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First Stand of *Paramecium octaurelia* in Europe and Molecular Characteristics of other Known Strains of this Species

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The first stand of *Paramecium octaurelia* in Europe (Germany) is described and interesting intra-specific polymorphism is compared within the species using strains originating from different continents (Europe, N. America and Asia). Sequenced fragments of 5' LSU rDNA and COI mtDNA revealed that the studied strains form two groups, one with strains from Germany and USA, and a second group from Israel.

Key words: *Paramecium aurelia* species complex, intra-specific polymorphism, distribution of species, sequencing of 5'LSU rDNA and COI mtDNA.

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Among the 15 species of the *P. aurelia* complex (SONNEBORN 1975; AUFDERHEIDE *et al*. 1983), *P. octaurelia* was known up to now from North America (USA) and Central America (Panama), Africa (Uganda) (SONNEBORN 1975), and Asia (Israel) (PRZYBOŚ *et al*. 2002). The present paper describes the first record of the species in Europe, although SONNEBORN's (1975) statement seems correct that *P. octaurelia* "…may be so around the world".

Since a new stand of the species was found in Europe, for the first time on this continent, a comparison of intra-specific polymorphism within *P. octaurelia* using strains originating from different continents is warranted.

Material and Methods

Material

The strains of the *Paramecium aurelia* species complex and *P. caudatum* 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. *P. caudatum* was used as an outgroup. The strains of *P. octaurelia* designated K8 and K9 were collected in 1996 by Beatrix Weber in Mackenbach (close to Kaiserslautern), Germany from a small pond located in a forest.

Methods

1. Identification and cultivation of strains

Paramecia cultivation and identification were performed according to SONNEBORN (1970). The paramecia were cultivated on a lettuce medium inoculated with *Enterobacter aerogenes*.

Identification of strains of the *P. aurelia* spp. complex (K8 and K9) was carried out on the basis of 95-100% conjugation between reactive (mature for conjugation) complementary mating types of the investigated strains with the mating types of the standard strain (138 from Florida, USA) of *P.octaurelia*. The survival of the hybrid clones was observed in both generations F1 (obtained by conjugation) and F2 (obtained by autogamy, using the method of daily isolation lines).

2. Crosses

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. Molecular methods

a. Isolation of DNA

Paramecium genomic DNA was isolated (200μl 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*. (2003). The strains used for sequencing: two new strains from Europe (Germany: K8, K9), the strain from N. America (USA, Florida: 138), and the strain from Asia (Israel: IEA) are presented in Table 1.

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 a universal eukaryotic primer (http://www.biology.duke.edu/fungi/mycolab/primers.htm). 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 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 gels for 45 min at 85 V with a DNA molecular weight marker (VI ™ Roche, France).

b. Amplification of gene fragments of mitochondrial cytochrome oxidase (COI)

Cox_L and Cox_H primers were used to amplify the CO I region (880bp) of mitochondrial DNA

Table 1

Paramecium octaurelia, other *P. aurelia* spp., and *P. caudatum* strains used in molecular studies

Species	Strain designation	Geographical origin	Reference	Accession number	
				5'LSU rDNA	COI mtDNA
P. octaurelia	K8	Germany, Mackenbach	Present paper	EU717658	EU717655
	K9			EU717659	EU717656
	138	USA, Florida	SONNEBORN 1974	EU717645	EU717636
	IEA	Israel, Ein Afek	PRZYBOŚ *et al*. 2002	EU717657	EU717654
P. tetraurelia	S	Australia, Sydney	SONNEBORN 1974	EU717652	EU717643
P. pentaurelia	87	USA, Pennsylvania	SONNEBORN 1974	EU086127	EU086118
P. novaurelia	510	Great Britain, Edinburgh	BEALE & SCHNELLER 1954	DQ837974	DQ837975
P. tredecaurelia	209	France, Paris	RAFALKO & SONNEBORN 1959	DQ 138112	EU729743
P. caudatum	PC	Cyprus, Akamas	PRZYBOŚ (unpublished)	DQ207375	DQ837977

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 mtDNA	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

Table 3

Paramecium octaurelia, survival in intra- and inter-strain crosses

Strain	F1 (by conjugation)	F2 (by autogamy)
138 x 138 (USA)	100	100
K8 x K8 (Germany)	100	100
K9 x K9 (Germany)	99	100
IEA x IEA (Israel)	100	100
K8 x 138 (Germany x USA)	94	84
K9 x 138 (Germany x USA)	98	96
K8 x IEA (Germany x Israel)	98	98
IEA x 138 (Israel x USA)	100	100

