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First American Stand of *Paramecium novaurelia* and Intra-specific Differentiation of the Species*

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A stand of *Paramecium novaurelia* was found in Boston Massachusetts, USA, the first on this continent. Molecular studies (RAPD and sequencing of rRNA [3'SSUrRNA-ITS1, 5' LSU rRNA] and COI mtDNA fragments) of *P. novaurelia* strains show intra-specific polymorphism within the species as strain clusters characterized by variable relationships.

Key words: *Paramecium aurelia* species complex, intra-specific differentiation, breeding system, geographical distribution, RAPD–PCR fingerprinting, sequencing of SSU-ITS1, LSU rRNA and COI mtDNA fragments.

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Among the 15 species of the *Paramecium aurelia* complex known world-wide (SONNEBORN 1975; AUFDERHEIDE *et al*. 1983), the majority also occur in the USA. The occurrence of some (*P. undecaurelia* and *P. sonneborni*) seems to be limited to this territory, other species (*P. primaurelia, P. biaurelia, P. tetraurelia, P. sexaurelia*) are cosmopolitan (SONNEBORN 1975; PRZYBOŚ & FOKIN 2000) or confined also to other territories (e.g. *P. septaurelia,* cf PRZYBOŚ *et al*. 2004, 2005).

P. novaurelia was regarded as restricted to Europe alone (SONNEBORN 1975) and a dominant species there (PRZYBOŚ & FOKIN 2000), later (PRZYBOŚ 1998) it was also recorded in Asia (Turkey, Anatolian Upland). This species has never been found again outside of Europe, in spite of numerous studied samples originating from different places around the world (cf PRZYBOŚ *et al*. 2006a). This is the first American record of *P. novaurelia*, identified in a sample collected in Boston, USA. A comparative analysis of molecular features of the American, European, and Asiatic strains of this species is also performed.

Material and Methods

Material

The strains designated USB1-4 were collected by I. Skoblo in Boston, USA from a natural ponds in 1994; the YR strain originated from Yellowstone National Park, Yellowstone River, USA, the water sample was collected by C. Vanini in 2004, and the strain was established by S. Fokin; the BR strains from Baton Rouge, USA were collected by S. Fokin in 2004.

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

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were used: strain 90 of *P. primaurelia*; strain Rieff, Scotland of *P. biurelia*; strain 87 of *P. pentaurelia;* strain 510 of *P. novaurelia.*

2. Strain crosses

In the intra and inter-strain crosses, the F_1 generation was obtained by conjugation and F_2 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

Paramecium genomic DNA was isolated (200 μl of cell culture was used for DNA extraction) from vegetative cells at the end of the exponential phase using the Qiamp DNA Kit (Qiagen™, Germany) as described by PRZYBOŚ *et al*. (2003a). All strains used for analysis are listed in Table 1.

a. Amplification of ribosomal DNA (rDNA)

The primers used for PCR reactions are listed in Table 2, they amplified two regions of rDNA: the 3' end of SSU rRNA – the ITS1 fragment (about 300 bp) and the 5' end of a fragment of LSU (450bp). One of the primers – LSU_R has the same sequence as in JEROME and LYNN (1996). The

Table 1

Paramecium spp. strains used in molecular studies

Species	Strain designation	Geographical origin	References	Accesion numbers		
				ITS1	5'LSU	COI
P. novaurelia	510	Great Britain, Edinburgh	BEALE & SCHNELLER 1954	DQ837976	DQ837974	DQ837975
P. novaurelia	CVH	Czech Republic, Eastern Sudetes Mts, Nizky Jesenik Mts	PRZYBOŚ & KOMALA 1992	DQ837980	DQ837978	DQ837979
P. novaurelia	FLU	France, Lafilorierre	BEALE & SCHNELLER 1954	DQ837983	DQ837981	DQ837982
P. novaurelia	PB	Poland, Białowieża	KOMALA *et al*. 1960	DQ837986	DQ837984	DQ837985
P. novaurelia	PO	Poland, Carpathians, Orava Valley, Lipnica Wielka	PRZYBOŚ & KOMALA 1996	DQ837989	DQ837987	DQ837988
P. novaurelia	PSO	Poland, Sudetes Mts, Orlickie Plateau, Kudowa	KOMALA & PRZYBOŚ 1989	DQ837992	DQ837990	DQ837991
P. novaurelia	SB	Spain, Pyrenees, las Bassotes village	PRZYBOŚ 1991	DQ837995	DQ837993	DQ837994
P. novaurelia	TB	Turkey, Beysahir	PRZYBOŚ 1998	DQ837998	DQ837996	DQ837997
P. novaurelia	USB	United States, Boston	Present paper	DQ838001	DQ837999	DQ838000
P. caudatum	PC	Cyprus, Akamas	TARCZ *et al*. 2006	DQ207387	DQ207375	DQ837977

Table 2

Primers used in this study

Amplified region	Primer	Sequence 5'-3'	References
3'SSU rDNA – ITS1	ITS1_F	5'-TAAACCTTATCACTTAGAGGA-3	TARCZ *et al*. 2006
	ITS1_R	5'-CGAAAATCTAATGTCTCGCA-3'	TARCZ *et al*. 2006
5'LSU rDNA	LSU_F	5'-CCCGTATTTGGTTAGGACT-3'	TARCZ *et al*. 2006
	LSU_R	5'-TTGGTCCGTGTTTCAAGACG-3'	JEROME & LYNN, 1996
CO I	CoxL11058	5'-TGATTAGACTAGAGATGGC-3'	BARTH *et al*. 2006
	CoxH10176	5'-GAAGTTTGTCAGTGTCTATCC-3'	BARTH *et al*. 2006

other 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 based on unpublished sequences of the ITS2 - 5'LSU fragment of *Paramecium dodecaurelia*. PCR amplification was carried out in a final volume of 30 μl containing: 2 μl of DNA, 1.5 U Taq-Polymerase (Qiagen™, Germany), 0.6 μl 10mM of each primer, 10x PCR buffer, 0.6 μl 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 (VI ™ Roche, France).

