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Molecular Polymorphism of Strains within *Paramecium septaurelia* (Ciliophora, Oligohymenophorea)

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RAPD-PCR fingerprinting and ARDRA riboprinting revealed polymorphism within *P. septaurelia* strains from Russia (4 strains from Lower Volga Basin), and one strain from USA, Florida. However, the first method showed the existence of four RAPD genotypes while the second revealed only two groups of strains with different band patterns. All studied strains had a high percentage of surviving hybrid clones in the inter-strain crosses, with little differentiation of strains within species. Intra-species differentiation of strains in RAPD band patterns may be connected with the degree of inbreeding for the studied species. Species of the *P. aurelia* complex can be arranged according to the degree of inbreeding characteristic for each, which is correlated with the degree of DNA polymorphism revealed by the RAPD method from extreme inbreeders (e.g. *P. sexaurelia)*, moderate inbreeders (e.g. *P. triaurelia)* to weak inbreeders (e.g. *P. pentaurelia*). *P. septaurelia* of the *P. aurelia* complex should be included in the group of extreme inbreeder.

Key words: *Paramecium septaurelia*, genetic crosses, RAPD fingerprinting, ARDRA, phylogenetic analysis.

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The *Paramecium aurelia* species complex is composed of 15 species (SONNEBORN 1975; AUFDERHEIDE *et al*. 1983). They are sibling species, morphologically similar but with isolated gene pools. Species of the complex are characterized by various degrees of inbreeding (SONNEBORN 1957; LANDIS 1986), having an effect on intra-species differentiation.

Studies of relationships among strains by classical inter-strain crosses within several species of the *P. aurelia* complex (*P. triaurelia, P. pentaurelia, P. sexaurelia, P. novaurelia*), together with a comparison of strain genotypes revealed by RAPD-PCR fingerprints analysis, revealed the genetic character of the complex (STOECK *et al*. 1998, 2000). It was shown that the species differ in degree of inbreeding, which was correlated with differentiation of DNA genotypes within species, despite lack of distinct differentiation of strains revealed by classical genetic methods (crosses). Strains of *Paramecium triaurelia* and *P. novaurelia* can hybridize and have a high percentage of surviving hybrid clones in F1 and F2 generations, however, RAPD fingerprint analysis distinguished three genotypes (band patterns) (STOECK *et al*. 1998) within *P. triaurelia* and four genotypes (band patterns) within *P. novaurelia* (STOECK *et al*. 2000). Therefore, it seems worth to investigate the intraspecific differentiation within particular species of the complex.

P. septaurelia was known only from Florida and Alabama (SONNEBORN 1975). New habitats of the species recorded for the first time outside the USA in the Lower Volga Region of Russia (PRZYBOŚ *et al*. 2004, 2005) prompted investigations of polymorphism within this species. The studies concerned intraspecific differentiation of strains originating from different continents (North America and Europe), as well as strains originating from neighboring habitats (Russia, the Lower Volga Basin). Differentiation of strain genotypes within *P. septaurelia* was studied by classical genetic methods (strain crosses and evaluation of percentage of surviving clones in F1 and F2 generations) and by molecular methods (RAPD-PCR fingerprints and ADRDA riboprinting).

Materials and Methods

Materials

The strains that originated from Russia were designated AZ and V. Strains AZ were collected in Astrahan Nature Reserve near Damchik (45.83N/47.85 E) and at the Caspian coast (45.7N/47.9E) – a zone between the Volga River and the Caspian Sea with many islands scattered in a vast fresh water area. The distance of AZ strain sites was up to 10 km. The following AZ strains were used: AZ 3-1, AZ 6-23, AZ 8-20.

The strain designated V 5-13 was collected in the flood lands of the Natural Reserve Complex Volga-Ahtuba, Volgograd region (approximately 48.7N/44.7E). Details about collection sites of the Russian strains can be found in (PRZYBOŚ *et al*. 2004), the strains were collected by M. Rautian and A. Potekhin (St. Petersburg University, Russia).