(according to BARTH *et al*. 2006). PCR amplification was carried out in the same volume as in the case of the 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 85 V with a DNA molecular weight marker (VI ™ Roche, France).

c. Purification and sequencing

30 μl of each PCR product was separated on a 1.8 % agarose gel (100 V/60 min). Then, the band representing the examined fragment was cut out and transferred into an 1.5 ml 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 Ex Terminator ™ (A&A Biotechnology, Poland) 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 was performed using ClustalW (THOMPSON *et al*. 1994) in the BioEdit program (HALL 1999). Phylogenetic trees were constructed for the studied fragments in Mega version 4.0 (TAMURA *et al*. 2007), using the Neighbor-Joining method (NJ) (SAITOU and NEI 1987) and Maximum Parsimony (MP) (NEI and KUMAR 2000), and Bayesian inference (RONQUIST & HUELSENBECK 2003). The NJ analysis was performed using a Kimura 2-parameter correction model (KIMURA 1980) and Jukes-Cantor method (JUKES and 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.

Results and Discussion

P. octaurelia is a rather rare species with restricted occurrence in the world, therefore a new record of the species from Europe extends the known range of the species. The intra-specific polymorphism within *P. octaurelia* was compared using strains originating from different continents, e.g. Europe, N. America and Asia. Inter-strain crosses in *P. octaurelia* showed a high percentage of survival in both generations (Table 3). However, the sequenced fragments of 5'LSU rDNA and COI mtDNA revealed that the studied strains of *P. octaurelia* belong to two clades (groups) in the trees constructed by all applied methods (Figs 1-2). Strains from Germany (K8 and K9) and

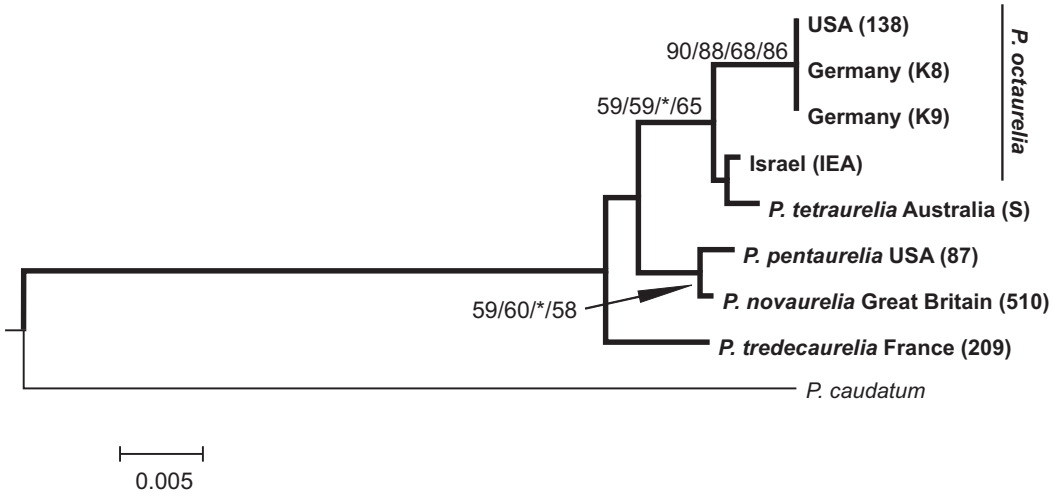

Fig. 1. Phylogenetic tree constructed for strains of *P. octaurelia*, *P. tetraurelia*, *P. pentaurelia*, *P. novaurelia*, *P. tredecaurelia* and *P. caudatum* (as an outgroup), based on a comparison of sequences from 5' LSU rDNA fragment using the NJ (neighbor joining) method (with the application of the Jukes-Cantor correction model and Kimura two-parameter), MP (maximum parsimony) analysis and Bayesian Inference (BI). Bootstrap values are presented as percentages (J-C/K2P/ MP/BI) for 1000 replicates. All positions containing gaps and missing data were eliminated from the dataset. There were a total of 349 positions in the final dataset. Phylogenetic analyses were conducted in MEGA 4.0.

