	.	.	---	.	G
APS	.	.	---	.	.	.	.	.	.	.	---	A	.	.	---	.	G

Table 4

Distance matrix presenting the number of base substitutions in *Paramecium bursaria* (see Table 1*) P.tetraurelia, P. caudatum* and *P. multimicronucleatum* strains, based on analyses of ITS1-5.8S-ITS2 rDNA sequences. Analyses were conducted using the Jukes-Cantor method (lower-left) and method Kimura 2-parameter (upper-right)

	IP	JT	UK	PB1	PB2	PB3	FP	GG	AW	APS	P. t	P. c	P. m
IP		0.002	0.008	0.014	0.014	0.014	0.018	0.012	0.002	0.002	0.263	0.280	0.260
JT	0.002		0.010	0.012	0.012	0.012	0.016	0.014	0.000	0.000	0.260	0.277	0.257
UK	0.008	0.010		0.006	0.006	0.006	0.016	0.004	0.010	0.010	0.268	0.286	0.257
PB1	0.014	0.012	0.006		0.000	0.000	0.014	0.002	0.012	0.012	0.264	0.274	0.253
PB2	0.014	0.012	0.006	0.000		0.000	0.014	0.002	0.012	0.012	0.264	0.274	0.253
PB3	0.014	0.012	0.006	0.000	0.000		0.014	0.002	0.012	0.012	0.264	0.274	0.253
FP	0.018	0.016	0.016	0.014	0.014	0.014		0.016	0.016	0.016	0.250	0.267	0.247
GG	0.012	0.014	0.004	0.002	0.002	0.002	0.016		0.014	0.014	0.267	0.278	0.256
AW	0.002	0.000	0.010	0.012	0.012	0.012	0.016	0.014		0.000	0.260	0.277	0.257
APS	0.002	0.000	0.010	0.012	0.012	0.012	0.016	0.014	0.000		0.260	0.277	0.257
P. t	0.207	0.205	0.210	0.207	0.207	0.207	0.199	0.210	0.205	0.205		0.058	0.075
P. c	0.218	0.215	0.220	0.212	0.212	0.212	0.210	0.215	0.215	0.215	0.055		0.085
P. m	0.208	0.205	0.205	0.202	0.202	0.202	0.200	0.205	0.205	0.205	0.070	0.079	

third contained the strain from France (FP). A correlation between place of origin and sequence pattern was observed in the strains from Poland and Austria. However, the strain from Japan had an identical genotype to the strains from Austria and similar to the strain from Italy. Slight differences were seen among strains from Poland, Germany, Ukraine in the first clade and Italy, Japan and Austria in the second clade.

Discussion

The relationships among the species of ciliates is very complicated because representatives of many genera show incredible species diversity revealed by classical genetic studies and different molecular techniques. Studies based on DNA fragment analysis (RAPD-fingerprinting, ARDRA-riboprinting) as well as comparison of gene sequences (rRNA) in protozoa were summarized by SCHLEGEL & MEISTERFELD (2003). For instance, RAPD was applied in *Diophrys* sp. (CHEN & SONG 2002), *Uronychia* sp. (CHEN *et al.* 2003), *Gonostomium affine* (FOISSNER *et al.* 2001) and in *Paramecium*: *Paramecium jenningsi* (PRZYBOŚ *et al*. 2003b), *Paramecium aurelia* complex (STOECK *et al.* 1998, 2000a; PRZYBOŚ *et al*. 2003a), *Paramecium caudatum* (STOECK *et al.* 2000b). *Paramecium bursaria* seems worthy of studies of molecular diversity as the species is unique among other species of *Paramecium,* being regarded as the first species that diverged within the genus (STRŰDER-KYPKE *et al.* 2000).