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

To amplify the CO I region (880bp) of mitochonrial 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 regions (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 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 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). Sequencing 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 study sequences was 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) and MP (Maximum Parsimony). The NJ analysis was performed using a KIMURA 2-parameter correction model (KIMURA 1980) by bootstrapping with 100 replicates (FELSENSTEIN 1985). The MP analysis was evaluated with Min-mini heuristic parameter (level =2) and bootstrapping with 100 replicates.

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 nucleotide sequence: 5' –GCAGAGAAGG- 3', using Taq polymerase (Qiagen). This primer was selected from STOECK & SCHMIDT (1998) after testing several dozen oligonucleotide primers because it gave "robust band patterns" in the *P. aurelia* species complex. It was also used in other studies carried out on the *P. aurelia* species complex (STOECK *et al*. 1998, 2000) and on *P. jenningsi* strains (PRZYBOŚ *et al*. 1999, 2003a; SKOTARCZAK *et al*. 2004 a, b) and *P. schewiakoffi* (FOKIN *et al*. 2004). RAPD-PCR was done in a Biometra thermocycler, products of PCR reactions were separated by electrophoresis in 1.5% agarose gels for 3.5 h at 85V together with a molecular weight marker VI™ (Roche™, France), 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++ ™ 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 strain from Boston, USA (USB1) originating from population AB8 was identified as *P. novaurelia* on the basis of strong conjugation with the standard strain of this species. A high percentage of surviving clones was observed in F_1 (86%) and F_2 (84%) generations of inter-strain crosses of the USB1 strain with the standard strain (510) from Scotland. This is the first stand of the species in the USA.

The other strains collected from the same locality and originating from one population AB7, designated USB2, USB3, and USB4 were identified as *P. biaurelia* on the basis of strong conjugation with the standard strain of the species.

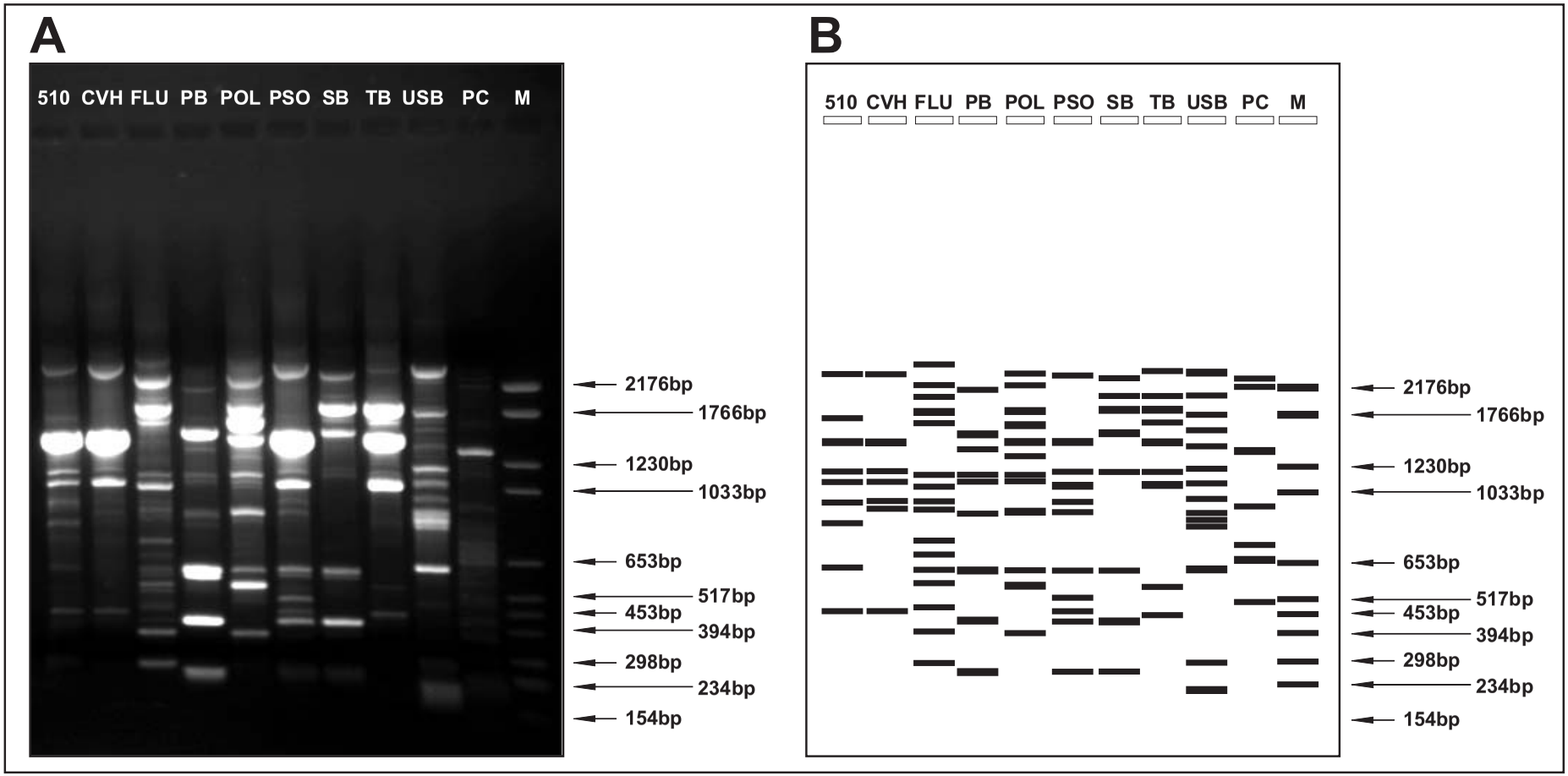

Fig. 1a. RAPD fingerprints (revealed by primer Ro 460-04) of the studied strains of *Paramecium novaurelia:* 510 – strain from Scotland, Edinburgh; CVH – strain from Czech Republic, Nizky Jesenik Mts; FLU – strain from France, Lafilorierre; PB – strain from Poland, Białowieża; POL – strain from Poland, Orava Valley; PSO – strain from Poland, Orlickie Plateau; SB – strain from Spain, las Bassotes village; TB – strain from Turkey, Beysahir; USB – strain from United States, Boston; *Paramecium caudatum*: PC – strain from Cyprus, Akamas. M – molecular weight marker (VI, Roche ™).
Fig. 1b. Schematic representation of Fig.1a showing specific band patterns representing different genotypes as revealed by RAPD-fingerprints.