The strain index includes: letters AZ and V, and two numbers – the first number represents the population and the second number after the dash represents the paramecium cell, e.g. AZ 3-1 means strain number 1 collected in Astrahan Nature Reserve, population 3. Strain 38 (T.M. SONNEBORN stock 38 = 30575 ATCC) from Florida, USA, standard *P. septaurelia,* was also used in the studies but restricted to one mating type (XIII) only. All T1 studied strains are presented in Table 1.

Methods

Cell culture

The methods of SONNEBORN (1970) were applied for the cultivation of strains, induction of conjugation, and autogamy in *P. septaurelia*. Paramecia were cultivated in a lettuce medium inoculated with *Enterobacter aerogenes.*

Strain crosses

In the strain crosses, the F1 generation was obtained by conjugation and F2 by autogamy (method of daily isolation lines). In the inter-strain crosses (50 pairs), the survival of hybrid clones in both generations was estimated by counting paramecia in them. According to CHEN (1956), clones may be considered survivors after passing 6-7 fissions during 72 hours after separation of partners of conjugation or postautogamous caryonids. The methods are described in detail in (PRZYBOŚ 1975). The percentage of surviving hybrid clones of both generations in inter-strain crosses was compared (Table 2).

Molecular methods

(a) RAPD-PCR fingerprinting

RAPD-PCR fingerprint analysis for *Paramecium aurelia* spp. was generally as in (STOECK & SCHMIDT 1998). Details are described in PRZYBOŚ *et al*. 2003. DNA was isolated from 5 strains of *P. septaurelia* (Table 1) using a QIAamp™ DNA Mini Kit (Qiagen™, Germany). RAPD-PCR

Table 1

Paramecium septaurelia strains

Strain designation and origin			
Country	Region	Habitat	Designation
Russia	Astrahan Nature Reserve	Canal Koklyuy	AZ 3-1
	Astrahan Nature Reserve	River Bystraya	AZ 6-23
	Astrahan Nature Reserve	Lotus field, coast	AZ 8-20
	Natural Reserve Complex Volga-Ahtuba	No information	V 5-13
USA	Florida, Broward Co.	No information	38 (30575 ATCC)

Table 2

Mean percentage of surviving hybrid clones in crosses of *P. septaurelia* strains

Strain	F1	F2
AZ3-1 (Russian) x 38 (USA)	98	94
AZ6-23 (Russian) x 38 (USA)	100	96
AZ8-20 (Russian) x 38 (USA)	100	94
AZ6-23 (Russian) x AZ8-20 (Russian)	100	97
AZ3-1 (Russian) x AZ6-23 (Russian)	100	87
V5-13 (Russian) x 38 (USA)	100	98
V5-13 (Russian) x AZ8-20 (Russian)	one mating type in both strains	

was performed with primer Ro-460 04 (Roth, Karsruhe, Germany), a 10mer random primer with nucleotide sequence: 5' –GCAGAGAAGG- 3', using Taq polymerase (Qiagen). The primer was selected by (STOECK & SCHMIDT 1998) after testing several dozen oligonucleotide primers as the one giving "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, 2003; SKOTARCZAK *et al*. 2004 a, b) and *P. schewiakoffi* (FOKIN *et al*. 2004). The products of the PCR reactions were separated by electrophoresis in 1.5% agarose gels for 3.5 h at 85V together with molecular weight marker XIV™ (Roche™, France), stained with ethidium bromide and visualized in UV light. The images were stored in computer memory using the program Biocapt™ (Vilbert Lourmat™, France). RAPD-PCR reactions were repeated for each strain and culture several times for verification.

