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Molecular Studies on Intraspecific Differentiation of *Paramecium dodecaurelia*, with Description of New Stands of the Species (Protozoa, Ciliophora)

Ewa PRZYBOŚ, Sebastian TARCZ, Magdalena GRECZEK-STACHURA, Marta SURMACZ, Alexey POTEKHIN and Maria RAUTIAN

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New stands of *Paramecium dodecaurelia* were found in Russia, Ukraine, Kazahstan, Poland, and Tenerife. Molecular studies (RAPD and sequencing of LSU rRNA and COI mtDNA gene fragments) of *P. dodecaurelia* strains depicted intraspecific polymorphism within the species, revealed as distinct differences between strains from the USA in comparison with strains from different regions of the Palearctic.

Key words: *Paramecium aurelia* species complex, *P. dodecaurelia*, intraspecific differentiation, geographical distribution, breeding system, RAPD-PCR fingerprinting, sequencing of LSU rRNA and COI mtDNA fragments.

Ewa PRZYBOŚ, Sebastian TARCZ, Marta SURMACZ, Department of Experimental Zoology, Institute of Systematics and Evolution of Animals, Polish Academy of Sciences, Sławkowska 17, 31-016 Kraków, Poland.
E-mail: przybos@isez.pan.krakow.pl
starcz@isez.pan.krakow.pl
Magdalena GRECZEK-STACHURA, Institute of Biology, Educational Academy, Podbrzezie 3, 31-054 Kraków, Poland.
E-mail: magresta@onet.pl
Alexey POTEKHIN, Maria RAUTIAN, Biological Research Institute, St. Petersburg State University, Old Peterhof, Oranienbaumskoye sch. 2, 198904 St. Petersburg, Russia.
E-mail: aurelia@fromru.com
mrautian@mail.ru

The genus *Paramecium* is a model organism in studies concerning speciation in Protozoa and generally in Eukaryota as several morphospecies are also subdivided into genetic species or syngens.

The *P. aurelia* complex is composed of 15 sibling species (SONNEBORN 1975; AUFDERHEIDE *et al.* 1983). Sibling species of the *P. aurelia* complex probably originated as a result of an explosion of speciation events which coincided with the most recent whole-genome duplication in paramecia as indicated by phylogenetic analysis (AURY *et al.* 2006).

Sibling species are morphologically similar but with isolated gene pools. They differ in temperature preferences, distribution, life cycle features and system of mating type inheritance. They are generally characterized by inbreeding occuring in various degrees in different species (SONNEBORN 1957; LANDIS 1986), however, they also show intraspecific differentiation. Different strain genotypes within species can be studied by genetic methods, both classical (strain crosses) and molecular (cf PRZYBOŚ *et al.* 2007a). At present, several different types of molecular analyses are used, e.g. PCR analyses (cf PRZYBOŚ *et al*. 2007a) as RAPD (random amplified polymorphic DNA), ARDRA (amplified ribosomal DNA restriction analysis), RFLP (restriction fragment length polymorphism) and sequencing of nuclear genes fragments such as histone *H4* (BERNHARD & SCHLEGEL 1998; PRZYBOŚ *et al*. 2006 b; MACIEJEWSKA 2007), *Hsp70* (PRZYBOŚ *et al*. 2003b; HORI *et al*. 2006) or rDNA (NANNEY *et al*. 1998; STRÜDER-KYPKE *et al*. 2000 a,b; COLEMAN 2005; TARCZ *et al*. 2006; PRZYBOŚ *et al*. 2007 b) as well as a fragment of the mitochondrial gene encoding cytochrome oxidase (COI mtDNA) (BARTH *et al.* 2006; PRZYBOŚ *et al*. 2007b). Unknown paramecia are identified as particular species of the *P. aurelia* complex by mating reaction (conjugation)

with the standard strains of the species (SONNEBORN 1975), but it is also possible to identify and characterize species by molecular data, e.g. by their band patterns obtained by RAPD analysis (STOECK & SCHMIDT 1998; STOECK *et al*. 1998, 2000). This method was also applied in the other species of *Paramecium* (cf PRZYBOŚ *et al*. 2007a). RAPD analysis has shown that the majority of species of the *P. aurelia* complex are differentiated intraspecifically, e.g. *P. primaurelia*, *P. biaurelia*, *P. triaurelia, P. tetraurelia*, *P. sexaurelia, P. novaurelia* and *P. dodecaurelia,* other species show a high similarity of genotypes (band patterns) such as *P. pentaurelia, P. decaurelia, P. tredecaurelia* and *P. quadecaurelia* (STOECK *et al*. 1998, 2000; PRZYBOŚ *et al*. 2005a, b; 2006 a; 2007b). Exceptional polymorphism was found in *P. octaurelia* and especially in *P. dodecaurelia*. The strains of the latter species (originating from the USA, Hawaii, Japan, Germany, and Elbe Island in Italy) belong to separate clusters in a dendrogram constructed on the basis of RAPD fingerprints of the strains representing all genotypes within the studied species (PRZYBOŚ *et al*. 2007a).

Other molecular methods (RFLP and ARDRA) also show the existence of groups of species within the *P. aurelia* complex, one group including species without polymorphism and the other with polymorphic species (PRZYBOŚ & TARCZ 2005; PRZYBOŚ *et al*. 2007a). High polymorphism in *P. dodecaurelia* may be connected with the characteristic degree of inbreeding in this species.

P. dodecaurelia (represented by strain 246 from the USA) also appeared as a clearly distinct branch from the clusters of other species of the *P. aurelia* complex, as shown by sequencing of the histone *H4* gene fragment (PRZYBOŚ *et al*. 2006b; MACIEJEWSKA 2007). The problem of high intraspecific polymorphism of *P. dodecaurelia* was later also studied by sequencing of rDNA fragments (3'SSU rRNA – ITS1 - 210bp long and 5' LSU rRNA -350bp long) in strains originating from remote collecting sites (TARCZ *et al*. 2006) such as strains from Europe (Germany; Elbe Island and Trento Italy), Japan, Hawaii, and USA (strain number 246). Alignment of both rDNA fragments revealed distinct polymorphism within the species, some strains of *P. dodecaurelia* differed as much as different species of the *P. aurelia* complex. The studies of macronuclear genomes of species of the *P. aurelia* complex by pulsed-field gel electrophoresis (PFGE) also showed exceptional features of *P. dodecaurelia*, as this species is characterized by the greatest polymorphism of PFGE profiles, while most other species demonstrated the same electrokaryotype for all analyzed strains (POTEKHIN *et al*. 2005; NEKRASOVA & POTEKHIN, unpublished data).

