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New European Stands of *Paramecium pentaurelia*, *Paramecium septaurelia*, and *Paramecium dodecaurelia*, Genetic and Molecular Studies*

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New stands of rare species of the *Paramecium aurelia* complex were found in Europe, i.e. *P. pentaurelia* and *P. dodecaurelia* in Italy and *P. septaurelia* in Germany. The species were identified by mating reactions with the standard strains of each species. Their relationships with some other known strains of particular species were studied by classical strain crosses (survival in F1 and F2 generations) and by comparison of RAPD-PCR fingerprints. The presence of the cosmopolitan species *P. tetraurelia* in Italy was also recorded.

Key words: *Paramecium aurelia* species complex, breeding system, genetic relationships, geographical distribution, RAPD–PCR fingerprinting.

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Among the 15 species of the *Paramecium aurelia* complex known world-wide SONNEBORN 1975; AUFDERHEIDE *et al*. 1983), at present 10 species are known from Europe (cf PRZYBOŚ & FOKIN 2000, 2003b; PRZYBOŚ *et al*. 2004; PRZYBOŚ 2005), i.e., *P. primaurelia, P. biaurelia, P. triaurelia, P. tetraurelia, P. pentaurelia, P. sexaurelia, P. septaurelia, P. novaurelia, P. dodecaurelia* and *P. tredecaurelia*. Of these, *P. primaurelia , P. biaurelia* and *P. novaurelia* are frequent in Europe. The occurrence of others, such as *P. triaurelia, P. tetraurelia, P. pentaurelia, P. sexaurelia* seems to be limited to certain climatic zones, while the distribution of other species is confined to certain environments, e.g. *P. septaurelia*, and even to habitats as in the case of *P. dodecaurelia* or *P. tredecaurelia.*

The frequency of the occurrence of species in Europe has been estimated on the basis of the number of investigated clones and habitats as well as the ratio value (r.v.), i.e. the number of habitats for a particular species to the total number of habitats in the area (country or zone) (PRZYBOŚ & FOKIN 2000; PRZYBOŚ 2005). Current knowledge on the occurrence of *P. aurelia* spp. in Europe has been reported by PRZYBOŚ (2005). In this work 474 habitats were taken for consideration. The dominant species is *P. novaurelia* (r.v. 0.39, found in 184 habitats), followed by *P. biaurelia* (r.v. 0.26, in 124 habitats), and by *P. primaurelia* (r.v. 0.24, in 112 habitats). Other species are rather rare, i.e. *P. tetraurelia* (r.v. 0.06 in 28 habitats), *P. triaurelia* (r.v. 0.04 in 21 habitats), or very rare as *P. pentaurelia* and *P. sextaurelia*, for both r.v. 0.02, found in 10 and 11 habitats, respectively. *P. dode-*

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caurelia was found in two localities in Europe, and two species are known in Europe from single habitats only, *P. septaurelia* found in Russia (Lower Volga Basin) (PRZYBOŚ *et al*. 2004, 2005), and *P. tredecaurelia* detected in France (Paris) (RAFALKO & SONNEBORN 1959). However, a different number of habitats were studied in the particular zones in Europe, the greatest number originating in the central zone, i.e. 362 among all 474 studied. In the southern zone only 52 habitats were studied, were *P. primaurelia* dominating (r.v. 0.40) over *P. biaurelia* (r.v. 0.35), and *P. novaurelia* (r.v.0.17). In the southern zone, the distribution of the *P. aurelia* complex has been studied in the following countries: Italy, Hungary, Bulgaria, Romania, Greece, Spain, and southern part of Russia (at the Caspian Sea) (PRZYBOŚ 2005).

In the present paper new European strains of *P. pentaurelia* and *P. dodecaurelia* from Italy as well as *P. septaurelia* from Germany are described using classical strain crosses and molecular characterization (RAPD-PCR fingerprinting). The presence of *P. tetraurelia* in Italy was also reported.

Material and Methods

Material

Strains designated ISN1, ISN2 and ISN8 were collected in Giardini Naxos, in Sicily, Italy, and the strain designated TR was collected in Trento (North of Italy), all were acquired by S. FOKIN in 2004.The strain designated GF was collected in Freiburg, Germany by A. Potekhin and I. Wishniakov in 1998 (Table 1).

Methods

Culturing and identification of strains

Species of the *P. aurelia* complex were cultured and identified according to the methods of SONNEBORN (1970). The paramecia were cultivated on a lettuce medium inoculated with *Enterobacter aerogenes.* Clones mature for conjugation were mated with the reactive mating types of standard strains of known species. The following standard strains were used: strain 90 of *P. primaurelia*; the Rieff strain, Scotland, of *P. biaurelia*; strain 324 of *P. triaurelia*; strain of *P. tetraurelia* from Sydney, Australia; strain 87 of *P. pentaurelia*; strain 159 of *P. sexaurelia*; strain 38 of *P. septaurelia*; strain 510 of *P. novaurelia*; strain 246 of *P. dodecaurelia.*

The recently studied strains were identified as *P. tetraurelia*, *P. pentaurelia*, *P. septaurelia*, and *P. dodecaurelia* on the basis of strong conjugation between the complementary mating types of the strains under examination with the corresponding ones of the particular species.