The strain YR, USA was identified as *P. biaurelia* on the basis of strong conjugation with the standard strain of the species. This is a cosmopolitan species.

The paramecia collected in Baton Rouge, Louisiana, USA were identified as *Paramecium multimicronucleatum* on the basis of analysis of the type and number of their micronuclei (VIVIER 1974) on slides stained using aceto-carmine and Giemsa's stain (after fixation and hydrolysis, cf PRZYBOŚ 1978).

RAPD-PCR analysis

Fingerprints (band patterns) of the studied *P. novaurelia* strains (the new one from the USA and the other selected strains) and the *P. caudatum* strain, revealed by DNA amplification with primer Ro 460-04, are presented in Figs 1a,b. Polymorphism within *P. novaurelia* is shown as several groups of genotypes. Strains from Scotland and the Czech Republic (510 and CVH) show about 60% similarity of band patterns, strains from Poland (PB, POL, PSO) and Spain (SB) compose the next group, the strain from the USA (USB) has a somewhat isolated position as its band pattern shows about 30% similarity to the previous strain groups, and strains from France and Turkey (FLU and TB) compose the next strain group. The *P. caudatum* band pattern is completely different from the patterns seen in *P. novaurelia* (Fig. 2).

Analysis of rDNA fragments and COI mtDNA gene

Results are generally similar, independent of the sequenced gene fragment.

Analysis of fragments of COI mtDNA (660bp), 3'SSU- ITS1 rDNA (210bp), and 5' LSU rDNA (350bp) fragments by NJ and MP methods revealed the existence of three main strain clusters (Figs 3-6), one cluster composed of strains from Scotland, Poland, USA and the second composed of strains from Spain, the Czech Republic, Turkey and again one strain from Poland, as well as a third cluster containing the strain from France. *P. caudatum* was used an an outgroup.

Ribosomal DNA analysis shows 24 haplotypes within *P. novaurelia* strains, 5 in the 3'SSU-ITS1 fragment and 19 in the 5' LSU fragment (Table 3a). The genetic distance between all the studied strains (*P. novaurelia* and *P. caudatum*) in the first ribosomal fragment is at the level of 6.2%, and between *P. novaurelia* as 2.4%. The 5'LSU fragment reveals higher diversity with 11.1% divergence between *P. novaurelia* and *P. caudatum* strains, and 5.4% divergence among *P. novaurelia* strains. Generally, 9 polymorphisms appear

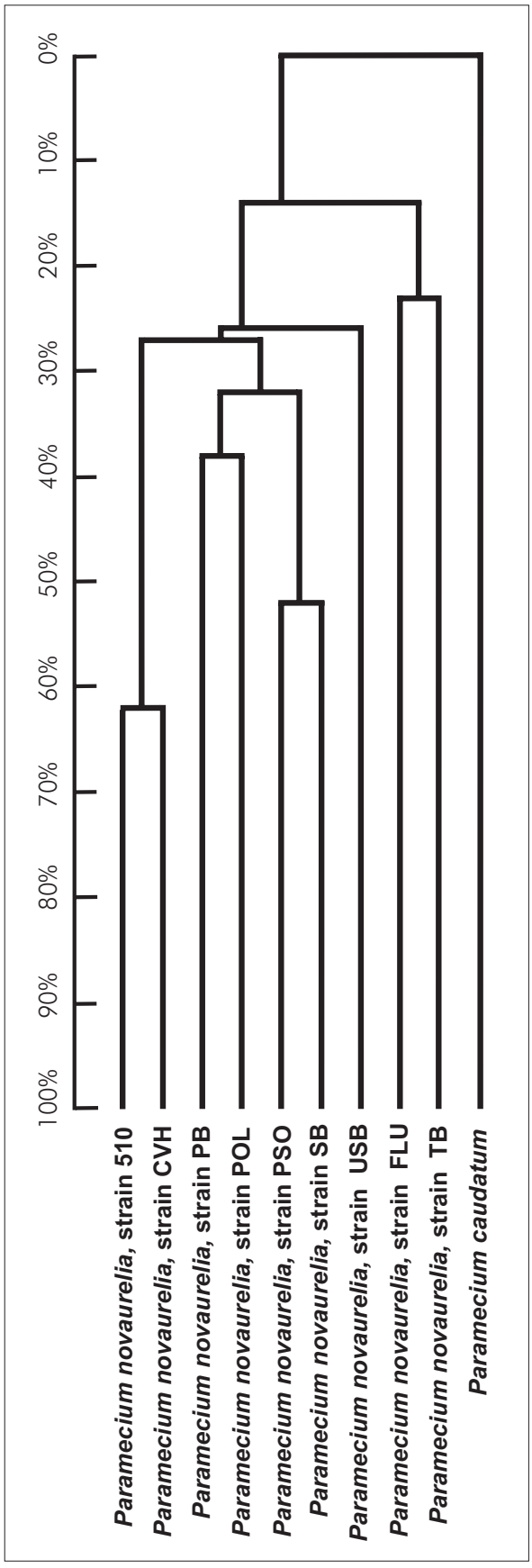

Fig. 2. Tree diagram of the cluster analysis of the RAPD fingerprint pattern similarity matrix of the studied *P. novaurelia* strains. Method used for analysis was UPGMA. Similarity index was calculated according to NEI and LI (1979).

