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Intraspecific Variation of Diagnostic rDNA Genes in *Paramecium dodecaurelia*, *P. tredecaurelia* and *P. quadecaurelia* (Ciliophora: Oligohymenophorea)*

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Summary. Fragments of the 3' end of SSU rRNA - ITS1 (210bp) and the 5' end of LSU rRNA (350bp) were analysed in a study of intraspecific polymorphism in species of the *Paramecium aurelia* complex. These species have shown various levels of intraspecific polymorphism in previous RAPD, RFLP and ARDRA analyses, i.e. *Paramecium dodecaurelia* (high intraspecific polymorphism), *P. quadecaurelia* and *P. tredecaurelia* (no intraspecific polymorphism). Studies were performed on six strains of *P. dodecaurelia* and in two strains each (only available) of *P. tredecaurelia* and *P. quadecaurelia*, originating from geographically remote collecting sites. Alignment of both rDNA gene fragments containing 3'SSU rRNA-ITS1 and 5' LSU rRNA revealed a distinct polymorphism within *P. dodecaurelia*, i.e six polymorphic sites were found in a fragment of rDNA at the 3' end of SSU rRNA-ITS1, only one site differentiating strains of *P. tredecaurelia*, and no changes in *P. quadecaurelia.* Similarly, at 5' LSU several polymorphisms characteristic for the particular strains of *P. dodecaurelia* were observed, three polymorphic sites when two strains of *P. tredecaurelia* were compared, and one polymorphic site in the case of two strains of *P. quadecaurelia*. Neighbor- joining and maximum parsimony phylogenies showed that *P. dodecaurelia* strains are scattered, particular strains differ as much as different species of the *P. aurelia* complex. Our present study confirmed previous results (RAPD, RFLP, and ARDRA analyses) which revealed such exceptional intraspecific differentiation in *P. dodecaurelia.* This is the first analysis of the 5'end fragment of LSU rRNA at the intraspecific level in the *P. aurelia* complex, showing that the fragment seems very useful at this level.

Key words: intraspecific polymorphism, ITS1 region, LSU rRNA gene sequences, *Paramecium aurelia* species complex, *P. dodecaurelia*, *P. quadecaurelia*, *P. tredecaurelia*, ribosomal DNA, sequence analyses, SSU rRNA gene sequences.

Abbreviations used: bp - base pairs, ITS1 - internal transcribed spacer, LSU - large subunit ribosomal RNA, SSU - small subunit ribosomal RNA.

INTRODUCTION

The *Paramecium aurelia* complex is composed of 15 species, 14 of which have been named by Sonneborn (1975) and the 15th - *P. sonneborni* by Aufderheide *et al.* (1983). The relationships of species of the complex as well as possible intra-specific polymorphism of particular species have been studied (Przyboś *et al.* 2006b). Genetic studies were carried out by classical strain crosses and molecular analyses such as RAPD fingerprinting, RFLP analysis, and ARDRA riboprinting. RAPD fingerprinting revealed intraspecific polymorphism (different band patterns characteristic for the studied strains) within several species. RFLP analysis and ARDRA riboprinting showed the existence of different groups of species within the complex, characterized by different band patterns as well as intraspecific polymorphism,

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depending on the enzyme used and the investigated species. High intraspecific polymorphism was revealed in *P. dodecaurelia* and high similarity of band patterns in *P. tredecaurelia* and *P. quadecaurelia*. However, high survival of inter-strain hybrids in both generations was observed in *P. dodecaurelia* and *P. quadecaurelia* but not in *P. tredecaurelia*. A fragment of rDNA of *P. dodecaurelia* strains (revealing high intra-specific polymorphism), *P. tredecaurelia,* and *P. quadecaurelia* (showing no intra-specific polymorphism) was sequenced with the aim to find explanation of such great differences between species as intra-specific polymorphism is concerned.