(b) ARDRA riboprintnig

A ribosomal DNA fragment of *Paramecium septaurelia* (fragment of small and large rRNA units) was amplified with primers: forward F rDNA**,** 5' GAAACTGCGAATGGCTC 3', and reverse R rDNA**,** 5' TTGGTCCGTGTTTCAAGACG 3' (IBB PAN [1]). PCR products (2400bp) were cut with restriction enzymes (Eco RV, Hinf I, Hha I, MspI, AluI, TaqI – Promega™, USA) and were separated by electrophoresis in 2% agarose gels for 3 h at 60V. The applied restriction enzymes (Eco RV, Msp I) were as in (CHEN *et al*. 2003) or were found on-line in REbase (Alu I, Hha I, Hinf I and TaqI, http://tools.neb.com/REBsites/index.php3). Sequences2 were obtained from GenBank (www.ncbi.nlm.nih.gov [2]) and from direct sequencing of the *Paramecium novaurelia* rDNA fragment. Phylogenetic analysis was performed by comparing the molecular weight of DNA obtained by the RAPD-PCR method using the Bio1D™ program (Vilbert Lourmat, France).

Results

Strain crosses

Results of strain crosses (Table 2) revealed a high percentage of surviving hybrid clones of both generations in inter-strain crosses of the Russian strains with an American T2 one, as well as between the Russian strains.

RAPD-PCR fingerprinting

RAPD-PCR fingerprints (band patterns) revealed by primer Ro 460-04 of all studied F1 strains of *Paramecium septaurelia* are presented in Fig 1. The basic band pattern appearing in all strains is comprised of the following bands: 290 bp, 390 bp, 590 bp, 610 bp, 980 bp,1010 bp and 1700 bp. It is characteristic for the species. However, a comparison of all band patterns of the particular strains distinguished four different genotypes within the species, revealing the F2 existence of DNA polymorphism of the Russian and American strains (Fig. 2). The first genotype (PsI) appears in strains AZ6-23 and AZ 3-1 and is the

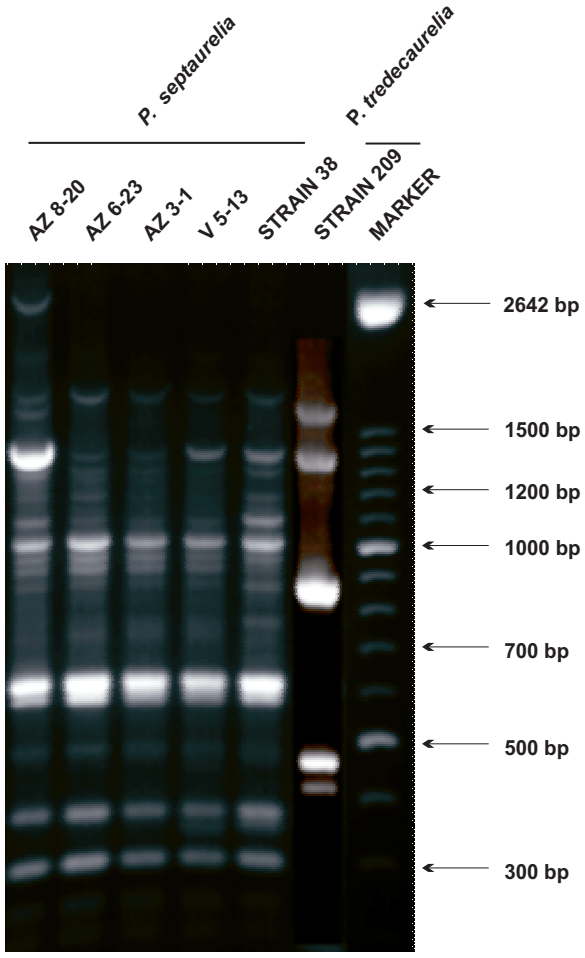


Fig. 1. RAPD fingerprint band pattern of the investigated *P. aurelia* strains on an agarose gel. Lanes 1-3 strains from Astrahan, Russia, lane 4 – strain from Volgograd region, Russia, lane 5 – strain 38 from Florida, USA, lane 6 – *P. tredecaurelia* strain 209 from Paris, France, lane 7 – molecular weight marker XIV (Roche, France).