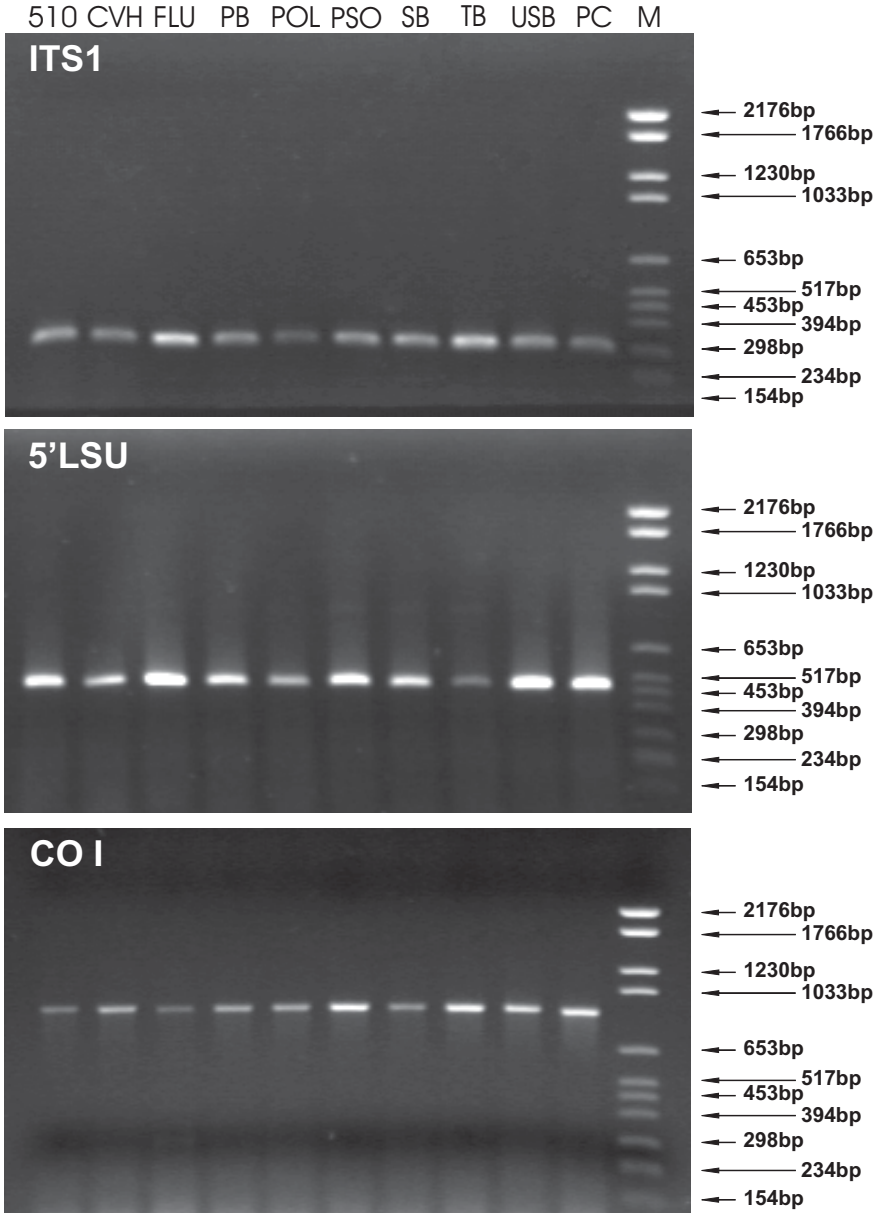

Fig. 3. Gel presenting the examined fragments: ITS1 – 3' end of the small subunit rRNA-internal transcribed spacer 1 fragment (310bp); 5'LSU – 5'end of the large subunit rRNA fragment (450bp); COI – fragment of COI gene (880bp). Designation of strains are the same as in Fig. 1a.

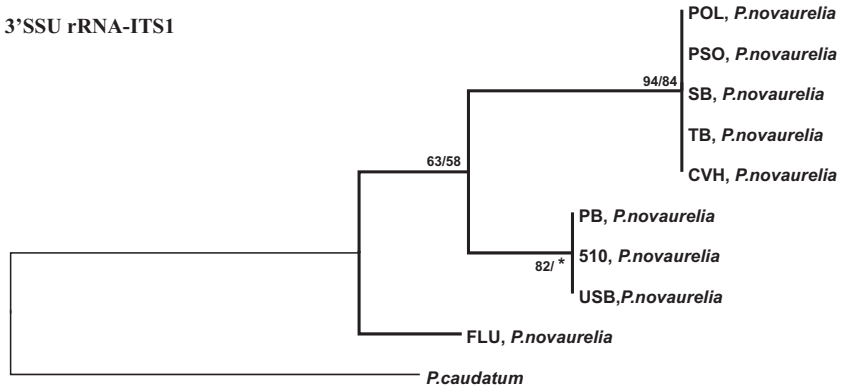

Fig. 4. Phylogenetic tree constructed for 9 strains of *P. novaurelia* and a single strain of *P. caudatum* as outgroup, based on a comparison of sequences from the 3' end of the small subunit rRNA-internal transcribed spacer 1 fragment using the NJ (neighbor joining) method with the application of the KIMURA two-parameter correction model and MP (maximum parsimony) analysis. Bootstrap values are presented as percentages (NJ/MP) for 100 comparisons. In the case of bootstrap values less than 50, the asterisk appears.

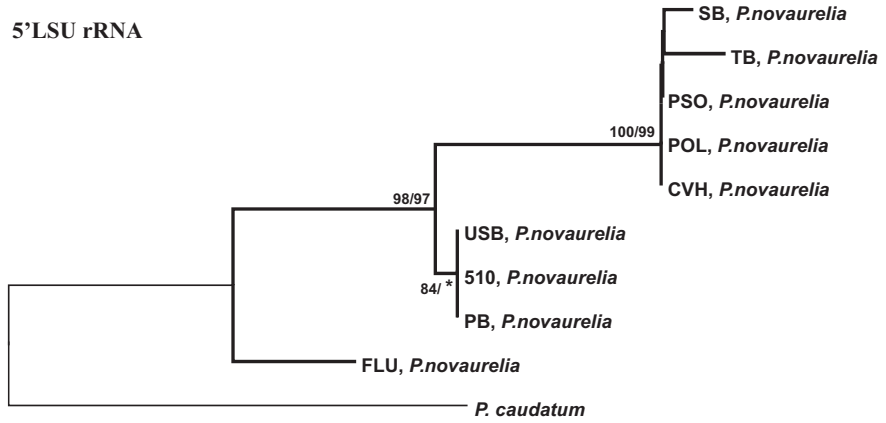

Fig. 5. Phylogenetic tree constructed for 9 strains of *P. novaurelia* and a single strain of *P. caudatum* as outgroup, based on a comparison of sequences from the 5'end of the large subunit rRNA fragment using the NJ (neighbor joining) method with the application of the KIMURA two-parameter correction model and MP (maximum parsimony) analysis. Bootstrap values are presented as percentages (NJ/MP) for 100 comparisons. In the case of bootstrap values less than 50, the asterisk appears.