DNA sequencing of the small subunit of ribosomal RNA (SSU rRNA) has recently been applied in systematics for comparisons at the specific and subspecific levels as well as for phylogenetic reconstruction (cf Hammerschmidt *et al.* 1996, Schlegel 2003). Sequence analyses of SSU rRNA gene have been frequently applied in ciliate taxonomy, e.g. in Heterotrichea (Fu *et al.* 2004), Spirotrichea (Bernhard *et al.* 2001, Chen and Song 2001, Petroni *et al.* 2002, Modeo *et al.* 2003), and recently 18 S rRNA gene phylogenies were compared with those based on morphological and ontogenetical data in the stichotrichines (Foissner *et al.* 2004) and in Oligotrichea (Agatha *et al.* 2005). In Oligohymenophorea within subclass Peniculia, the SSU rRNA genes were sequenced also in *Paramecium* (Strüder-Kypke *et al.* 2000b). The authors concluded that "the *Paramecium* species form at least four clades with the *Paramecium aurelia* subgroup being the most derived". The same authors also studied phylogenetic relationships of the genus *Paramecium* based on sequences from the small subunit rRNA gene (Strüder-Kypke *et al.* 2000a) in the species: *P. bursaria, P. calkinsi, P. duboscqui, P. jenningsi, P. nephridiatum, P. primaurelia, P. polycaryum.* The genus seems to be a monophyletic group. Within the *P. aurelia* species complex, *P. primaurelia* and *P. tetraurelia* differed by five nucleotides from each other and from *P. jenningsi* by six or seven nucleotides, respectively.The SSU rRNA sequence analysis placed the new species *P. schewiakoffi* into the monophyletic "aurelia" subgroup as the sister species of *P. jenningsi* (Fokin *et al.* 2004).

Ribosomal DNA (rDNA) composed of conserved and variable regions is useful for taxonomic analyses. Particularly useful are ITS (ITS - internal transcribed spacer) regions containing DNA sequences between genes of particular ribosomal subunits "as they are non-coding regions that evolve at a high rate" (Li and Graur 1991). Studies have been carried out in different organisms for testing the validity of species, e.g. for the dragonfly species *Cordulegaster bilineata* the entire ITS-1 region of rDNA was applied (Pilgrim *et al.* 2002). In another paper, ITS-1, 5.8 S and ITS-2 r DNA sequences were used as diagnostic tools for assessing the variability within amoeboflagellate species of the genus *Naegleria* (De Jonckheere 2004), and a new species *Naegleria angularis* was identified based on differences in ITS1 and ITS2 sequences when compared with *N. pussardi* (De Jonckheere and Brown 2005). In ciliates intraspecific variation in *Cryptocaryon irritans* (cl. Oligohymenophorea, subcl. Hymenostomatia) was examined using sequences of ITS-1 rDNA combined with developmental and morphological characters (Diggles and Adlard 1997), characterizing four strains. At present, the *Paramecium tetraurelia* genome has also been sequenced (e.g. Sperling *et al.* 2004). Coleman (2005) has recently sequenced the ITS region, of the nuclear ribosomal cistron of 13 species of the *P. aurelia* complex for analysis of their genetic relatedness based on one or two strains for species.

Here we examine two rRNA gene fragments in six unique strains of *P. dodecaurelia* and two strains each of *P. tredecaurelia* and *P. quadecaurelia*, all originating from remote collecting sites with hope to find the proper marker (DNA fragment) presenting intraspecific differentiation. The studied strains of *P. dodecaurelia* are very different as shown by our previous studies (RAPD, RFLP and ARDRA analyses) (Przyboś *et al.* 2006b) so worth of examination.

MATERIALS AND METHODS

The strains of *Paramecium dodecaurelia*, *P. tredecaurelia*, and *P. quadecaurelia* examined here (listed in Table 1) have been kept in the collection of the Institute of Systematics and Evolution of Animals, Polish Academy of Sciences, Kraków.

DNA isolation, amplification, and electrophoresis

Paramecia were cultivated on lettuce medium inoculated with *Enterobacter aerogenes* according to Sonneborn (1970). Genomic DNA was isolated from vegetative cells, representing 12 strains listed in Table 1, at the end of the exponential phase using the QIamp DNA Kit (Qiagen™, Germany) as described by Przyboś *et al.* (2003c); 200µl of cell culture was used for the DNA extraction. The primers amplified two regions of rDNA: the 3' end of SSU rRNA - the ITS1 fragment and the fragment which contains 5' end of LSU rRNA. The primers used for the PCR reaction are listed in Table 2. One of the

primers - LSU_R has the same sequence as in Jerome and Lynn (1996). The others primers were constructed using Oligoanalyzer 3.0 (http://scitools.idtdna.com/analyzer/). Primer construction was based on data from Genbank (Accesion numbers: X03772 - for ITS1_F primer, and AY833401 - for ITS1_R). Primer LSU_F was constructed basing on unpublished sequences of ITS2 fragment of *Paramecium dodecaurelia*.

PCR amplification was carried out in a final volume of 30 µl containing: 2 µl of template, 1.5 U Taq-Polymerase (Qiagen™, Germany); 0.6 µl 10mM of each primer; 10× PCR buffer; 0.6 µl of 10mM dNTPs in a Tpersonal thermocycler (Biometra GmbH, Germany). The amplification protocol consisted of initial denaturation at 94°C, followed by 34 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 60 s, and extension at 72°C for 60 s, with final extension at 72° for 5 min. After amplification the PCR products were electrophoresed in 1% agarose gel for 45 min at 85V with a DNA molecular weight marker (XIV ™ Roche, France) (Fig. 1).