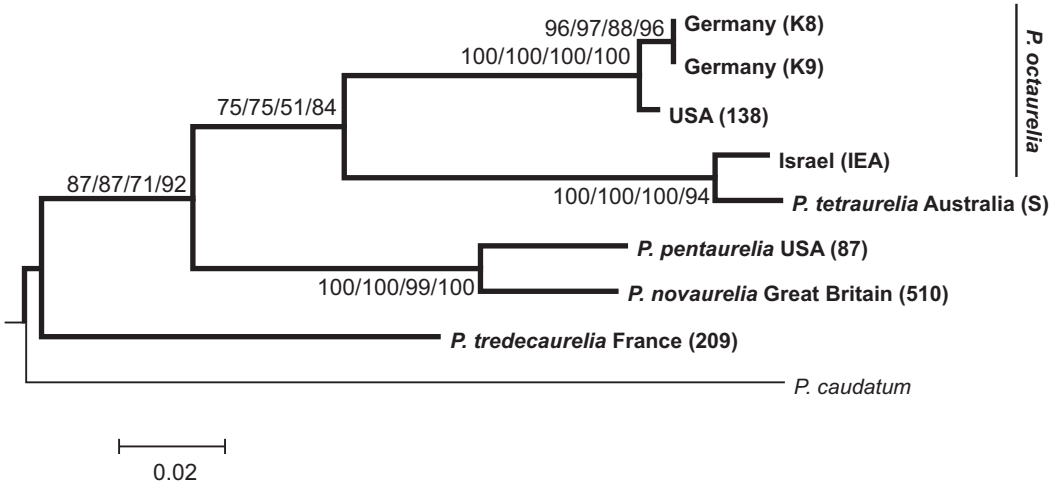

Fig. 2. Phylogenetic tree constructed for strains of *P. octaurelia*, *P. tetraurelia*, *P. pentaurelia*, *P. novaurelia*, *P. tredecaurelia* and *P. caudatum* (as an outgroup), based on a comparison of sequences from CO I mtDNA fragment using the NJ (neighbor joining) method (with the application of the Jukes-Cantor correction model and Kimura two-parameter), MP (maximum parsimony) analysis and Bayesian Inference (BI). Bootstrap values are presented as percentages (J-C/K2P/ MP/BI) for 1000 replicates. All positions containing gaps and missing data were eliminated from the dataset. There were a total of 309 positions in the final dataset. Phylogenetic analyses were conducted in MEGA 4.0.

strain 138 from USA form one group and the strain from Israel belongs to the second group.

The relationship of *P. octaurelia* strains was compared with strains representing the other species of the complex, i.e. *P. pentaurelia* and *P. novaurelia* which represent species characterized by a caryonidal system of mating type inheritance (group A), *P. tetraurelia* with a clonal type of mating type inheritance (group B), and *P. tredecaurelia* , its .." two mating types are determined by different alleles at the same locus" (group C) according to SONNEBORN (1975). *P. pentaurelia* and *P. novaurelia* also differ in intra-specific differentiation. The first species did not show such differentiation in molecular characters (RAPD; PRZYBOŚ *et al*. 2005; PRZYBOŚ *et al*. 2007a) and the second species showed deep intra-specific differentiation (STOECK *et al*. 2000; PRZYBOŚ *et al*. 2006; PRZYBOŚ *et al*. 2007b). In the presented trees (Figs 1-2), independently of the DNA fragment used or applied statistical method, the *P. tetraurelia* strain from Australia (Sydney–S) is very close to the *P. octaurelia* strain (IEA- Israel).

HORI *et al*. (2006) compared the relationships of species of the *P. aurelia* complex on the basis of the *hsp70* fragment, using the NJ method. These authors found that *P. octaurelia* strains (137 and 138 both from Florida, USA) appear very close to each other in the tree and to the *P. tetraurelia* strains, as in our tree.

According to SONNEBORN (1975) both species are characterized by a clonal system of mating type inheritance, and two mating types of *P. tetraurelia* give strong mating reactions with the complementary mating types of *P. octaurelia*. However, a high proportion of *P. tetraurelia* reacting cells of type E can conjugate in the mixture of type O of *P. octaurelia*, but only a small percentage of cells conjugate in the reciprocal combination, and these crosses are nonviable. Mating type E of both species react but not conjugate with type O of *P. decaurelia* and *P. dodecaurelia*. *P. tetraurelia*, *P. octaurelia* and *P. dodecaurelia* cells are the smallest in the *P. aurelia* complex. A weak mating reaction, without conjugation, can occur in both mating type combinations of *P. octaurelia* with *P. primaurelia*, and between type E of *P. octaurelia* and type O of *P. septaurelia*, and type O of *P. triaurelia*.

Similarly, *P. tetraurelia* and *P. octaurelia*, which can inter-mate, are close related on the tree based on sequencing the ITS region of the nuclear ribosomal cistron (COLEMAN 2005). Our previous studies (PRZYBOŚ *et al*. 2007a) concerning the relationships of species of the *P. aurelia* complex based on RAPD and ARDRA analyses, also showed that *P. tetraurelia*, *P. octaurelia* and *P. dodecaurelia* belong to the same group of species with a characteristic band pattern.