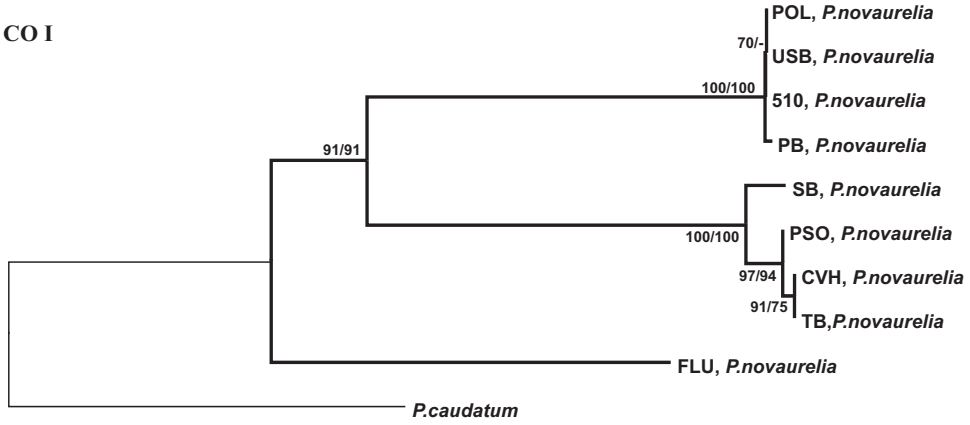

Fig. 6. Phylogenetic tree constructed for 9 strains of *P. novaurelia* and a single strain of *P. caudatum* as outgroup, based on a comparison of sequences from the fragment of CO I gene using the NJ (neighbor joining) method with the application of the KIMURA two-parameter correction model and MP (maximum parsimony) analysis. Bootstrap values are presented as percentages (NJ/MP) for 100 comparisons.

Table 3a

Polymorphisms in 3'SSU rRNA – ITS1 and 5'LSU rRNA fragments of *P. novaurelia*

Strain designation	# 152	# 165	# 180	# 181	# 188	# 136	# 139	# 147	# 149	# 155	# 158	# 171	# 192	# 193	# 201	# 203	# 213	# 214	# 216	# 227	# 265	# 272	# 284	# 301
510	***T***	**C**	**T**	**T**	**T**	**T**	**A**	**A**	**T**	**A**	**C**	**A**	**G**	**C**	**C**	**G**	**G**	**C**	**G**	**A**	**C**	**G**	**C**	**G**
CVH	**C**	**T**	**.**	**.**	**A**	**.**	**.**	**.**	**.**	**.**	**.**	**G**	**A**	**T**	**T**	**.**	**C**	**.**	**.**	**.**	**T**	**A**	**.**	**A**
FLU	**C**	**.**	**C**	**A**	**.**	**C**	**G**	**G**	**C**	**T**	**T**	**.**	**.**	**.**	**.**	**.**	**.**	**T**	**A**	**G**	**.**	**.**	**T**	**.**
PB	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**
POL	**C**	**T**	**.**	**.**	**A**	**.**	**.**	**.**	**.**	**.**	**.**	**G**	**A**	**T**	**T**	**.**	**C**	**.**	**.**	**.**	**T**	**A**	**.**	**A**
PSO	**C**	**T**	**.**	**.**	**A**	**.**	**.**	**.**	**.**	**.**	**.**	**G**	**A**	**T**	**T**		**C**	**.**	**.**	**.**	**T**	**A**	**.**	**A**
SB	**C**	**T**	**.**	**.**	**A**	**.**	**.**	**.**	**.**	**.**	**.**	**G**	**A**	**T**	**T**	**A**	**C**	**.**	**.**	**.**	**T**	**A**	**.**	**A**
TB	**C**	**T**	**.**	**.**	**A**	**.**	**.**	**.**	**.**	**.**	**.**	**G**	**A**	**T**	**T**		**C**	**.**	**.**	**.**	**T**	**A**	**.**	**A**
USB	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**
	3'SSU rRNA – ITS1					5'LSU rRNA																		