Recently, eight new strains of *P. dodecaurelia* were identified in several stands in Russia (European Russia : Vologda, Yaroslavl, Myshkin, Asian Russia: Ułan Ude, Selenga River), in Vinnitsa, Ukraine, Kazakhstan, Poland (Kraków), and Tenerife of the Canary Islands. In this paper we investigate the level molecular of intraspecific differentiation of the new strains originating from geographically very distant sites (continents) and compare it with the previously studied strains of the species from the North America (strain 246 from the USA) and from Europe (strain from Elbe Island, Italy). We present the genetic makeup of *P. dodecaurelia* strains, as the results of crosses and molecular analyses (RAPD and sequences of fragments of LSU rDNA and mitochondrial gene encoding for cytochrome oxidase COI).

Material and Methods

Material

The strains (Table 1) designated RV, RY, and RM were collected by M. RAUTIAN in the surroundings of Vologda, Yaroslavl, and Myshkin in the European part of Russia in 2004 and 2003; the RS strain was collected by A. NADACHOWSKI in Ulan Ude, Eastern Siberia, Asian part of Russia in 2006; the strain UV was collected by V. YAKOVLEV in Vinnitsa, Ukraine,1991; the TE strain was collected by E. PRZYBOŚ in Loro Park, Puerto de la Cruz, Tenerife, Canary Is. in 2006; the KA strain was collected by A. O. SMUROV in Sarbas, Aral region, Kazakhstan in 2003; strain PK was collected by M. SURMACZ in Jordan's Park, Kraków, Poland in 2006.

Methods

1. Culturing and identification of paramecia

Species of the *P. aurelia* complex were cultured and identified according to the methods of SONNEBORN (1970). Paramecia were cultivated on a lettuce medium inoculated with *Enterobacter aerogenes.* Clones mature for conjugation were mated with the reactive mating types of standard strains of known species. The following standard strains were used: strain 90 of *P. primaurelia*; strain Rieff, Scotland of *P. biurelia*; strain 324 of P. *triaurelia*, strain 87 of *P. pentaurelia;* strain 159 of *P. sexaurelia*, strain 38 of *P. septaurelia*, strain 138 of *P. octaurelia*, strain 510 of *P. novaurelia,* strain 223 of *P. decaurelia*, strain 219 of P. *undecaurelia*, strain 246 of *P. dodecaurelia*, strains 209 and 321 of *P. tredecaurelia*, and strain 328 of *P.*

Table 1

New strains (RV, RY, RS, RM, UV, PK, KA, TE) of *Paramecium dodecaurelia*, and other *P. dodecaurelia*, *P. pentaurelia* and *P. caudatum* strains used in molecular analyses

Species	Strain designation	Geographical origin	Reference	Accesion number	
				5'LSU rDNA	COI
P. dodecaurelia	RV	Russia, Vologda region	This paper	EU086123	EU086114
P. dodecaurelia	RY	Russia, Yaroslavl region	This paper	EU086124	EU086115
P. dodecaurelia	RS	Russia, Ulan Ude, Selenga River	This paper	EU086122	EU086113
P. dodecaurelia	RM	Russia, Myshkin	This paper	EU086121	EU086112
P. dodecaurelia	UV	Ukraine, Vinnitsa	This paper	EU086126	EU086117
P. dodecaurelia	TE	Canary Is., Tenerife, Puerto de la Cruz	This paper	EU086125	EU086116
P. dodecaurelia	KA	Kazakhstan, Aral region, Sarbas	This paper	EU086119	EU086110
P. dodecaurelia	PK	Poland, Kraków, Jordan's Park	This paper	EU086120	EU086111
P. dodecaurelia	246	USA, southern state	Sonneborn, 1974	DQ207369	EU086108
P. dodecaurelia	IE	Italy, Elbe Island	Przyboś & Fokin 2003	DQ207372	EU086109
P. pentaurelia	87	USA, Pennsylvania	Sonneborn 1974	EU086127	EU086118
P. caudatum	Pc	Cyprus, Akamas	Przyboś, unpublished	DQ207375	DQ837977

quadecaurelia. The studied strains were identified on the basis of strong conjugation with the standard strain of the particular species.

2. Strain crosses

In the intra- and inter-strain crosses, the F_1 generation was obtained by conjugation and the F_2 generation by autogamy (using the method of daily isolation lines). The occurrence of the desired stage of autogamy (specimens at the stage of two macronuclear anlagen) was examined on preparations stained with aceto-carmine. Survival of clones in both generations was estimated as percentages. According to CHEN (1956), clones can be considered as surviving after passing 6-7 fissions during 72 hours after separation of partners of conjugation or postautogamous caryonids. The methods were described in detail in PRZYBOŚ (1975).

3. Methods used in molecular studies

a. Amplification of ribosomal DNA (rDNA)

The primers used for PCR reactions are listed in Table 2. They amplified the 5' end of a fragment of LSU rDNA (450bp). The forward primer was constructed using Oligoanalyzer 3.0 (http://scitools.idtdna.com/analyzer/). The reverse primer – LR6 is the universal eukaryotic primer (http://www.biology.duke.edu/fungi/mycolab/primers.htm). PCR amplification was carried out in a final volume of 30 l containing: 2l of DNA, 1.5 U Taq-Polymerase (Qiagen™, Germany), 0.6l 10mM of each primer, 10x PCR buffer, 0.6l 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

Table 2

Primers used in this study

Amplified region	Primer	Sequence 5'-3'	References
5'LSU rDNA	LSU_F	5'-CCCGTATTTGGTTAGGACT-3'	TARCZ *et al*. 2006
	LR6	5'-CGCCAGTTCTGCTTACC-3'	universal eukaryotic primer*
CO I	CoxL11058	5'-TGATTAGACTAGAGATGGC-3'	BARTH *et al*. 2006
	CoxH10176	5'-GAAGTTTGTCAGTGTCTATCC-3'	BARTH *et al*. 2006

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

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 (VI ™ Roche, France).

b. Amplification of a fragment of the mitochodrial cytochrome oxidase gene (COI)

To amplify the CO I region (880bp) of mitochondrial DNA, Cox_L and Cox_H primers were used (according to BARTH *et al*. 2006). PCR amplification was carried in the same volume as in the case of rDNA region (see above), and the protocol followed BARTH *et al*. (2006). After amplification, the PCR products were electrophoresed in 1% agarose gels for 45 min at 85V with a DNA molecular weight marker (VI ™ Roche, France).

c. Sequencing

30 l 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 transferred into an 1.5ml Eppendorf tube. Purification was performed according to the Qiaquick Gel Extraction Kit ™ protocol (Qiagen). Cycle sequencing was done in both directions using the BigDye Terminator v3.1™ chemistry (Applied Biosystems, USA). Sequenced products were precipitated using sodium acetate/ethanol and separated on an ABI PRISM 377 DNA Sequencer™ (Applied Biosystems, USA).

d. Data analysis

Sequences were examined using Chromas Pro (Technelysium™, Australia). Alignment and consensus of the studied sequences were performed using ClustalW (THOMPHSON *et al*. 1994) in the BioEdit program (HALL 1999). Phylogenetic trees were constructed for the studied fragments in Mega version 3.1 (KUMAR *et al*. 2004), using NJ (Neighbor-joining method) (SAITOU & NEI 1987), MP (Maximum Parsimony) and Bayesian Interference (BI) analysis. The NJ analysis was performed using the 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 Min-mini heuristic parameter (level =2) and bootstrapping with 1000 replicates. Markov Chain Monte Carlo (MCMC) analyses, were performed in MrBayes 3.1.2 (RONQUIST & HUELSENBECK 2003). The analysis was run for 5,000,000 generations and trees were sampled every 100 generations. All trees were depicted with TreeView 1.6.6 (PAGE 1996).