[1]IBB PAN – Institute of Biochemistry and Biophysics, Polish Academy of Sciences.

[2]Sequences accession number: AJ548821, AF255362, AF255361, AF255360, AF217655, AF100315, AF100314, AF100313, AF100312, AF100311, AF100310, AF010352.

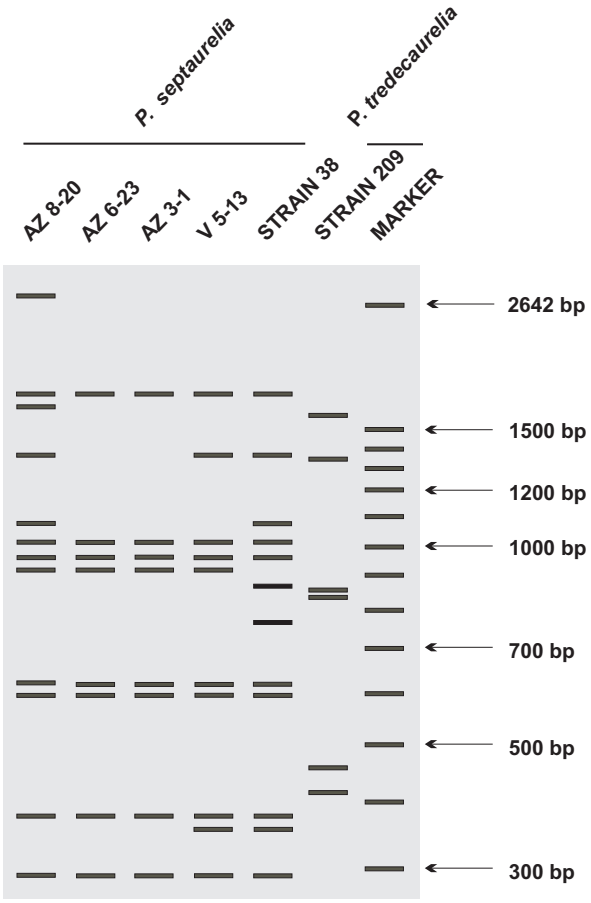


Fig. 2. Scheme of different *P. septaurelia* genotypes (basic band patterns) of the studied strains revealed by the primer Ro 460-04 (Roth, Germany). Molecular weight of marker bands are given in bp. Lanes 1-3 strains from Astrahan, Russia, lane 4 – strain from Volgograd region, Russia, lane 5 – strain 38 from Florida, USA, lane 6 – *P. tredecaurelia* strain 209 from Paris, France, lane 7 – molecular weight marker XIV (Roche, France).