Table 3b

Polymorphisms in COI mtDNA fragment of *P. novaurelia*

	# 8	# 11	# 14	# 17	# 20	# 23	# 29	# 32	# 38	# 40	# 41	# 44	# 47	# 50	# 53	# 56	# 62	# 65	# 68	# 71	# 77	# 80	# 89	# 95	# 98	# 107	# 122	# 125	# 128	# 129	# 131	# 132	# 134	# 155	# 158	# 161	# 164	# 167	# 170	# 173	# 176
P. novaurelia, strain 510	***A***	**T**	**G**	**A**	**C**	**C**	**A**	**T**	**C**	**A**	**C**	**C**	**G**	**T**	**A**	**C**	**A**	**T**	**A**	**G**	**T**	**T**	**A**	**T**	**T**	**C**	**C**	**T**	**T**	**T**	**G**	**C**	**A**	**G**	**G**	**C**	**T**	**T**	**A**	**A**	**T**
P. novaurelia, strain CVH	**G**	**.**	**T**	**C**	**.**	**T**	**.**	**C**	**A**	**.**	**T**	**T**	**A**	**A**	**.**	**.**	**G**	**C**	**.**	**.**	**.**	**A**	**G**	**C**	**C**	**T**	**G**	**C**	**C**	**.**	**A**	**T**	**G**	**A**	**A**	**T**	**C**	**.**	**.**	**G**	**C**
P. novaurelia, strain FLU	**T**	**G**	**A**	**T**	**T**	**T**	**T**	**C**	**T**	**.**	**T**	**T**	**A**	**.**	**C**	**T**	T	**.**	**G**	**A**	**C**	**A**	**G**	**.**	**C**	**T**	**A**	**C**	**.**	**C**	**T**	**T**	**.**	**.**	**.**	**G**	**A**	**A**	**G**	**.**	**.**
P. novaurelia, strain PB	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**
P. novaurelia, strain POL	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**
P. novaurelia, strain PSO	**.**	**.**	**.**	**C**	**.**	**T**	**.**	**C**	**A**	.	**T**	**T**	**A**	**A**	**.**	**.**	**G**	**C**	**.**	**.**	**.**	**A**	**G**	**C**	**C**	**T**	**G**	**C**	**C**	**.**	**A**	**T**	**G**	**A**	**A**	**T**	**C**	**.**	**.**	**G**	**C**
P. novaurelia, strain SB	**.**	**.**	**.**	**C**	**.**	**T**	**.**	**C**	**A**	—	**T**	**T**	**A**	**A**	**.**	**.**	**G**	**C**	**.**	**.**	**.**	**A**	**G**	**C**	**C**	**T**	**G**	**C**	**C**	**.**	**A**	**T**	**G**	**A**	**A**	**T**	**C**	**.**	**G**	**G**	**C**
P. novaurelia, strain TB	**.**	**.**	**T**	**C**	**.**	**T**	**.**	**C**	**A**	**.**	**T**	**T**	**A**	**A**	**.**	**.**	**G**	**C**	**.**	**.**	**.**	**A**	**G**	**C**	**C**	**T**	**G**	**C**	**C**	**.**	**A**	**T**	**G**	**A**	**A**	**T**	**C**	**.**	**.**	**G**	**C**
P. novaurelia, strain USB	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**

	# 179	# 182	# 185	# 188	# 191	# 200	# 203	# 206	# 209	# 218	# 219	# 221	# 224	# 227	# 230	# 239	# 245	# 246	# 248	# 251	# 254	# 263	# 266	# 269	# 284	# 287	# 290	# 293	# 299	# 302	# 308	# 317	# 326	# 332	# 335	# 338	# 341	# 344	# 345	# 347	# 350
P. novaurelia, strain 510	***C***	**T**	**G**	**C**	**T**	**A**	**A**	**T**	**T**	**C**	**C**	**C**	**T**	**G**	**C**	**T**	**A**	**A**	**G**	**A**	**A**	**T**	**T**	**T**	**G**	**T**	**G**	**T**	**C**	**T**	**G**	**C**	**A**	**T**	**T**	**A**	**C**	**C**	**A**	**C**	**C**
P. novaurelia, strain CVH	**.**	**C**	**.**	**.**	**.**	**.**	**G**	**A**	**C**	**T**	**.**	**T**	**C**	**.**	**T**	**C**	**.**	**.**	**.**	**C**	**G**	**.**	**C**	**C**	**.**	**C**	**.**	**.**	**T**	**C**	**A**	**T**	**G**	**C**	**C**	**G**	**G**	**.**	**.**	**.**	**.**
P. novaurelia, strain FLU	**T**	**.**	**A**	**T**	**C**	**G**	**.**	**C**	**.**	**T**	**T**	**G**	**C**	**A**	**.**	**.**	G	**G**	**A**	**G**	**G**	**C**	**C**	**.**	**A**	**.**	**A**	**C**	**T**	**.**	**A**	**.**	**.**	**A**	**C**	**G**	**A**	**.**	**T**	**T**	**T**
P. novaurelia, strain PB	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**
P. novaurelia, strain POL	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**
P. novaurelia, strain PSO	**.**	**C**	**.**	**.**	**.**	**G**	**A**	**C**	T	**.**	**T**	**C**	**.**	**T**	**C**	**.**	**.**	**.**	**.**	**C**	**G**	**.**	**C**	**C**	**.**	**C**	**.**	**.**	**T**	**C**	**A**	**T**	**G**	**C**	**C**	**G**	**G**	**.**	**.**	**.**	**.**
P. novaurelia, strain SB	**.**	**C**	**.**	**.**	**.**	**G**	**A**	**C**	**T**	**.**	**T**	**C**	**.**	**T**	**C**	**.**	**.**	**.**	**.**	**T**	**G**	**.**	**C**	**C**	**.**		**.**	**.**	**T**	**C**	**A**	**T**	**G**	**C**	**C**	**G**	**G**	**T**	**G**	**.**	**.**
P. novaurelia, strain TB	**.**	**C**	**.**	**.**	**.**	**G**	**A**	**C**	**T**	**.**	**T**	**C**	**.**	**T**	**C**	**.**	**.**	**.**	**.**	**C**	**G**	**.**	**C**	**C**	**.**	**C**	**.**	**.**	**T**	**C**	**A**	**T**	**G**	**C**	**C**	**G**	**G**	**.**	**.**	**.**	**.**
P. novaurelia, strain USB	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**