RAPD - PCR fingerprint method was generally performed in accordance with STOECK & SCHMIDT (1998), details are described in PRZYBOŚ *et al*. (2003a). RAPD-PCR was performed with a 10mer random primer Ro-460 04 (Roth, Karsruhe, Germany), with the nucleotide sequence: 5'-GCAGAGAAGG- 3', using Taq polymerase (Qiagen). This primer was selected by STOECK & SCHMIDT (1998) after testing several dozen oligonucleotide primers because it gave a "robust band patterns" in the *P. aurelia* species complex. It was also used in other studies carried out on the *P. aurelia* species complex: *P. jenningsi*, *P. schewiakoffi* (cf PRZYBOŚ *et al.* 2007a). RAPD-PCR was done in a Biometra thermocycler, products of PCR reactions were separated by electrophoresis in 1% agarose gels for 1.5 h at 85V together with a molecular weight marker pGEM ™ DNA(Promega, Madison, USA), then stained with ethidium bromide and visualized in UV light. The images were stored in computer memory using the Scion Image™ program (Scion Corporation™, USA). Three repetitions of the PCR reaction were performed in order to assess the reproducibility of the data. Analysis of similarity was carried out by comparing the molecular mass of DNA band patterns obtained by the RAPD method (the Bio1D++ ^{TM} 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.

Results

Strain identification based on mating reaction

The strains from Russia, Ukraine, Kazahstan, Poland, and Tenerife (Table 1) were identified as *P. dodecaurelia* on the basis of strong conjugation with the standard strain of this species. A high percentage of surviving clones was observed in F_1 and F_2 generations of all inter-strain crosses of the strains (Table 3), in spite of strain origin from distant locations.

Strain characteristics based on preparations

Paramecia from all strains were observed on slides stained using Giemsa's stain, after fixation and hydrolysis (cf PRZYBOŚ 1978), vegetative cells/individuals as well as autogamous individuals were used. All strains have a nuclear apparatus characteristic for species of the *P. aurelia* complex with two vesicular micronuclei, and two macronuclear anlagen in autogamous individuals (VIVIER 1974).

Table 3

Mean percentage of surviving hybrid clones in crosses of the *Paramecium dodecaurelia* strains

Species	F1 (by conjugation)	F2 (by autogamy)
RV x RS, Russia,Vologda x Russia, Selenga	100	96
RS x TE, Russia, Selenga x Tenerife	96	92
TE x KA, Tenerife x Kazahstan	88	84
PK x KA, Poland, Kraków x Kazahstan	94	76
PK x RV, Poland, Kraków x Russia, Vologda	100	94
RV x IE, Russia, Vologda x Italy, Elbe Island	96	82

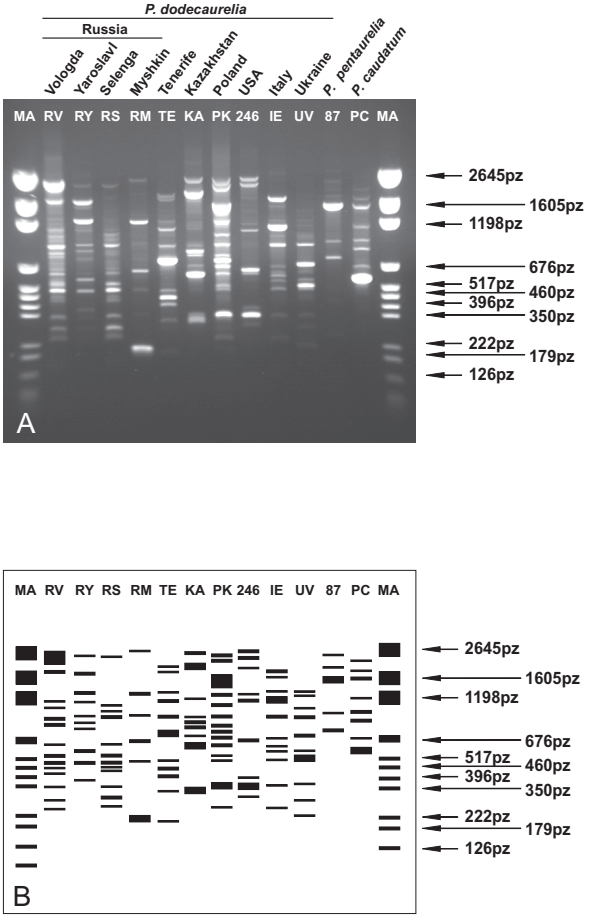

Fig. 1A. RAPD fingerprints (revealed by primer Ro 460-06) of *P. dodecaurelia* strains: RV – Russia, Vologda; RY – Russia, Yaroslavl; RS – Russia, Selenga River; RM – Russia, Myshkin; TE – Tenerife; KA – Kazakhstan; PK – Poland, Kraków; 246 – USA IE – Italy, Elbe Island; UV – Ukraine, Vinnitsa; 87– *P. pentaurelia* (strain 87); PC – *P. caudatum* strain from Akamas, Cyprus. MA – molecular weight marker. Fig. 1B. Schematic representation of Fig.1A showing specific band patterns representing genotypes revealed by RAPD-fingerprints.

RAPD-PCR analysis

Fingerprints (band patterns) of the studied *P. dodecaurelia* strains, one strain of *P. pentaurelia* and one of *P. caudatum*, revealed by DNA amplification with primer Ro 460-04, are presented in Figs 1A,B. The first strain is representative of species of the *P. aurelia* showing no intraspecific differences, and *P. caudatum* is representative of different morphospecies of the genus *Paramecium*. A dendrogram (Fig. 2) presents the relationships of strains within *P. dodecaurelia*. Band patterns of strains from Russia RV, RS, RY are similar in about 50%, and strain 246 from the USA belongs to the same cluster. The second cluster is composed of strains from Myshkin, Russia and Tenerife as well as strains from Kazahstan, Poland, Italy (Elbe Island), and Ukraine. Similarity of the studied *P. dodecaurelia* strains presented by fingerprints ranges from 10 to 50%, showing intraspecific variation. Strains of *P. pentaurelia* and *P. caudatum* were used as outgroups.

Analysis of rDNA fragments

Analysis of the sequenced gene fragment of the large ribosomal DNA subunit (5' LSU rDNA)

Fragments of 5' LSU rDNA (350 bp long) of ten strains of *Paramecium dodecaurelia* were compared. As an outgroup, standard strain 87 of *P. pentaurelia* was used as representative of species of the *P. aurelia* complex showing no intraspecific differentiation, and *P. caudatum* as representative of different morphospecies in *Paramecium*.