Sequencing

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

Data analysis

Sequences were examined using Chromas Lite (Technelysium ™, Australia). Alignment and consensus of the study sequences was performed by ClustalW (Thomphson *et al.* 1994) in BioEdit program (Hall 1999). Phylogenetic trees were constructed for both studied fragments in Mega version 3.1 (Kumar *et al*. 2004), using NJ (Neighbor-joining method) (Saitou and Nei 1987) and MP (Maximum Parsimony). The NJ analysis was performed using a Kimura 2-parameter correction model (Kimura 1980) by bootstrapping with 1000 replicates (Felsenstein 1985). The MP analysis was evaluated with Min-mini heuristic (level = 2) and bootstrapping with 1000 replicates.

RESULTS

Two rDNA fragments were analyzed: one fragment is 210 bp long and contains the 3' end of SSU rRNA and ITS1 and the second fragment is 350 bp long and contains the 5' end of LSU rRNA (Fig. 2). Both sequences were compared with sequences available in Genbank (AF149979, AY833401, AY833394, AY833386). Alignment of the fragment containing 3'SSU rRNA-ITS1 revealed 23 polymorphisms in ITS1 in three studied *Paramecium* species (*P. cadatum*, *P. multimicronucleatum* and the *P. aurelia* complex), however, in the *P. aurelia* spp. only six polymorphic sites were observed (Table 3a). In turn, 50 polymorphisms were found in all studied *Paramecium* spp. In the fragment of 5' end of LSU rRNA, among which 26 polymorphisms in *P. aurelia* spp. were found. A 130bp stretch of DNA at the 5' end did not harbour polymorphism (Table 3b) in the LSU fragment. We found 88.5 % identity in the 3' SSU rRNA-ITS1 fragment of all studied strains belonging to the investigated species of the *P. aurelia* complex and 97.1 % between *P. dodecaurelia* strains. In the 5' LSU 85.7 % of nucleotides were identical among all studied species (Tables 3a,b) and in *P. dodecaurelia* 94.3% were identical. Strains of *Paramecium caudatum* and *P. multimicronucleatum* were used as outgroups. Polymophism is highly recognizable within the *P. dodecaurelia* strains, as well as in 3'SSU rRNA - ITS1 fragment and especially in 5' LSU rRNA fragment. All sequences were deposited in Genbank (accession numbers in Table 1).

SSU rRNA - ITS1 fragment

6 polymorphic sites were found in the fragment of rDNA at the 3' end of SSU rRNA – ITS1 in the studied strains of *P. dodecaurelia*. They appear at positions #90, #152, #165, #180, #181, #188. Site #180 seems interesting as three different bases can be found there (C in 246 and HHS strain, A in JU strain, and T in the other strains). At site #188, an A appears in strains G and IE. No differences were found within *P. tredecaurelia* *P. quadecaurelia* (Table 3a).

5' LSU fragment of rRNA

Particular strains of *P. dodecaurelia* can be characterized by the type of polymorphism. Several polymorphisms characteristic for the European strains G and IE were found at sites #192, #193, #201, #213, #265, #301 at 5' LSU rRNA as well as polymorphisms differentiating European strains from the others at #158, #227, #284. Strain HHS from Hawaii, most distant from the other strains, is also characterized by the most numerous changes characteristic for this strain only, at #136, #139, #147, #149, #155, #214, #216.

Three polymorphic sites (#287, #311, #316) are characteristic only for two *P. tredecaurelia* strains and they differentiate them from the other species of the complex, polymorphic site at #205 is characteristic for two strains of *P. quadecaurelia* (Table 3a). There are also three variable sites (#147, #167, #195) within *P. quadecaurelia* strains.

Table 1. Strains of the *Paramecium aurelia* complex, *P. caudatum* and *P. multimicronucleatum* used in analyses.