	# 353	# 356	# 359	# 362	# 368	# 372	# 374	# 381	# 383	# 389	# 392	# 395	# 401	# 404	# 407	# 411	#413	# 416	# 419	# 420	# 422	# 425	# 437	# 440	# 444	# 449	# 455	# 458	# 461	# 464	# 467	# 476	# 479	# 482	# 485	# 488	# 494	# 497	# 500	# 503	# 512
P. novaurelia, strain 510	***C***	**C**	**C**	**T**	**A**	**C**	**A**	**C**	**A**	**C**	**C**	**C**	**T**	**C**	**C**	**T**	**G**	**G**	**T**	**C**	**A**	**T**	**T**	**T**	**A**	**A**	**A**	**A**	**T**	**T**	**G**	**G**	**T**	**G**	**T**	**T**	**T**	**A**	**A**	**G**	**A**
P. novaurelia, strain CVH	**.**	**T**	**T**	**C**	**G**	**T**	**.**	**T**	**.**	**T**	**.**	**T**	**C**	**.**	**T**	**.**	**.**	**.**	**C**	**.**	**C**	**.**	**.**	**G**	**C**	**G**	**G**	**C**	**C**	**C**	**C**	**A**	**C**	**.**	**.**	**.**	**.**	**G**	**T**	**A**	**T**
P. novaurelia, strain FLU	**T**	**T**	**.**	**C**	**C**	**.**	**C**	**.**	**T**	**T**	**T**	**T**	**.**	**T**	**T**	**C**	T	**A**	**C**	**T**	**G**	**A**	**A**	**C**	**.**	**.**	**G**	**C**	**.**	**A**	**.**	**A**	**C**	**A**	**C**	**A**	**A**	**.**	**T**	**A**	**.**
P. novaurelia, strain PB	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**
P. novaurelia, strain POL	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**
P. novaurelia, strain PSO	**.**	**T**	**T**	**C**	**G**	**T**	**.**	**T**	**.**	T	**.**	**T**	**C**	**.**	**T**	**.**	**.**	**.**	**C**	**.**	**C**	**.**	**.**	**G**	**C**	**G**	**G**	**C**	**C**	**C**	**C**	**A**	**C**	**.**	**C**	**.**	**.**	**G**	**T**	**A**	**T**
P. novaurelia, strain SB	**.**	**T**	**T**	**.**	**G**	**T**	**.**	**T**	**.**	**T**	**.**	**T**	**C**	**.**	**T**	**.**	**.**	**.**	**C**	**.**	**C**	**.**	**.**	**A**	**C**	**G**	**G**	**C**	**C**	**C**	**C**	**A**	**C**	**.**	**C**	**.**	**.**	**G**	**T**	**A**	**T**
P. novaurelia, strain TB	**.**	**T**	**T**	**C**	**G**	**T**	**.**	**T**	**.**	**T**	**.**	**T**	**C**	**.**	**T**	**.**	**.**	**.**	**C**	**.**	**C**	**.**	**.**	**G**	**C**	**G**	**G**	**C**	**C**	**C**	**C**	**A**	**C**	**.**	**C**	**.**	**.**	**G**	**T**	**A**	**T**
P. novaurelia, strain USB	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**

	# 515	# 524	# 527	# 530	# 542	# 545	# 548	# 551	# 554	# 557	# 560	# 563	# 564	# 566	# 572	# 573	# 575	# 578	# 581	# 582	# 587	# 593	# 596	# 599	# 602	# 608	# 611	# 614	# 617	# 620	# 621	# 623	# 626	# 632	# 638	# 639	# 641	# 644	# 647	# 650	# 653
P. novaurelia, strain 510	***C***	**C**	**A**	**T**	**T**	**T**	**T**	**T**	**T**	**G**	**T**	**C**	**C**	**C**	**A**	**G**	**T**	**T**	**T**	**T**	**A**	**C**	**C**	**G**	**A**	**C**	**T**	**C**	**C**	**C**	**C**	**C**	**A**	**G**	**C**	**T**	**G**	**T**	**T**	**T**	**C**
P. novaurelia, strain CVH	**A**	**.**	**T**	**C**	**.**	**C**	**C**	**A**	**C**	**.**	**.**	**T**	**T**	**A**	**.**	**.**	**C**	**C**	**C**	**C**	**T**	**T**	**T**	**A**	**C**	**T**	**C**	**A**	**.**	**T**	**.**	**.**	**C**	**A**	**G**	**C**	**.**	**A**	**A**	**A**	**T**
P. novaurelia, strain FLU	**A**	**T**	**.**	**.**	**G**	**.**	**C**	**A**	**A**	**.**	**C**	**A**	**.**	**T**	**T**	**T**	.	**.**	**.**	**.**	**.**	**.**	**T**	**C**	**C**	**T**	**.**	**T**	**T**	**T**	**T**	**A**	**T**	**.**	**T**	**C**	**A**	**G**	**C**	**C**	**.**
P. novaurelia, strain PB	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**A**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**
P. novaurelia, strain POL	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**
P. novaurelia, strain PSO	**A**	**.**	**T**	**C**	**.**	**.**	**C**	**A**	**C**	**.**	**.**	**T**	**T**	**A**	**.**	**.**	**C**	**C**	**C**	**C**	**T**	**T**	**T**	**A**	**C**	**T**	**C**	**A**	**.**	**T**	**.**	**.**	**C**	**A**	**G**	**C**	**.**	**A**	**A**	**A**	**T**
P. novaurelia, strain SB	**A**	**.**	**T**	**C**	**.**	**C**	**.**	**A**	**C**	**.**	**.**	**T**	**T**	**A**	**.**	**.**	**C**	**C**	**C**	**C**	**G**	**T**	**T**	**A**	**C**	**T**	**C**	**A**	**.**	**T**	**.**	**.**	**C**	**A**	**G**	**C**	**.**	**A**	**A**	**A**	**.**
P. novaurelia, strain TB	**A**	**.**	**T**	**C**	**.**	**C**	**C**	**A**	**C**	**.**	**.**	**T**	**T**	**A**	**.**	**.**	**C**	**C**	**C**	**C**	**T**	**T**	**T**	**A**	**C**	**T**	**C**	**A**	**.**	**T**	**.**	**.**	**C**	**A**	**G**	**C**	**.**	**A**	**A**	**A**	**T**
P. novaurelia, strain USB	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**

in both fragments, grouping the examined strains into two main clusters, and there are also 12 haplotypes characteristic only for the FLU strain.

COI mtDNA analysis**.** Much higher divergence was observed in the case of the COI mtDNA fragment, 215 haplotypes (for all studied strains) and 164 substitutions were found in the *P. novaurelia* strains comparison (Table 3b). The distance between *P. novaurelia* and *P. caudatum* strains is about 32.7%, and 24.8% among *P. novaurelia* strains.

When *P. novaurelia* was compared with the other *Paramecium* spp. (*P. caudatum, P. multimicronucleatum, P. schewiakoffi*) some variable positions characteristic for all studied species of the genus were found. A geographical correlation between genotypes was not observed, and most haplotypes occurred at the third codon position.