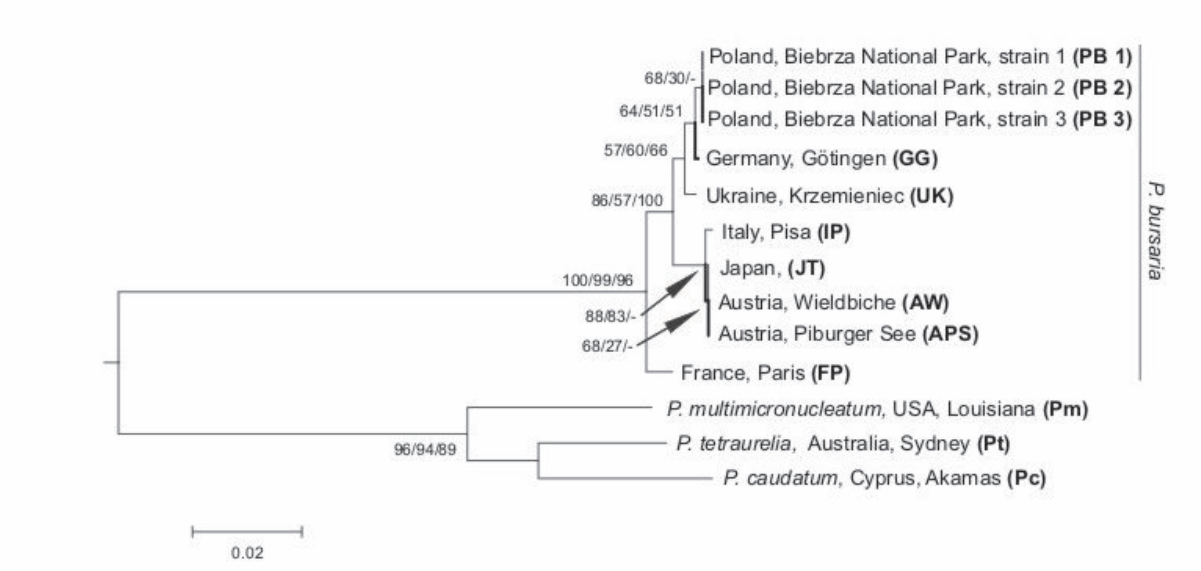

Fig. 4. Phylogenetic tree constructed for 10 strains of *P. bursaria* and *P. tetraurelia, P. caudatum*, *P. multimicronucleatum* strains as outgroups, based on a comparison of sequences from ITS1-5.8S-ITS2 rDNA fragment using the NJ (neighbor joining) method (with the application of Maximum Composite Likelihood), MP (Maximum Parsimony) analysis and Bayesian Interference (BI). Bootstrap values are presented as percentages (NJ/MP/BI) for 1000 replicates. All positions containing gaps and missing data were eliminated from the dataset. There were a total of 509 positions in the final dataset. Phylogenetic analyses were conducted in MEGA 4.1.

Our results of RAPD analysis showed that strains of *Paramecium bursaria* harbour intraspecific polymorphism. The strains from Japan, Ukraine and France could be characterized by the most discriminate band patterns. Dendrograms constructed on the basis of RAPD-PCR analysis distinguished some groups of genotypes (Fig. 1). The strains from Austria and the strains from Poland represented very similar genotypes (similarity within these groups was about 80% to 100%).

RAPD-bands are subject to different interpretations because the intensity of the bands varies in parallel probes. Sometimes, it is difficult to interpret band patterns (STOECK *et al*. 2000a) and RAPD data on their own are not sufficient to determine the relationships between strains. STOECK *et al.* (2000b) applied two molecular methods: RAPD-fingerprinting and ARDRA-riboprinting to try to confirm the hypothesis of sibling species in *Paramecium caudatum* and in fact they revealed their abscence.

The method of ARDRA riboprinting (using a highly conserved rDNA fragment about 3085bp) with the application of restriction enzymes *Eco*RV, *Dra*I and *Pst*I distinguished the strain from Japan with different band patterns. The enzyme *Msp*I produced quite different band patterns in DNA of the strain from Ukraine. Results of ARDRA – riboprinting analysis confirmed the great polymorphism of DNA in *Paramecium bursaria* strains. Both methods (RAPD and ARDRA) confirmed significant genetic distance between strains from Japan and Ukraine to the other studied strains of *P. bursaria*.

Polymorphism in *P. bursaria* is higher than in *Paramecium aurelia* complex (PRZYBOŚ *et al*. 2007a,b) in which some enzymes, e.g. *Dra* I and *Pst* I, did not cleave the used fragment of rDNA, but *Eco* RV did not produce different restriction patterns in the studied species. These differences could be connected with the length of the studied rDNA fragments (previously 2400bp and in present studies 3085bp).