Species	Locality	Reference	GenBank Accession No.	
			3'SSU rRNA - ITS1	5'LSU rRNA
P. dodecaurelia, strain G	Germany, Münster.	Przyboś and Fokin 2003b	DQ 207378	DQ 207370
P. dodecaurelia, strain IE	Italy, Elbe Island, Azurro	Przyboś and Fokin 2003b	DQ 207380	DQ 207372
P. dodecaurelia, strain JU	Japan, Honsiu Island, Ube city	Przyboś *et al*. 2003a	DQ 207381	DQ 207373
P. dodecaurelia, strain 246	USA, southern state	Sonneborn 1974	DQ 207377	DQ 207369
P. dodecaurelia, strain HHS	Hawai, Honolulu	Przyboś and Fokin 2003a	DQ 207379	DQ 207371
P. dodecaurelia, strain TR	Italy, Trento	Przyboś *et al*. 2005	DQ 207382	DQ 207374
P. tredecaurelia, strain 209	France, Paris	Rafalko and Sonneborn 1959	DQ 207383	DQ 138112
P. tredecaurelia, strain IKM	Israel, Kinet Motzkin	Przyboś *et al*. 2002	DQ 207384	DQ 138113
P. quadecaurelia, strain 328	Austraila, Emily Gap	Sonneborn 1975	DQ 207385	DQ 138114
P. quadecaurelia, strain AN	Africa, Namibia	Przyboś *et al*. 2003b	DQ 207386	DQ 138115
P. caudatum, strain PC	Cyprus, Akamas	Przyboś (unpublished)	DQ 207387	DQ 207375
P. multimicronucleatum, strain PM	USA, Louisiana	Przyboś (unpublished)	DQ 207388	DQ 207376