Discussion

Polymorphism within *P. novaurelia,* appearing as differentiated RAPD fingerprints (genotypes), was first revealed by STOECK *et al*. (2000). The genotypes seemed not to be closely associated with the geographical origin of the studied strains, also a high percentage of surviving clones in F_1 and F_2 generations was observed in inter-strain hybrids. Later, RAPD fingerprints of the newly identified strains of *P. novaurelia* from Russia were compared with band patterns of other European strains of the species and intra-specific polymorphism was confirmed (PRZYBOŚ *et al*. 2006b). Several genotypes within *P. novaurelia* were also observed in the present study when strains originating from different continents (North America, Europe, and Asia) were compared, and two main groups of strains were revealed (Figs 1a,b, 2). The band pattern characteristic for *P. novaurelia* is different from the band pattern of *Paramecium caudatum*.

STOECK *et al*. (1998, 2000) suggested that such polymorphism may be associated with a degree of inbreeding which is characteristic (SONNEBORN 1957; LANDIS 1986) for the species of the complex and may cause intra-specific differentiation. This correlation was confirmed by studies concerning polymorphism within *P. dodecaurelia* (PRZYBOŚ *et al*. 2005) and by studies carried out on several strains of other species of the *P. aurelia* complex (PRRZYBOŚ *et al*. 2006 a). Species characterized by inbreeding (e.g. *P. tetraurelia*, *P. dodecaurelia)* showed higher intra-specific polymorphism in band pattern than did species characterized by weak inbreeding such as *P. pentaurelia.*

Polymorphism within *P. novaurelia* revealed by the RAPD method was in turn confirmed and analyzed by comparison of rDNA and COI mt DNA gene sequences. As a result, the existence of various strain clusters within species was revealed, however they are different from those obtained by RAPD analysis, only the distinctness of the FLU strain is similar. Lack of correlation (revealed by analysis of rDNA fragments and COI fragment of mtDNA) between different strain genotypes and their distribution (geographical origin) is in consensus with previous studies carried out by STOECK *et al*. (2000) with application of a RAPD marker. Perhaps this may be connected with the manner of expansion (spreading) of paramecia around the world, they can be transported for long distances by birds (SONNEBORN 1957; COLEMAN 2005).

Intra-specific polymorphism within *P. novaurelia* was at a level of 2.4% in 3'SSU-ITS1 and 5.4% in 5'LSU fragments of rDNA as well as 13.9% divergence in the case of COI mtDNA. Many highly variable positions and polymorphic sites characteristic for the strain groups (Table 3b) were found in COI mtDNA in all studied strains of *P. novaurelia* as well as in other species of the *Paramecium* genus. Three polymorphic sites were localized in single stranded nucleotide positions of ITS1. A much higher divergence was observed in the case of the COI mtDNA fragment than in the rDNA fragments. It is worth mentioning that the fragment of COI mtDNA investigated here has been recently used as a bar-code, i.e a standard fragment of DNA appearing in the majority of living organisms enabling the analysis of phylogenetic relationships on low and high taxonomic levels (HEBERT *et al*. 2003). However, our analyses showed the limited usefulness of this mtDNA fragment for investigations of broad relationships, it seems useful on rather low taxonomic levels as revealed by the high variability at intra-specific or intra-population levels. Analysis of the COI mtDNA fragment revealed intra-specific differentiation not only in *Paramecium* but also in the butterfly *Astraptes fulgerator* (HEBERT *et al*. 2004), as the existence of ten cryptic species within the previous single species.

Information on intra-specific molecular diversity within *P. aurelia* species is rare. However, studies carried out within *P. dodecaurelia* showed that its intra-specific diversity was as high as that between different species of the *P. aurelia* complex based on analysis of sequences of rDNA fragments, i.e. six polymorphic sites were found in a fragment of rRNA at the 3' end of SSU and several sites at the 5' end of LSU (TARCZ *et al*. 2006). In *P. novaurelia* 24 polymorphisms appear in both rRNA fragments. Similarly, analysis of sequences of hsp70 (HORI *et al*. 2006) and the H4 histone (PRZYBOŚ *et al*. 2006c) genes also showed the isolated position of *P. dodecaurelia* within the tree constructed for species of the *P. aurelia* complex. Intra-specific differentiation within *P. novaurelia* is not as substantial as that existing within

P. dodecaurelia. Intra-specific polymorphism was also studied in *P. quadecaurelia* (PRZYBOŚ *et al*. 2003b) by comparison of cytosol-type hsp 70 gene sequences but the base sequence of both strains were 99.2% identical.

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 intra-specific divergences up to 7% in *P. caudatum* and 9.5% in *P. multimicronucleatum.* 13.9% divergence in COI mtDNA was found in *P. novaurelia* strains in this study.

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. Intra-specific differentiation 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". Here, polymorphism within *P. novaurelia* was revealed. To evaluate the level of polymorphism in *P. novaurelia,* the variabilty should be compared with results of other studies carried out on the *P. aurelia* complex with the application of SSU rRNA gene sequences, e.g. *P. primaurelia* and *P. tetraurelia* differed by five nucleotides from each other (STRÜDER-KYPKE *et al*. 2000a,b). Another species of the *Paramecium* genus, *Paramecium schewiakoffi*, had 99.3% similarity with the sequences of *P. jenningsi* and a 98.9% similarity with sequences of *P. tetraurelia* and *P. primaurelia* (FOKIN *et al*. 2004) based on 18S rRNA sequences. 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 no more than two strains of the particular species. Hsp 70 gene sequences were also used by HORI *et al*. (2006) for comparison of species of the *P. aurelia* complex and *P. caudatum* syngens. They found that non-synonymous substitutions were frequent in *P. triaurelia*, *P. septaurelia*, *P. dodecaurelia* (11, 10, 5 respectively in these species), *P. novaurelia*, *P. tredecaurelia*, and *P. quadecaurelia* have also one non-synonymous substitution in the same position. However, the authors did not study intraspecific differentiation.

In the future, application of the ITS2 fragment of rRNA in investigations concerning the intraspecific differentiation within *Paramecium* species of the *aurelia* complex will bring new data.

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