38 variable nucleotide positions were found when all studied strains (*P. dodecaurelia, P. pentaurelia, P. caudatum*) were compared, and 11 within *P.*

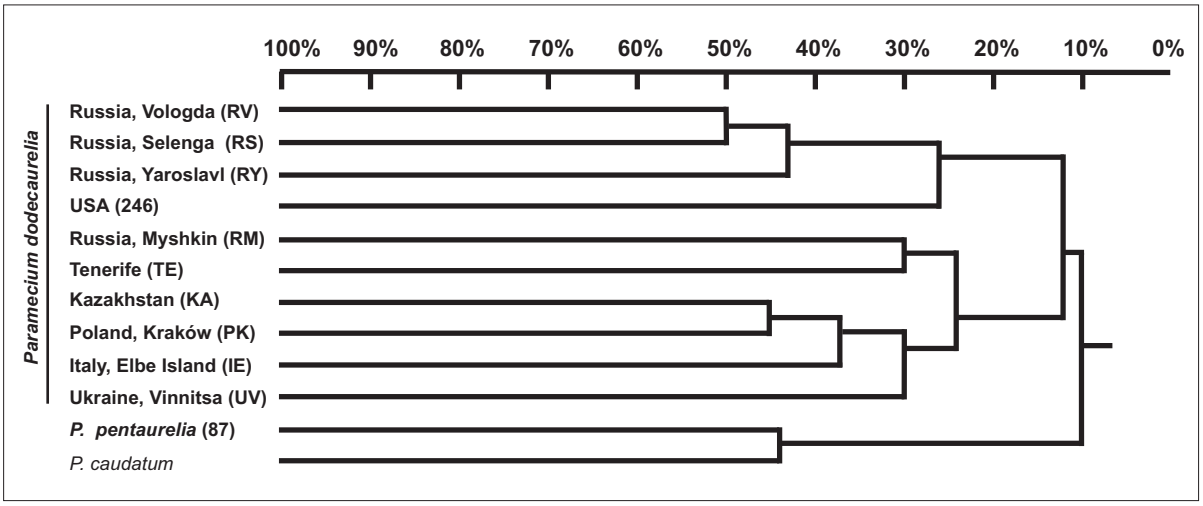

Fig. 2. Tree diagram of the cluster analysis of the RAPD fingerprint pattern similarity matrix of the studied *P. dodecaurelia* strains. Method used for analysis was UPGMA. Similarity index was calculated according to NEI and LI (1979).

dodecaurelia, i.e. # 151, #158, #192, #193, #201, #213, #227, #265, #272, #284, #301 (Table 4).

Trees (based on analyses by NJ, MP, and Bayesian methods) showed that *P. dodecaurelia* strains are divided into two clusters, one comprising strain 246 from the USA, and the second with all other strains, i.e. new strains identified from Russia (RS, RY, RV, RM), Ukraine (UV), Kazahstan (KA), Poland (PK), Tenerife (TE) and the previously known European strain from Italy (IE). The *P. pentaurelia* strain is associated with the second cluster of *P. dodecaurelia* strains (Fig. 3 A,B,C).

The genetic distance between *P. dodecaurelia* strains is at the level of 3.2% (based on the distance matrix, Table 5), both according to Kimura and Jukes-Cantor models. Diversity between *P. dodecaurelia* strains and *P. pentaurelia* ranges from 1.7 to 2% , and between *P. caudatum* and *P. dodecaurelia* – 9.1 to 10.1%.

Analysis of the fragment of mitochondrial cytochrome oxidase gene (COI mtDNA)

Fragments of COI mtDNA (430 bp long) of ten strains of *P. dodecaurelia*, and single strains of *P. pentaurelia* (87) and *P. caudatum* were sequenced and compared, the latter two strains were used as outgroups.

141 variable nucleotide positions were revealed (Table 6) in comparisons of *P. dodecaurelia*, *P. pentaurelia* and *P. caudatum* strains, and 76 variable positions were found within *P. dodecaurelia*.

Table 4

Polymorphisms in 5' LSU rDNA fragments in *P. Dodecaurelia* and *P. pentaurelia*

Strain designation	# 133	# 136	# 147	# 149	# 150	# 151	# 155	# 158	#165	# 166	# 167	# 171	# 192	# 193	# 196	# 197	# 201	# 207	# 212	# 213	# 214	# 216	# 227	# 228	# 250	# 265	# 267	# 272	# 281	# 284	# 287	# 288	# 289	# 290	# 301	# 311	# 314	# 316
P. dodecaurelia, 246	A	T	A	T	T	A	A	T	T	G	C	G	G	C	T	A	C	C	T	G	C	G	G	T	C	C	A	G	C	T	C	G	C	T	G	C	G	C
P. dodecaurelia, IE						T		C					A	T			T			C			A			T		A		C					A			
P. dodecaurelia, KA						T		C					A	T			T			C			A			T		A		C					A			
P. dodecaurelia, PK						T		C					A	T			T			C			A			T		A		C					A			
P. dodecaurelia, RM						T		C					A	T			T			C			A			T		A		C					A			
P. dodecaurelia, RS						T		C					A	T			T			C			A			T		A		C					A			
P. dodecaurelia, RV						T		C					A	T			T			C			A			T		A		C					A			
P. dodecaurelia, RY						T		C					A	T			T			C			A			T		A		C					A			
P. dodecaurelia, TE						T		C					A	T			T			C			A			T		A		C					A			
P. dodecaurelia, UV						T		C					A	T			T			C			A			T		A		C								
P. pentaurelia, 87						T		C				A											A					A		C								
Paramecium caudatum	G	C	G	C	A	T	T	C	A	T	T				G	T		T	A		T	A		C	T		G	A	T	C	T	A	T	C		T	A	T