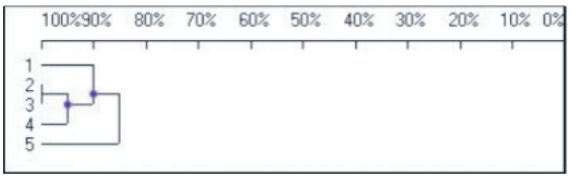
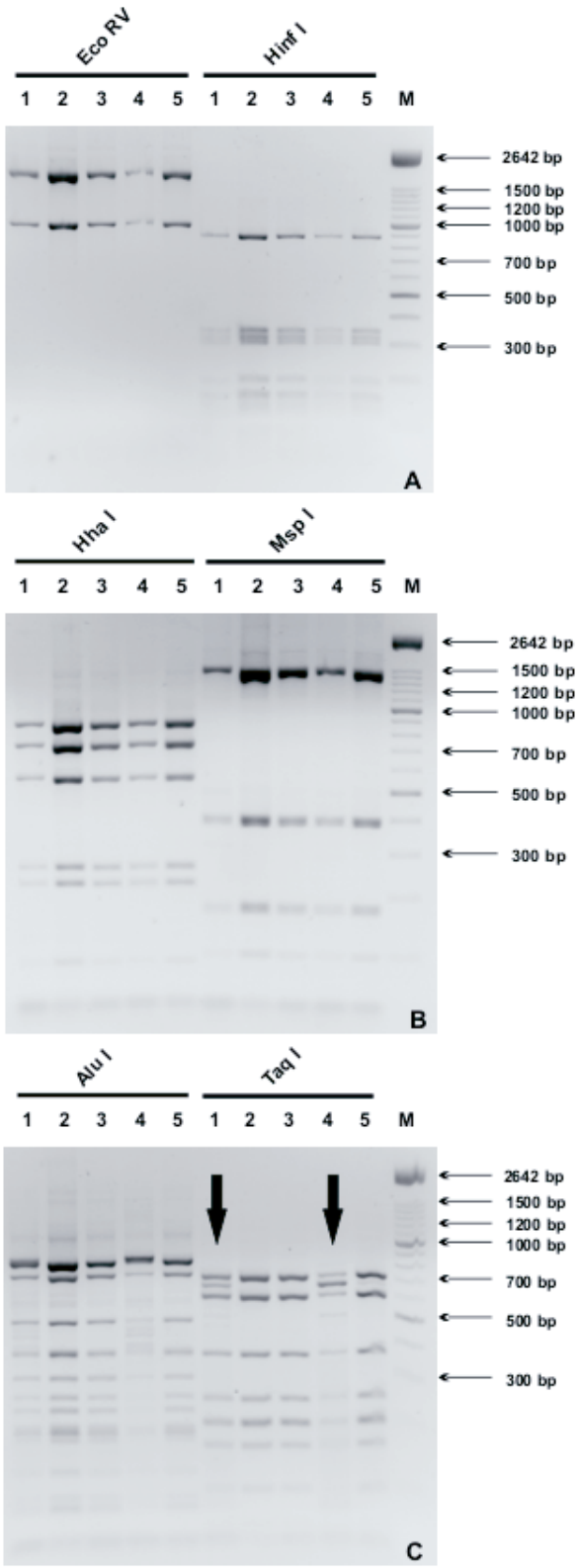


Fig. 3. Tree diagram of the cluster analysis of RAPD fingerprint pattern similarity matrix of the studied *P. septaurelia* strains. Method used for analysis was UPGMA. Similarity index was calculated according to NEI & LI (1979). 1 – strain AZ 8-20, 2 – strain AZ 6-23, 3 – strain AZ 3-1, 4 – strain V 5-13 (all strains from Russia, Lower Volga Basin), 5 – strain number 38 from Florida, USA.

same as the one characteristic for the species. The second genotype (PsII), represented by strain AZ8-20, is characterized by four extra bands in comparison with PsI. Bands at 1600 and 2600 bp are characteristic for Ps II only. Band 1100 bp appears in AZ 8-20 (PsII) and strain 38 (PsIV), band 1370 bp appears in all strains except PsI genotype. Strains from Russia collected in the Astrahan region are characterized by two different genotypes.



Figs 4 (a,b,c). ARDRA band patterns of the investigated *P. aurelia* strains on agarose gel after digestion by restriction enzymes. 1 – AZ 8-20, 2 – AZ 6-23, 3 – AZ 3-1 all three strains from Astrahan, Russia, lane 4 – V 5-13 -strain from Volgograd region, Russia, lane 5 – strain 38 from Florida, USA, M – molecular weight marker XIV(Roche, France).