ARDRA analysis can be a useful tool for the rapid identification and assessment of relatedness among species. Different band patterns of strains originating from different geographical regions may be connected with the type of breeding system characteristic for the species. *Paramecium bursaria* is an extreme outbreeder which is reflected in the high level of polymorphism.

RAPD-fingerprinting and ARDRA-riboprinting revealed the existence of groups of species within the *P. aurelia* complex (PRZYBOŚ *et al*. 2007a). Molecular techniques applied in the *P. aurelia* complex (PRZYBOŚ *et al*. 2006), *Euplotes* sp. (KUSCH & HECKMAN 1996), and *Gonostomium* sp. (FOISSNER 2001) revealed within the studied ciliates several distinct genotypes independent of geographical origin. Certain genotypes of *P. jenningsi* (PRZYBOŚ *et al.* 2003b), *P. quadecaurelia* (PRZYBOŚ *et al*. 2003a) and *P. schewiakoffi* (FOKIN *et al*. 2004) are restricted to particular geographical region. Genotypes of *P. bursaria* (from

Austria and Poland) seem to be correlated with geographical origin.

For a comparison of our results, ITS sequences of ten strains representing two syngens of *P. bursaria* originating from Japan, China, Australia, Germany and UK, deposited in GenBank (HOSHINA *et al.* 2006), were used. The results of HOSHINA *et al*. (2006) have shown that the mean variability was at the level of 0.7%. The molecular analysis carried out at present showed similar differentiation (0.9%) among the studied *P. bursaria* strains.

The sequenced short fragment (550bp) of the *P. bursaria* genome also presents greater intraspecific differentiation than in the *P. aurelia* species complex (COLEMAN 2005) in which the mean genetic distance was at a level of 0.6%. In *P. caudatum* no genetic differentiation (BARTH *et al*. 2006) was observed. Mean sequence divergence between the studied strains of *P. bursaria* was lower than in *P. multimicronucleatum* (2%) (BARTH *et al*. 2006).

There were no differences between ITS sequences (550bp) obtained for the strain from Japan and strains from Austria, whereas ARDRA analysis of the 3085bp fragment showed that the strain from Japan presented different band patterns. Therefore, further studies should emphasize the fragment which was used in the ARDRA analysis and contained the variable LSU rDNA region. This variable rDNA fragment was used to show the large intraspecific differentiation in the *Paramecium aurelia* complex (TARCZ *et al*. 2006, PRZYBOŚ *et al*. 2007a; TARCZ 2009).

In the present work we used *P. bursaria* strains collected in the Botanical Gardens in Pisa (Italy) and Paris (France), so the different genotype (ITS sequence analysis) for the strain from France could correspond to the collection place. The same situation occurred (RAPD analysis) in the case of the Italian strain. There is greater probability that such strains could be transported with tropical plants. The problem of the presence of ciliate species in ponds of Botanical Gardens was studied previously by KOMALA and PRZYBOŚ (2001). In this paper they described a strain of *P. tetraurelia*, which could have been transferred to the Cracow Botanical Garden with aquatic plants.

The studies on intraspecific variability in *Paramecium bursaria* will continue in the future using more strains originating from other parts of the world, i.e. Australia and more from Japan. The results obtained at present have only a preliminary character and were planned to check the sensitivity of the applied methods and level (if any) of intraspecific diversity of *P. bursaria* strains.

Acknowledgements

The authors are grateful for *Paramecium bursaria* samples from Prof. S. I. FOKIN, Biological Research Institute, St. Petersburg State University, Russia (strains from France and Italy), Dr. A. POTEKHIN, Faculty of Biology and Soil Science, St. Petersburg State University, Russia (strain from Japan), Dr. B. SONNTAG, Laboratory of Aquatic Photobiology and Plankton Ecology, Institute of Ecology, University of Innsbruck, Austria (strains from Austria), Dr. M. LORENZ, Culture Collection of Algae (SAG), University of Gottingen, Germany (strain from Germany).

The authors are also grateful Ms. Marta SURMACZ for excellent technical assistance.

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