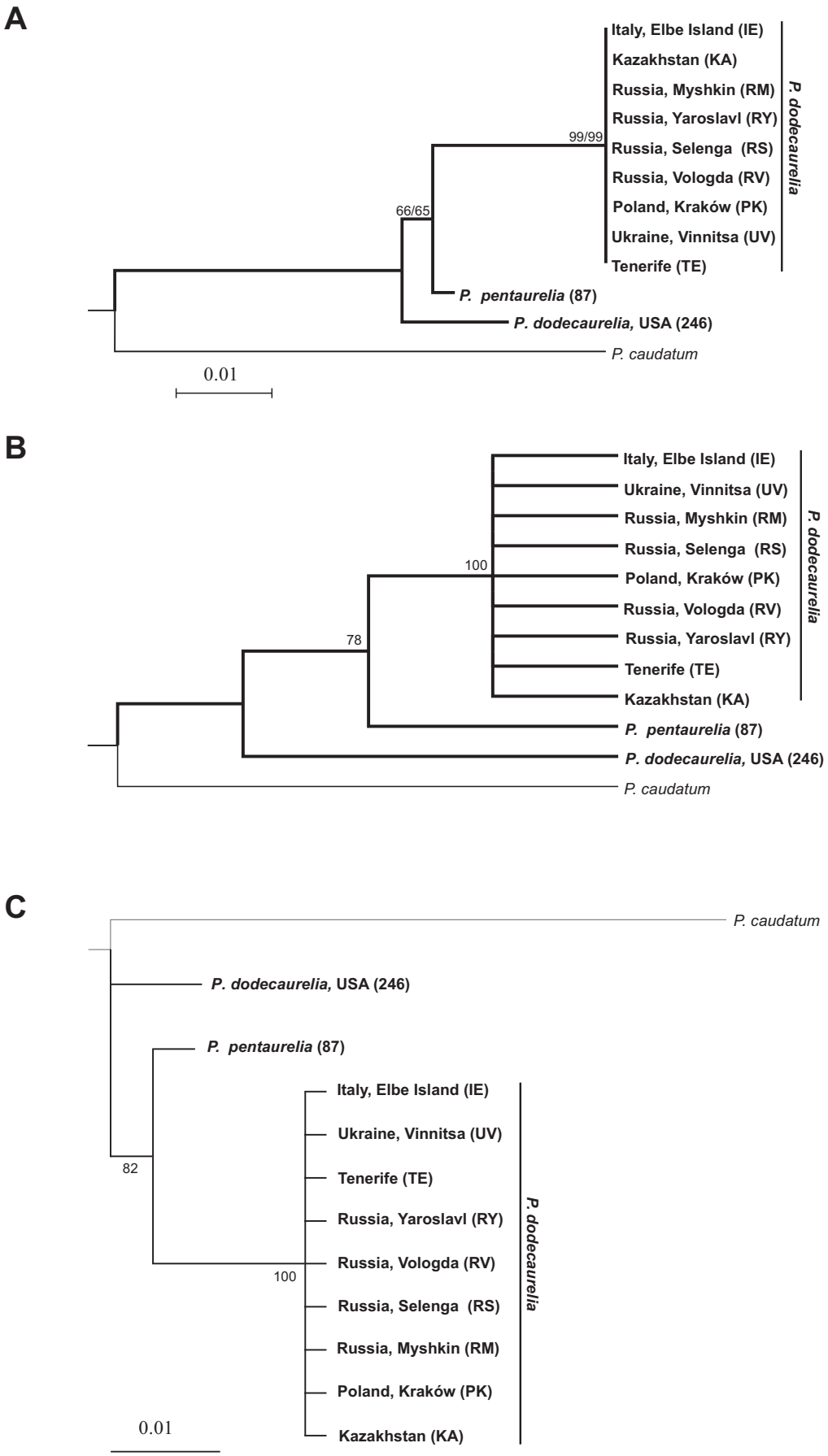

Fig. 3A,B,C. Trees constructed for *P. dodecaurelia*, *P. pentaurelia* and *P. caudatum* strains, based on comparison of sequences from 5' LSU rDNA fragment using NJ (neighbor joining) – A; MP (maximum parsimony) – B; and Bayesian analyses – C.

Table 5

Distance matrix presenting the number of base substitutions in *Paramecium dodecaurelia, Paramecium pentaurelia* and *Paramecium caudatum* strains, based on analyses of 5'LSU rDNA sequences. Analyses were conducted using the Kimura 2-parameter method (Lower-left) and Jukes-Cantor method (Upper-right)

5'LSU rDNA	*Paramecium dodecaurelia*_246	*Paramecium dodecaurelia*_IE	*Paramecium dodecaurelia*_KA	*Paramecium dodecaurelia*_PK	*Paramecium dodecaurelia*_RM	*Paramecium dodecaurelia*_RS	*Paramecium dodecaurelia*_RV	*Paramecium dodecaurelia*_RY	*Paramecium dodecaurelia*_TE	*Paramecium dodecaurelia*_UV	*Paramecium pentaurelia*_87	*Paramecium caudatum*
*Paramecium dodecaurelia*_246		0.032	0.032	0.032	0.032	0.032	0.032	0.032	0.032	0.032	0.017	0.091
*Paramecium dodecaurelia*_IE	0.032		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.020	0.101
*Paramecium dodecaurelia*_KA	0.032	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.020	0.101
*Paramecium dodecaurelia*_PK	0.032	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.020	0.101
*Paramecium dodecaurelia*_RM	0.032	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.020	0.101
*Paramecium dodecaurelia*_RS	0.032	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.020	0.101
*Paramecium dodecaurelia*_RV	0.032	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.020	0.101
*Paramecium dodecaurelia*_RY	0.032	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.020	0.101
*Paramecium dodecaurelia*_TE	0.032	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.020	0.101
*Paramecium dodecaurelia*_UV	0.032	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.020	0.101
*Paramecium pentaurelia*_87	0.017	0.020	0.020	0.020	0.020	0.020	0.020	0.020	0.020	0.020		0.085
Paramecium caudatum	0.092	0.103	0.103	0.103	0.103	0.103	0.103	0.103	0.103	0.103	0.086	

Trees obtained by NJ, MP, and Bayesian methods showed that *P. dodecaurelia* strains are divided into two clusters, one with strain 246 from the USA only, and the second with all other strains (Fig. 4). However, several subgroups may be seen within the second cluster, as strains from Italy and Tenerife are in one sub-group, strains from Russia RY, RV,RM, Ukraine and Poland in a second, and strains from Kazahstan and the Selenga River in Russia are in the third cluster (Fig. 4 A,B). *P. pentaurelia* appears in the trees obtained by NJ and Bayesian methods (Fig. 4A, C) in a disparate position, and between two main clusters of *P. dodecaurelia* strains when MP method was applied (Fig. 4B).

The variability within *P. dodecaurelia* (genetic distance, Table 7) is equal to 4% according to the Kimura model and 5% according to the Jukes-Cantor model. In turn, genetic distance between *P. dodecaurelia* strains and strain 87 of *P. pentaurelia* ranges from 17 to 18%, and between *P. dodecaurelia* strains and *P. caudatum* from 28% to 30%.

Discussion

Molecular methods based on PCR analyses (RAPD, RFLP, ARDRA) have been used in the last two decades for species identification and for studies of genetic polymorphism in different organisms (cf PRZYBOŚ *et al.* 2007a) including ciliates (LYNCH *et al*. 1995; CLARK 1997; STOECK & SCHMIDT 1998; FOKIN *et al.* 1999; PRZYBOŚ *et al*. 1999; STOECK *et al.* 2000; CHEN *et al*. 2000; YI *et al*. 2006; MACIEJEWSKA 2006; PRZYBOŚ *et al*. 2007b).