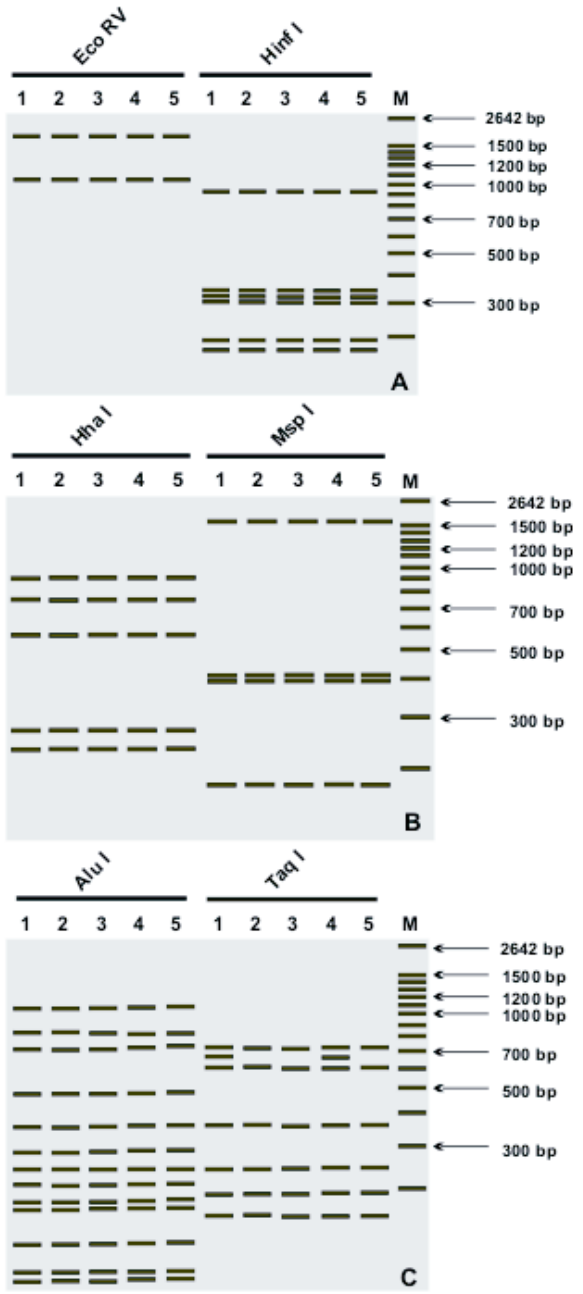


Fig. 5 (a,b,c). Schematic representation of band patterns obtained by restriction enzyme cleavage of the *P. aurelia* strains. 1 – AZ 8-20, 2 – AZ 6-23, 3 – AZ 3-1 all three strains from Astrahan, Russia, lane 4 – V 5-13 strain from Volgograd region, Russia, lane 5 – strain 38 from Florida, USA, M – molecular weight marker XIV (Roche, France).

The third genotype (PsIII), appearing in strain V5-13 (Volgograd), differs from PsI by one extra band at 370 bp. The fourth genotype (PsIV) appears in strain 38 from Forida, USA. It is characterized by three extra bands, two at 370 bp, 770 bp and one 880 bp. The last two bands are characteristic for PsIV. In comparison with the other genotypes Ps IV has not 960 bp band.

The basic band pattern characteristic for *P. septaurelia* was compared with the band pattern characteristic for the other species, *P. tredecaurelia* (strain 209 from Paris, France – from the paper PRZYBOŚ *et al*. 2005), for confirmation of dissimilarity of basic band patterns revealed by RAPD-PCR fingerprinting of two species of the *P. aurelia* complex.

A diagram constructed on the basis of cluster analysis of fingerprint similarity (NEI F3 & LI 1979) presents the relationships of strains within *P. septaurelia* (Fig. 3). Strains AZ6-23 and AZ 3-1 (genotype PsI) show high similarity (95%) to strain V5-13 (PsIII), and all of them reveal 90% similarity to strain AZ8-20 (PsII). The Russian strains AZ and V show a similarity of 85 % of band patterns to strain 38 from Florida, USA (PsIV) which is the most distant strain on the diagram.