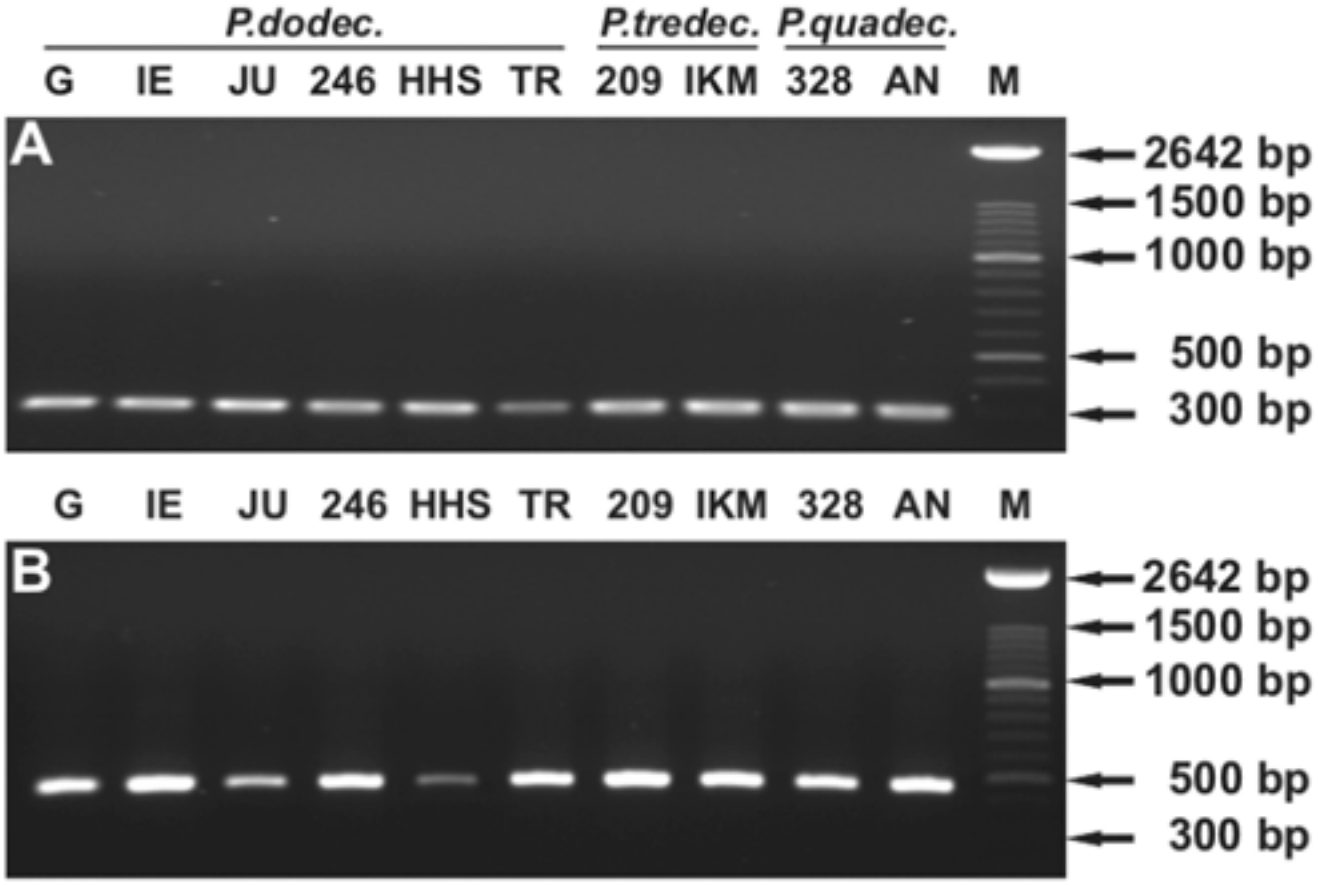

Fig. 1. Gel presenting the examined fragments: A- 3' end of small subunit rRNA-internal transcribed spacer 1 fragment (310bp); B- 5'end of large subunit rRNA LSU rRNA fragment (450bp). Designation of strains: G - Germany, Münster, IE- Italy, Elbe Island, JU- Japan, Honsiu Island, 246- USA, southern state, HHS - Hawaii, Honolulu, TR- Italy, Trento, 209- France, Paris, IKM- Israel, Kinet Motzkin, 328- Australia, Emily Gap, AN- Africa, Namibia, M- weight marker (XIV, Roche ™).

Table 2. Sequences of applied primers

Amplified region	Primer designation	Primer sequence	References
3' SSU rRNA - ITS1	ITS1_F	5'-TAAACCTTATCACTTAGAGGA-3'	This study
	ITS1_R	5'-CGAAAATCTAATGTCTCGCA-3'	This study
5' LSU rRNA	LSU_F	5'-CCCGTATTTGGTTAGGACT-3'	This study
	LSU_R	5'-TTGGTCCGTGTTTCAAGACG-3'	Jerome and Lynn 1996

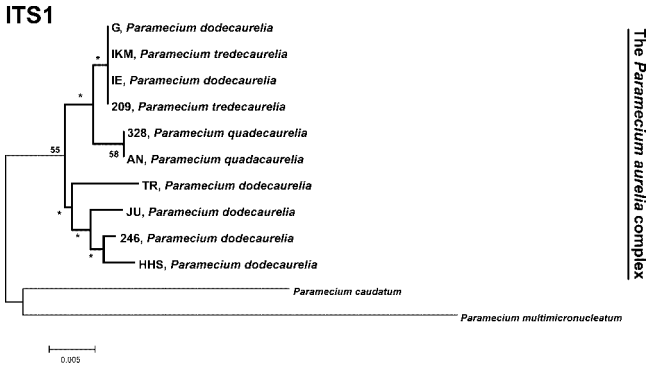

Fig. 2. Phylogenetic tree constructed for 12 strains of the *P. aurelia* complex with a single strain of *P. caudatum* and *P. multimicronucleatum* as outgroups, based on a comparison of sequences the 3' end of small subunit rRNA-internal transcribed spacer 1 fragment using the NJ (neighbor joining) method with the application of the Kimura two-parameter correction model. Bootstrap values are presented as percentages for 1000 comparisons. In the case of bootstrap values less than 50, the asterisks appear.

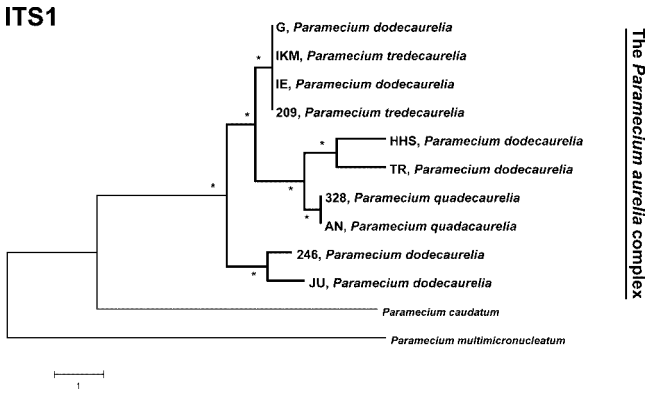

Fig. 3. Phylogenetic tree constructed for 12 strains of the *P. aurelia* complex with a single strain of *P. caudatum* and *P. multimicronucleatum* as outgroups based on a comparison of sequences the 3' end of small subunit rRNA-internal transcribed spacer 1 fragment using MP (maximum parsimony). Bootstrap values are presented as percentages for 1000 comparisons. In the case of bootstrap values less than 50, the asterisks appear.