Recently sequencing of gene fragments, especially rDNA, has been applied in studies on ciliate phylogeny, based mainly on small subunit rDNA (SSU rDNA), e. g. in *Euplotes elegans* (SCHWARTZ *et al*. 2007), in Stichotrichia (SCHMIDT *et al*. 2006b; 2007), studies of ribotypes have shown the existence of multiple ecotypes (FINLAY 2004; FINLAY *et al.* 2006), other studies applied LSU rDNA (NANNEY *et al.* 1998); hsp70 gene sequences (HORI *et al*. 2006); a fragment of the histone *H4* gene (BERNHARD & SCHLEGEL 1998) or mitochondrial cytochrome oxidase (BARTH *et al*. 2006; PRZYBOŚ *et al*. 2007b).

Several studies concerned comparisons of species within the *P. aurelia* complex. NANNEY *et al*. (1998) compared sequence differences in a variable 23S rRNA domain among several species of the *P. aurelia* complex (without *P. novaurelia*) and found that pairs of species are separated by four or more changes. Intraspecific differentiation

Table 6

Polymorphism in COI mtDNA fragments of *Paramecium dodecaurelia, P. pentaurelia, P. caudatum*

Strain designation	# 5	# 8	# 11	# 14	# 17	# 19	# 20	# 23	#29	# 32	# 38	# 41	# 44	# 47	# 50	# 53	# 59	# 62	# 65	# 68	# 71	# 80	# 89	# 90	# 92	# 95	# 98	# 101	# 107	# 110	# 113	# 116	# 119	# 122	# 125	# 128
P. dodecaurelia, 246			C	C	T	T	C	C	A	C	T	C	C	G	A	C	C	A	A	C	A	A	G	C	T	T	T	T	G	C	G	C	C	C	T
P. dodecaurelia, IE			T	G	C			T			A	T	T	A		A		G	C		G					C	C				T			G		C
P. dodecaurelia, KA			T	G	C			T			A	T	T	A		A		G	C		G					C	C				T			G		C
P. dodecaurelia, PK			T	G	C			T			A	T	T	A		A		G	C		G					C	C				T			G		C
P. dodecaurelia, RM			T	G	C			T			A	T	T	A		A		G	C		G					C	C				T			G		C
P. dodecaurelia, RS			T	G	C			T			A	T	T	A		A		G	C		G					C	C				T			G		C
P. dodecaurelia, RV			T	G	C			T			A	T	T	A		A		G	C		G					C	C				T			G		C
P. dodecaurelia, RY			T	G	C			T			A	T	T	A		A		G	C		G					C	C				T			G		C
P. dodecaurelia, UV			T	G	C	A		T			A	T	T	A		A		G	C		G					C	C				T			G		C
P. dodecaurelia, TE			T	G	C			T			A	T	T	A		A		G	C		G					C	C				T			G		C
P. pentaurelia, 87	C	A	T	G	C					G					T				T		G	T	A				C	C	C		T			T	T	
Paramecium caudatum		T	A	T	A		T	T	T	T		T	T	A		T	T		T	T		T	A	T	A	C				A	T	A	A	T	T	

Strain designation	# 129	# 131	# 132	# 134	# 137	# 140	# 143	# 149	#155	# 158	# 161	# 164	# 167	# 173	# 176	# 179	# 182	# 185	# 188	# 203	# 206	# 209	# 212	# 215	# 217	# 218	# 219	# 221	# 224	# 227	# 228	# 230	# 233	# 237	# 239	# 242
P. dodecaurelia, 246	T	A	C	T	T	A	A	T	G	G	A	G	C	A	C	C	T	G	T	G	C	C	C	G	C	T	C	T	C	G	G	C	C	A	T	C
P. dodecaurelia, IE			T	G			G		A	A	T	C	T	G		T			C		A											T	T		C	T
P. dodecaurelia, KA			T	G			G		A	A	T	C	T	G			C		C		A											T	T		C	T
P. dodecaurelia, PK			T	G			G		A	A	T	C	T	G			C		C		A											T	T		C	T
P. dodecaurelia, RM			T	G			G		A	A	T	C	T	G			C		C		A											T	T		C	T
P. dodecaurelia, RS			T	G			G		A	A	T	C	T	G			C		C		A											T	T		C	T
P. dodecaurelia, RV			T	G			G		A	A	T	C	T	G			C		C		A											T	T		C	T
P. dodecaurelia, RY			T	G			G		A	A	T	C	T	G			C		C		A											T	T		C	T
P. dodecaurelia, UV			T	G			G		A	A	T	C	T	G			C		C		A											T	T		C	T
P. dodecaurelia, TE			T	G			G		A	A	T	C	T	G		T			C		A											T	T		C	T
P. pentaurelia, 87		G		A			G				C	T	T			T			C	A	T	T	T	A		C		C					T		C	T
Paramecium caudatum	C	T	T	A	C	T		A	A	A		C	T		T	T		A		A	A	T	T	A	T		G		T	A	A	T	T	C		T

Strain designation	# 247	# 248	# 251	# 254	# 257	# 263	# 266	# 267	# 269	#275	# 276	# 278	# 281	# 284	# 287	# 290	# 291	# 292	# 293	# 294	# 296	# 299	# 300	# 302	# 308	# 311	# 314	# 317	# 320	# 323	# 326	# 331	# 332	# 335	# 336	# 338
P. dodecaurelia, 246	C	G	G	G	T	C	T	C	A	G	A	G	C	A	C	G	T	C	C	C	G	T	C	T	G	A	A	A	G	G	G	C	T	C	A	A
P. dodecaurelia, IE	T		C			T	C		C					G					T	T				C		G		T	A	A			C			G
P. dodecaurelia, KA	T		C			T	C		C					G					T	T				C	A	G		T	A	A		A	C			G
P. dodecaurelia, PK	T		C			T	C		C					G					T	T				C	A	G	G	T	A	A			C			G
P. dodecaurelia, RM	T		C			T	C		C					G					T	T				C	A	G	G	T	A	A			C			G
P. dodecaurelia, RS	T		C	A	C	T	C		C					G					T	T				C		G		T	A	A			C			G
P. dodecaurelia, RV	T		C			T	C		C					G					T	T				C	A	G	G	T	A	A			C			G
P. dodecaurelia, RY	T		C			T	C		C					G					T	T				C	A	G	G	T	A	A			C			G
P. dodecaurelia, UV	T		C			T	C		C					G					T	T				C	A	G	G	T	A	A			C			G
P. dodecaurelia, TE	T		C			T	C		C					G					T	T				C		G		T	A	A			C		C	G
P. pentaurelia, 87	T		A			T	C		T					G					T	T		C				G	G	C	A	A	A		C			
Paramecium caudatum		T	A			T	C	T	T	A	C	A	T	G	T	A	G	T	T	T	A		T	A	A		G		A	A	A		A	T		