Other species of the *P. aurelia* complex, i.e. *P. pentaurelia* and *P. novaurelia*, are close in our trees, *P. tredecaurelia* is less related (Figs 1-2). The genetic distance among *P. octaurelia* strains varies from 0% to 0.9%, and distance of *P. octaurelia* strains to other *P. aurelia* spp. varies from 0.3% to 1.7% (Table 4, 5'LSU rDNA). In turn, the distance of *P. octaurelia* strains evaluated on the basis of CO I mtDNA (Table 5) varies from 0% to 15.4%, but the divergence to other *P. aurelia* spp. varies from 2.3% to 22.7%.

The strains from Germany (K8 and K9) were earlier identified as *P. octaurelia* based on their

Table 4

Distance matrix presenting the number of base substitutions in *Paramecium octaurelia*, *P. tetraurelia*, *P. pentaurelia*, *P. novaurelia*, *P. tredecaurelia*, and *P. caudatum* strains, based on analyses of 5'LSU rDNA sequences. Analyses were conducted using the Jukes-Cantor method (lower-left) and method Kimura 2-parameter (upper-right). *P. octaurelia* is marked grey

5'LSU	*P. tetr.*, S	*P. pent.*, 87	*P. oct.*, 138	*P. oct.*, IEA	*P. oct.*, K8	*P. oct.*, K9	*P. nov.*, 510	*P. tredec.*, 209	*P. caud.*
P. tetraurelia, S		0.012	0.006	0.003	0.006	0.006	0.009	0.012	0.099
P. pentaurelia, 87	0.012		0.017	0.009	0.017	0.017	0.003	0.017	0.086
P. octaurelia, 138	0.006	0.017		0.009	0.000	0.000	0.014	0.017	0.092
P. octaurelia, IEA	0.003	0.009	0.009		0.009	0.009	0.012	0.014	0.096
P. octaurelia, K8	0.006	0.017	0.000	0.009		0.000	0.014	0.017	0.092
P. octaurelia, K9	0.006	0.017	0.000	0.009	0.000		0.014	0.017	0.092
P. novaurelia, 510	0.009	0.003	0.014	0.012	0.014	0.014		0.014	0.089
P. tredecaurelia, 209	0.012	0.017	0.017	0.014	0.017	0.017	0.014		0.089
P. caudatum	0.098	0.085	0.091	0.095	0.091	0.091	0.088	0.088	

Table 5

Distance matrix presenting the number of base substitutions in *Paramecium octaurelia*, *P. tetraurelia*, *P. pentaurelia*, *P. novaurelia*, *P. tredecaurelia*, and *P. caudatum* strains, based on analyses of CO I mtDNA sequences. Analyses were conducted using the Jukes-Cantor method (lower-left) and method Kimura 2-parameter (upper-right). *P. octaurelia* is marked grey

CO I	*P. tetr.*, S	*P. pent.*, 87	*P. oct.*, 138	*P. oct.*, IEA	*P. oct.*, K8	*P. oct.*, K9	*P. nov.*, 510	*P. tredec.*, 209	*P. caud.*
P. tetraurelia, S		0.227	0.142	0.023	0.138	0.138	0.216	0.209	0.278
P. pentaurelia, 87	0.216		0.162	0.217	0.167	0.167	0.054	0.172	0.274
P. octaurelia, 138	0.138	0.158		0.150	0.010	0.010	0.149	0.212	0.290
P. octaurelia, IEA	0.023	0.207	0.146		0.154	0.154	0.216	0.195	0.278
P. octaurelia, K8	0.134	0.162	0.010	0.150		0.000	0.162	0.227	0.274
P. octaurelia, K9	0.134	0.162	0.010	0.150	0.000		0.162	0.227	0.274
P. novaurelia, 510	0.207	0.054	0.146	0.207	0.158	0.158		0.195	0.269
P. tredecaurelia, 209	0.199	0.166	0.203	0.186	0.216	0.216	0.186		0.225
P. caudatum	0.265	0.265	0.279	0.265	0.265	0.265	0.260	0.220	

RAPD fingerprint (according to STOECK & SCHMIDT 1998).

The discovery of a new stand of *P. octaurelia* in Europe calls for a revision of the problem of paramecia distribution around the world. As cysts are not known in this genus, they may be transferred by birds or "human activities… played an important role" (FOISSNER 2006).

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