ARDRA riboprinting

Ribosomal DNA fragments of all studied strains of *P. septaurelia* were digested by six restriction enzymes (HhaI, MspI, EcoRV, HinfI, AluI, and TaqI) (Figs 4-5), but only F4-5 TaqI cleaved different band patterns between strains of *P. septaurelia* (Figs 4c,5c). This enzyme revealed the existence of two genotypes, one in strains from Russia AZ6-23 and AZ3-1 as well as in strain 38 from Florida, USA, the second in strains AZ8-20 and V5-13, both from Russia but from different localities. The second genotype is characterized by an extra band at 680 bp.

Discussion

DNA polymorphism was revealed within *P. septaurelia*. Strains of this species showed different band patterns (RAPD genotypes PsI, PsII, PsIII, and PsIV). Notably, such polymorphism is characteristic for the strains designated AZ (genotypes PsI, PsII) and V (genotypes PsIII), originating from the same region (the Lower Volga Region, Russia), and also between strains designated AZ originating from the Astrahan region. The collection sites in the latter region are situated only up to 10 km from each other. The standard strain of the species, strain 38 from Florida, is characterized by a RAPD genotype (PsIV) different from band patterns of the Russian strains. However, all the studied strains showed a high percentage of surviving hybrid clones in the inter-strain crosses, suggesting a low level of differentiation among strains.

ARDRA riboprinting also revealed some differences between the studied strains of *P. septaurelia.* Among six applied enzymes, only TaqI gave

an extra band in strains AZ8-20 and V5-13, grouping them in one genotype, the other strains (AZ6-23, AZ3-1, and strain 38) showed the same band pattern (genotype) and were grouped together.

Both methods revealed polymorphism within *P. septaurelia.* However, the first method showed the existence of four RAPD genotypes and the second only two. 90% of band pattern similarity was found by RAPD analysis of strains V5-13 (genotype PsIII) and AZ8-20 (genotype PsII) (Fig. 3), which according to results of ARDRA riboprinting, are grouped together in one band type. Intraspecific differentiation of strains in RAPD band patterns may be connected with the degree of inbreeding for the studied species. Species of the *P. aurelia* complex can be arranged according to their degree of inbreeding, which is in turn correlated with the degree of DNA polymorphism revealed by the RAPD method from extreme inbreeders (e.g. *P. sexaurelia*) by moderate inbreeders (e.g. *P. triaurelia*) to weak inbreeders (e.g. *P. pentaurelia*) (cf STOECK *et al*. 1998, 2000). *P. septaurelia* of the *P. aurelia* complex should be included in the group of extreme inbreeders. A high degree of genetic polymorphism was also revealed by analysis of molecular data obtained by RAPD and RFLP in natural populations of *Euglena gracilis* (ZAKRYŚ 1997). According to the author "a high degree of genetic polymorphism is maintained between the clones, which is the result of intense microevolutionary processes on the intraspecific level". DNA fingerprints also revealed a heterogeneous distribution of four genotypes in the species *Stentor coeruleus* (KUSCH 1998).

RFLP and RAPD markers were also used in studies revealing genetic variation in *Avicennia marina* (mangrove plants) (PARANI *et al*. 1997; GIANG *et al*. 2003). The genetic diversity of *Echinacea* species was revealed by application of RFLP polymorphism markers (KIM *et al*. 2004). RAPD and ARDRA methods were used for separation and identification of *Uronychia* spp. (CHEN *et al*. 2003). Recently, ARDRA analysis (REGENSBOGENOVA *et al*. 2004) was also used for rapid identification of rumen Protozoa.

RAPD fingerprinting seems to be a good method for studies on population (strain) polymorphism within species, which could be presented as strain-specific band patterns as in the case of markers within *P. septaurelia.* The technique is based on the amplification of undefined fragments of the genome by PCR using single arbitrary primers. Polymorphism can be found in any region of the genome. Lack of differentiation within studied species of the *P. aurelia* complex in the case of ARDRA riboprinting may be caused by use of only limited DNA fragment (about 2400 bp) and strictly limited to sites recognized by the particular restriction enzymes.

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