Strain designation	# 341	# 344	# 347	# 350	# 353	# 356	# 359	# 362	#365	# 366	# 368	# 372	# 373	# 380	# 381	# 386	# 389	# 392	# 395	# 398	# 401	# 404	# 407	# 410	# 411	# 412	# 413	# 416	# 419	# 420	# 422	# 425	# 428			
P. dodecaurelia, 246	G	T	T	T	T	T	C	C	C	C	A	T	T	T	T	T	T	T	T	C	T	T	T	T	C	T	A	G	C	T	A	C	C			
P. dodecaurelia, IE		C		C	C		T							C		C		C		T	C	C		C			G			C	C	T				
P. dodecaurelia, KA			C	C	C		T				G			C		C		C			C	C		C	T		G			C	C	T				
P. dodecaurelia, PK		C	C	C	C		T				G			C		C		C			C	C		C	T		G			C	C	T				
P. dodecaurelia, RM		C	C	C	C		T				G			C		C		C			C	C		C	T		G			C	C	T				
P. dodecaurelia, RS			C	C	C		T				G			C		C		C			C	C		C	T		G			C	C	T				
P. dodecaurelia, RV		C	C	C	C		T				G			C		C		C			C	C		C	T		G			C	C	T				
P. dodecaurelia, RY		C	C	C	C		T				G			C		C		C			C	C		C	T		G			C	C	T				
P. dodecaurelia, UV		C	C	C	C		T				G			C		C		C			C	C		C	T		G			C	C	T				
P. dodecaurelia, TE		C	C	C	C		T						G	C		C		C		T	C	C		C		G	G			C	C	T				
P. pentaurelia, 87	C	C	C			C						C		C	C				C		C		C	C	T		G		T	C						
Paramecium caudatum	T		A			C		T	A	T						C	A			T				C	T		G	A	T			A	T			

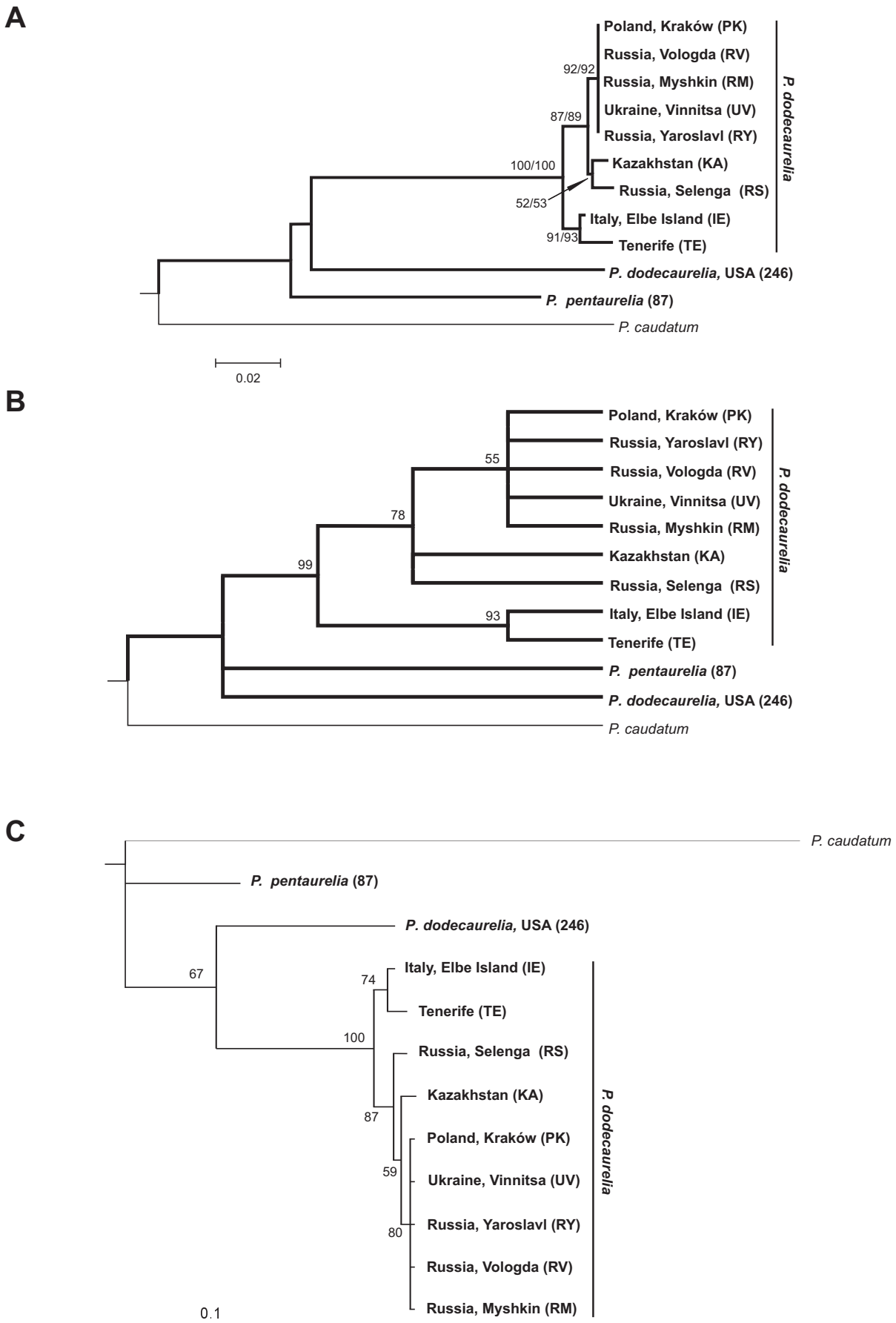

Fig. 4 A,B,C. Trees constructed for *P. dodecaurelia*, *P. pentaurelia* and *P. caudatum* strains, based on comparison of sequences from COI mt DNA gene fragment using NJ (neighbor joining) – A; MP (maximum parsimony) – B, and Bayesian analyses – C.

Table 7

Distance matrix presenting the number of base substitutions per site from analyses of *P. dodecaurelia, P. pentaurelia* and *P. caudatum* CoI mtDNA. Analyses were conducted using the Kimura 2-parameter method (lower left) and Jukes-Cantor method (upper right)