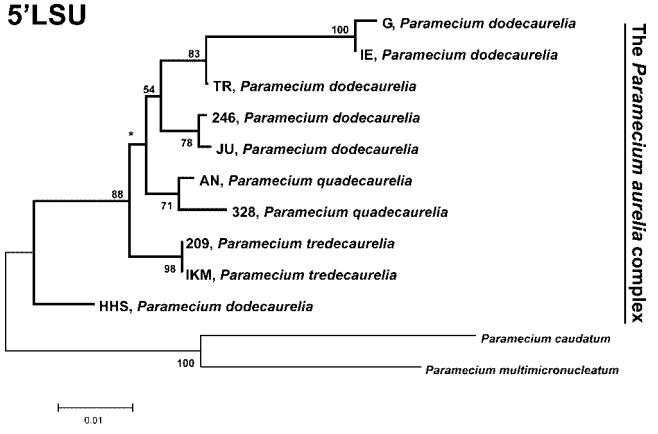

Fig. 4. Phylogenetic tree constructed for 12 strains of the *P. aurelia* complex with a single strain of *P. caudatum* and *P. multimicronucleatum* as outgroups based on a comparison of sequences the 5' end of large subunit rRNA fragment using the NJ (neighbor joining) method with the application of the Kimura two-parameter correction model. Bootstrap values are presented as percentages for 1000 comparisons. In the case of bootstrap values less than 50, the asterisks appear.

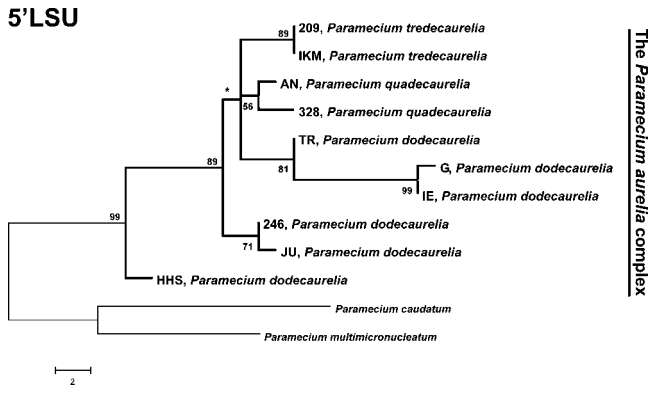

Fig. 5. Phylogenetic tree constructed for 12 strains of the *P. aurelia* complex with a single strain of *P. caudatum* and *P. multimicronucleatum* as outgroups based on comparison of sequences 5' end of large subunit rRNA fragment using MP (maximum parsimony). Bootstrap values are presented as percentages for 1000 comparisons. In the case of bootstrap values less than 50, the asterisks appear.

Table 3a. Polymorphisms in 3' SSU rRNA-ITS1 studied fragment.

	# 73	# 86	# 90	# 92	# 105	# 106	# 108	# 137	# 152	# 156	# 157	# 158	# 160	# 164	# 165	# 175	# 178	# 180	# 181	# 184	# 186	# 188	# 189
246, *P. dodecaurelia*	T	G	A	C	T	T	A	A	C	T	C	-	C	T	T	C	G	C	T	A	T	T	A
G, *P. dodecaurelia*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	A	.
HHS, *P. dodecaurelia*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	A	.	.	.	.
IE, *P. dodecaurelia*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	A	.
JU, *P. dodecaurelia*	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.
TR, *P. dodecaurelia*	.	.	.	.	.	.	.	.	T	.	.	.	.	.	C	.	.	T	.	.	.	.	.
209, *P .tredecaurelia*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	A	.
IKM, *P. tredecaurelia*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	A	.
328, *P. quadecaurelia*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	T	-	.	.	A	.
AN, *P. quadecaurelia*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	T	-	.	.	A	.
P. caudatum	.	T	.	A	A	-	C	G	T	.	T	A	.	.	.	.	.	.	A	.	.	.	.
P. multimicronucleatum	C	T	.	.	.	.	G	.	.	C	T	A	A	A	.	T	A	T	.	-	A	A	C

We suppose that there are some sites in rDNA fragments (e.g. #108 in 3'SSU rRNA-ITS1 fragment and #150 in 5'LSU fragment) which could be recognized as characteristic markers for variability in genus *Paramecium*, as they appeared in the studied *P. aurelia* species as well as *P. caudatum* and *P. mulitmicronucleatum* (Tables 3a, b).

Trees

A gene phylogeny constructed on the basis of the 3'SSU-ITS1 fragment of rRNA, using the NJ method (Fig. 2), reveals a very close relationship of *P. quadecaurelia* strains (328 and AN), strains of *P. tredecaurelia* also appear together (IKM and 209), while *P. dodecaurelia* strains are scattered: European strains G and IE cluster with *P. tredecaurelia*, the other European strain TR is in a different group with the strain JU (Japan), 246 (USA), HHS (Hawaii). They are paraphyletic.

A phylogeny reconstructed on the basis of the 5' LSU fragment of rRNA using the NJ method similarly discriminates between *P. caudatum*, *P. multimicronucleatum* and the species of the *P. aurelia* complex. Strains of *P. dodecaurelia* are scattered, European strains appear together: G, IE and TR, and together strains 246 (USA) and JU (Japan). The strain HHS (Hawaii) is distant. Strains of *P. tredecaurelia* and *P. quadecaurelia* appear in two species specific clusters (Fig. 4).

Analysis was also performed using MP (Maximum Parsimony) (Figs 3, 5). Figure 3 presents relationship of species based on analysis of the 3'SSU rRNA - ITS1 fragments of rRNA. *P. caudatum* and *P. multimicronucleatum* are distant, *P. quadecaurelia* strains cluster together. Again strains of *P. dodecaurelia* are scattered, European strains G and IE are close, as well as strains from Japan (JU) and USA (246), but strain TR and HHS are less related. Strains of *P. tredecaurelia* (209 and IKM) form one cluster with strains G and IE of *P. dodecaurelia.* Analysis of LSU rRNA (Fig. 5) shows that strains of *P. tredecaurelia* and *P. quadecaurelia* form their own clusters. Strains of *P. dodecaurelia* appear in different clusters.

DISCUSSION

Coleman (2005) recently studied relationships between species of the *P. aurelia* complex based on the ITS region of the nuclear ribosomal cistron in 13 species. According to this author, polymorphic sites should not be numerous in the ITS region (non coding fragment of rDNA) of the species of the *P. aurelia* complex. This was confirmed by our analysis of a 120 bp long ITS1 fragment in which only 6 polymorphic sites were found, mainly within *P. dodecaurelia*.

The sequences we obtained for *P. dodecaurelia*, *P. tredecaurelia*, and *P. quadecaurelia* were compared

Table 3b. Polymorphisms in 5' LSU rRNA studied fragment.

	# 104	# 133	# 136	# 139	# 147	# 149	# 150	# 151	# 155	# 158	# 165	# 166	# 167	# 171	# 192	# 193	# 195	# 196	# 197	# 201	# 203	# 205	# 207	# 212	# 213
246, *P. dodecaurelia*	G	A	T	A	A	T	T	A	A	T	T	G	C	G	G	C	G	T	A	C	G	G	C	T	G
G, *P. dodecaurelia*	.	.	.	.	.	.	.	T	.	C	.	.	.	.	A	T	.	.	.	T	A	.	.	.	C
HHS, *P. dodecaurelia*	.	.	C	G	G	C	.	T	T	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.
IE, *P. dodecaurelia*	.	.	.	.	.	.	.	T	.	C	.	.	.	.	A	T	.	.	.	T	.	.	.	.	C
JU, *P. dodecaurelia*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
TR, *P. dodecaurelia*	.	.	.	.	.	.	.	T	.	C	.	.	.	A	.	.	.		.	.	.	.	.	.	.
209, *P. tredecaurelia*	.	.	.	.	.	.	.	T	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.
IKM, *P. tredecaurelia*	.	.	.	.	.	.	.	T	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.
328, *P. quadecaurelia*	.	.	.	.	.	.	.	T	.	.	.	.	T	A	.	.	A	.	.	.	.	A	.	.	.
AN, *P. quadecaurelia*	.	.	.	.	G	.	.	T	.	.	.	.	.	A	.	.	.	.	.	.	.	A	.	.	.
P. caudatum	.	G	C	.	G	C	A	T	T	C	A	T	T	.	.	.	.	G	T	.	.	.	T	A	.
P. multimicronucleatum	A	G	.	.	G	C	G	T	T	C	.	.	.	A	.	.	.	.	.	.	.	.	T	A	.

	# 214	# 216	# 225	# 227	# 228	# 246	# 248	# 250	# 253	# 265	# 267	# 272	# 273	# 281	# 284	# 287	# 288	# 289	# 290	# 291	# 301	# 311	# 314	# 316	# 318
246, *P. dodecaurelia*	C	G	G	G	T	C	C	C	G	C	A	G	G	C	T	C	G	C	T	G	G	C	G	C	G
G, *P. dodecaurelia*	.	.	.	A	.	.	.	.	.	T	.	A	.	.	C	.	.	.	.	.	A	.	.	.	.
HHS, *P. dodecaurelia*	T	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
IE, *P. dodecaurelia*	.	.	.	A	.	.	.	.	.	T	.	A	.	.	C	.	.	.	.	.	A	.	.	.	.
JU, *P. dodecaurelia*	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.
TR, *P. dodecaurelia*	.	.	.	A	.	.	.	.	.	.	.	A	.	.	C	.	.	.	.	.	.	.	.	.	.
209, *P. tredecaurelia*	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	T	.	T	.
IKM, *P. tredecaurelia*	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	T	.	T	.
328, *P. quadecaurelia*	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AN, *P. quadecaurelia*	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. caudatum	T	A	.	.	C	.	.	T	.	.	G	A	.	T	C	T	A	T	C	.	.	T	A	T	.
P. multimicronucleatum	T	A	A	.	C	T	T	T	A	.	G	.	C	.	.	T	.	.	.	A	.	T	A	T	A