COI	*Paramecium dodecaurelia*_246	*Paramecium dodecaurelia*_IE	*Paramecium dodecaurelia*_KA	*Paramecium dodecaurelia*_PK	*Paramecium dodecaurelia*_RM	*Paramecium dodecaurelia*_RS	*Paramecium dodecaurelia*_RV	*Paramecium dodecaurelia*_RY	*Paramecium dodecaurelia*_TE	*Paramecium dodecaurelia*_UV	*Paramecium pentaurelia*_87	*Paramecium caudatum*
*Paramecium dodecaurelia*_246		0.032	0.032	0.032	0.032	0.032	0.032	0.032	0.032	0.032	0.017	0.091
*Paramecium dodecaurelia*_IE	0.168		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.020	0.101
*Paramecium dodecaurelia*_KA	0.177	0.024		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.020	0.101
*Paramecium dodecaurelia*_PK	0.181	0.019	0.009		0.000	0.000	0.000	0.000	0.000	0.000	0.020	0.101
*Paramecium dodecaurelia*_RM	0.181	0.019	0.009	0.000		0.000	0.000	0.000	0.000	0.000	0.020	0.101
*Paramecium dodecaurelia*_RS	0.178	0.021	0.012	0.012	0.012		0.000	0.000	0.000	0.000	0.020	0.101
*Paramecium dodecaurelia*_RV	0.181	0.019	0.009	0.000	0.000	0.012		0.000	0.000	0.000	0.020	0.101
*Paramecium dodecaurelia*_RY	0.181	0.019	0.009	0.000	0.000	0.012	0.000		0.000	0.000	0.020	0.101
*Paramecium dodecaurelia*_TE	0.181	0.019	0.009	0.000	0.000	0.012	0.000	0.000		0.000	0.020	0.101
*Paramecium dodecaurelia*_UV	0.183	0.012	0.031	0.026	0.026	0.029	0.026	0.026	0.026		0.020	0.101
*Paramecium pentaurelia*_87	0.178	0.169	0.178	0.169	0.169	0.178	0.169	0.169	0.169	0.177		0.085
Paramecium caudatum	0.272	0.270	0.277	0.274	0.274	0.285	0.274	0.274	0.274	0.283	0.257	

was not studied, but the authors wrote "since multiple clones of the same species were not examined, D2 polymorphism within *aurelia* species remains a possibility". The application of SSU rRNA gene sequences (STRÜDER-KYPKE *et al*. 2000a,b) showed that two species of the complex, i.e. *P. primaurelia* and *P. tetraurelia*, differed by only five nucleotides from each other. In turn, COLEMAN (2005) investigated the rRNA ITS region in the *P. aurelia* complex and found strain variability as "variant nucleotide positions" only within *P. primaurelia* and *P. tredecaurelia. P. tetraurelia* and *P. novaurelia* and also *P. octaurelia* and *P. dodecaurelia* were identical in the entire ITS2 sequence. However, the author only studied at the most two strains of each species. Hsp 70 gene sequences were used by HORI *et al*. (2006) for comparison of species of the *P. aurelia* complex and *P. caudatum* syngens. They found that nonsynonymous substitutions were frequent in *P. triaurelia*, *P. septaurelia*, *P. dodecaurelia* (11, 10, 5 respectively in these species), while *P. novaurelia*, *P. tredecaurelia*, and *P. quadecaurelia* also have one nonsynonymous substitution in the same position. However, the authors did not study intraspecific differentiation.

Studies carried out within *P. dodecaurelia* based on analysis of sequences of rDNA fragments showed that its intraspecific diversity was as high as that between different species of the *P. aurelia* complex, i.e. six polymorphic sites were found in a fragment of rRNA at the 3' end of SSU-ITS1 (210 bp) and several sites at the 5' end of LSU when strains originating from Europe (Elbe Island and Trento, Italy; Münster, Germany), North America (USA), Asia (Japan), and Hawaii were studied. In trees constructed on the basis of 5'LSU using the NJ and MP methods, strains of *P. dodecaurelia* were scattered, European strains clustered together, and strains from USA and Japan were found in separate branchs, while the strain from Hawaii was distant (TARCZ *et al*. 2006). In turn, analyses of sequences of the hsp70 gene in *P. dodecaurelia* strains 251 from a southern state of the USA and from Hawaii (HORI *et al*. 2006) and the H4 histone gene fragment in strain 246, USA (PRZYBOŚ *et al*. 2006b) showed the isolated position of *P. dodecaurelia* within the tree constructed for all species of the *P. aurelia* complex.

The present study based on sequences of the 5' LSU fragment (350bp long) of new *P. dodecaurelia* strains and previously known strains from Italy and USA, showed 11 base substitutions between strains. Their genetic distance (based on a distance matrix) is at the level of 3.2%, larger than between *P. dodecaurelia* and *P. pentaurelia* strains (1.7-2%).

However, trees constructed by NJ, MP, and Bayesian methods revealed an interesting situation. Only the strain from the USA appeared in separate cluster, others formed one group (common cluster). It seems interesting that these *P. dodecaurelia* strains originating from such remote places as Tenerife, Italy, Poland, Ukraine, Kazahstan and Russia (European and Asian parts) appear in one cluster in the rDNA tree (Fig.3). They originated from collecting sites still within the boundaries of the Palaearctic Region (as proposed by RAZOWSKI 2007). The strain from the USA, being in separate cluster in the tree, may be representative of the Nearctic Region.

Similarly, SCHMIDT *et al*. (2006a) reported in *Stylonychia lemnae* (Spirotrichea) (having a global distribution) a distinct difference within the small subunit ribosomal DNA gene only in clones from the USA in comparison with clones from different regions in Europe.

Recently, the mitochondrial cytochrome c oxidase I (COI) gene was used for intraspecific investigations in *P. caudatum* and *P. multimicronucleatum* and results were compared with obtained sequences of the ITS regions (BARTH *et al*. 2006). The mitochondrial sequences revealed higher variation in both species with intraspecific divergences up to 7% in *P. caudatum* and 9.5% in *P. multimicronucleatum.* Similarly, 13.9% divergence in COI mtDNA was found in *P. novaurelia* strains (PRZYBOŚ *et al*. 2007 P), a much higher divergence than in the rDNA fragment. It is worth mentioning that the investigated fragment of COI mtDNA has recently been used as a "barcode" of life, i.e. a standard fragment of DNA appearing in the majority of living organisms (HEBERT *et al*. 2003) and was also tested in *Tetrahymena* species (LYNN & STRÜDER-KYPKE 2006; CHANTANGSI & LYNN 2006).

The COI mtDNA fragment was also sequenced in investigations on intraspecific differentiation of *P. dodecaurelia* strains. Much higher intraspecific differentiation within this species was revealed by analyses of COI mtDNA (4% in Kimura model and 5% in Jukes-Cantor model, and 76 variable nucleotide positions) than obtained by analysis of 5' LSU rDNA (differentiation was equal to 3.2%, and 11 variable positions were found). However, two main strain clusters were similar in the case of both analyzed DNA fragments, more detailed relationships of strains appeared in the tree based on COI mtDNA.

High intraspecific polymorphism of some species of the *P. aurelia* complex may be associated according to STOECK *et al*. (1998, 2000) with a degree of inbreeding which is characteristic (SONNEBORN 1957; LANDIS 1986) for species of the complex and which causes intraspecific differentiation. This correlation was confirmed by studies concerning polymorphism within *P. dodecaurelia* (PRZYBOŚ *et al*. 2005a; TARCZ *et al*. 2006, and the present paper) as well as by studies carried out on several strains of other species of the *P. aurelia* complex (PRZYBOŚ *et al*. 2007 a,b). Species characterized by extreme inbreeding (e.g. *P. tetraurelia, P. dodecaurelia)* showed higher intraspecific polymorphism than did species characterized by weak inbreeding, such as *P. pentaurelia.*

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