with a similar fragment studied by Coleman (2005) in 18 strains representing different species of the *P. aurelia* complex. The six polymorphic sites which appeared in our strains of *P. dodecaurelia* were also found in all species of the *P. aurelia* complex studied by Coleman. They appears in single stranded nucleotides positions (except #152).

Coleman (2005) used only one or two strains representing the particular species, we were able to use up to six strains in the case of the very polymorphic *P. dodecaurelia*. It is worth emphasizing that our strains of *P. dodecaurelia* originate from distant and geographically isolated collecting sites making intra-specific differentiation possible.

Nanney *et al.* (1998) compared sequence differences in a variable D2 domain of 23 rRNA (190 bp long) among several species of the *P. aurelia* complex. They found that genetic species in the *P. aurelia* complex are separated from each other by one or more site changes "but constitute a dense evolutionary cluster".

The 5' LSU rRNA fragment seems very useful in studies concerning relationships within species of the *P. aurelia* complex as well as within the genus. Similar studies using 5'LSU rRNA have also been carried out within *P. novaurelia* (Tarcz unpublished), however, only a few polymorphic sites were found and differences between strains were not so striking as those found within *P. dodecaurelia*. This may be caused by different characteristics of the species and different distances between places of strain origin in the *P. novaurelia* and *P. dodecaurelia* comparison. The first species is known mainly from Europe, so the divergence between strains

is not as great as between strains of *P. dodecaurelia* (from Europe, Hawaii, Japan, USA).

Here we have shown that much polymorphism exists within *P. dodecaurelia*. Our studies confirmed previous results showing exceptional intra-specific differentiation in this species when compared to other species of the *P. aurelia* complex. A high level of intra-specific polymorphism has also been shown by the application of RAPD, RFLP, and ARDRA methods (Przyboś *et al.* 2006b). It seems that intra-specific differentiation within *P. dodecaurelia* is as great as that between different species of the *P. aurelia* complex. Studies in which hsp70 (Hori *et al.* 2006) and H4 histone (Przyboś *et al.* 2006a) genes were sequenced also showed the isolated (distant) position of the species within the phylogenetic tree constructed for species of the *P. aurelia* complex. There are no strong correlation between the geographical origin and molecular differentiation of *P. dodecaurelia* strains, however, some kind of correlation exists, e.g. strain HHS from Hawaii is very distant from the other strains of the species, and the European strains (G, IE, TR) are in one cluster (5'LSU rRNA fragment).

A gene phylogeny constructed on both studied fragments rDNA reveals a very close relationship of *P. quadecaurelia* (328 and AN) and of *P. tredecaurelia* strains (IKM and 209) in the present paper. Similarly, studies of cytosol-type *hsp70* (Przyboś *et al.* 2003b) also revealed a close cluster of *P. quadecaurelia* and *P. tredecaurelia*. It is worth to mention that two strains of *P. quadecaurelia* show 99% similarity in cytosol-type *hsp70* and in studied presently 5' LSU rRNA fragment.

The 5'LSU rRNA fragment is a variable region at the species level and even within species as in the case of *P. dodecaurelia*. At present however, it is impossible to check if it is a good marker for phylogenetic studies (as the ITS1 fragment is) because no comparative data are available in the other *Paramecium* species. It seems worth testing its usefulness also at higher taxonomic levels in Ciliophora. Analysis of the 5' fragment of the LSU rRNA is the first at the intraspecific level in the species of the *P. aurelia* complex. Previous studies (cf Strüder-Kypke 2000a, b; Coleman 2005) concerned relations between the particular genera within Oligohymenophorea, between species within genus *Paramecium*, and between the particular species of the *P. aurelia* complex in which the fragments SSU rRNA, ITS1-5.8S, and ITS1 were analysed. However, there is no resolution in the ITS1 trees, concerning *P. dodacaurelia*. In future studies we are going to use the ITS2 fragment as well for comparison of intraspecific differentiation of *P. dodacaurelia* strains. Probably, strains of that species are some kind of subspecies?

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