Strain crosses

In the intra and inter-strain crosses, 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. According to CHEN (1956), the clones could be recognized as surviving after passing 6-7 fissions during 72 hours after separation of partners of conjugation or postautogamous caryonids.

Table 1

List of strains of the *Paramecium aurelia* species complex used in genetic studies

Species	Strain designation	Geographical origin	References
Paramecium pentaurelia	87 (standard of the species)	USA, Pennsylvania	SONNEBORN 1974
	ISN2	Italy, Sicily, Giardini Naxos	Present paper
	ISN8	Italy, Sicily, Giardini Naxos	Present paper
	HB	Hungary, Balatonfüzfo	KOŚCIUSZKO 1964
	ALT	Russia, Altay, plain part	Present paper
Paramecium septaurelia	38 (standard of the species)	USA, Florida	SONNEBORN 1975
	RA	Russia, Astrahan Nature Reserve	PRZYBOŚ *et al*. 2004
	GF	Germany, Freiburg	Present paper
Paramecium dodecaurelia	246 (standard of the species)	USA, south	SONNEBORN 1974
	TR	Italy, Trento	Present paper
	IE	Italy, Elba Island	PRZYBOŚ & FOKIN 2003
	G	Germany, Münster	PRZYBOŚ & FOKIN 2003

Inter- and intra-strain crosses within species were done and the percentage of surviving hybrid clones in intra- and inter-strain crosses were compared. The methods were described in details in PRZYBOŚ (1975).

Methods used in molecular studies – random amplified polymorphic DNA-PCR (RAPD-PCR) analysis

Paramecium genomic DNA was isolated from vegetative cells being at the end of exponential phase using a QIA[a]mp DNA Mini Kit (Qiagen Germany) as described in PRZYBOŚ *et al*. (2003b). DNA was stored at –20° C until analysis. All strains of respective species used for analysis are listed in Table 1.

PCR amplification of genomic DNA for RAPD analysis was carried out using one oligonucleotide 10 mer random primer characterized by the sequence 5'- GCAGAGAAGG - 3'. This primer was chosen from series of ten 10 mer random primers (Ro 460 Roth, Karsruhe, Germany) because it gave easily distinguishable banding patterns of species and strains in *Paramecium jenningsi* (SKOTARCZAK *et al*. 2004 a,b). The same primer was also used in several studies carried out on the *P. aurelia* spp. (STOECK *et al*. 1998, 2000) and on *P. jenningsi* (PRZYBOŚ *et al*. 1999; PRZYBOŚ *et al*. 2003b). Each amplification reaction mixture of 20 μl contained 2 μl of DNA template, 1 x reaction QIAGEN PCR buffer, 2.5 mM $MgCl_2$, 200 μM dNTPs, 1.5 μM primer, 1.5U of Taq DNA polymerase (QIAGEN). PCR reactions were performed according to the program described by STOECK and SCHMIDT (1998).

PCR products (15μl), along with the pGEM DNA molecular weight marker (Promega) were run on 1.5% TBE agarose gels stained with 0.5 μg ml^{-1} ethidium bromide. Generally, three repetitions of the PCR reaction were performed in order to assess the reproducibility of the data. All RAPD parameters were carefully standardized.

Results

Strain identification based on mating reaction and results of inter-strain crosses within the studied species

The strain ISN1 (Naxos, Sicily) was identified as *P. tetraurelia* on the basis of conjugation with the standard strain of the species (Table 2).

Table 2

New localities of species of the *Paramecium aurelia* complex in Europe

Species and strain designation	Habitat
Paramecium tetraurelia, ISN1	Italy, Sicily, River in Giardini Naxos
Paramecium pentaurelia, ISN2, ISN8	Italy, Sicily, River in Giardini Naxos
Paramecium septaurelia, GF	Germany, Freiburg
Paramecium dodecaurelia, TR	Italy, Trento

Strains ISN2 and ISN8, also from the same habitat (Naxos, Sicily), were identified as *P. pentaurelia* on the basis of conjugation with the standard strain of the species (Table 2). A high percentage of surviving clones was observed in F1 and F2 generations of inter-strain crosses (Table 3) of ISN strains (ISN2 and ISN8) with the standard strain (87), the strain HB from Hungary and the ALT strain from the Altay region in Russia.

Table 3

Mean percentage of surviving hybrid clones in crosses of the *P.* a*urelia* species

Species	Strain	F1 (by conjugation)	F2 (by autogamy)
Paramecium pentaurelia	ISN2 x ISN2	100	100
	ISN8 x ISN8	100	100
	ISN2 x 87	100	100
	ISN8 x 87	100	100
	ISN2 x ISN8	100	100
	ISN2 x HB	100	92
	ISN2 x ALT	90	94
Paramecium septaurelia	GF x GF	One mating type only	100
	GF x 38	The same mating type	–
	GF x RA	98	84
Paramecium dodecaurelia	TR x TR	One mating type only	100
	TR x 246	98	96
	TR x IE	100	100
	TR x G	The same mating type	–

The strain GF (Freiburg, Germany) was identified as *P. septaurelia* (Table 2) on the basis of conjugation with the strain of the species from Astrahan Nature Reserve (RA) in Russia. Strain GF and the standard strain (38) of the species both represent the same mating type so conjugation between them was not possible. In the inter-strain crosses (GF x RA strains), the high percentage of surviving clones was observed (Table 3).

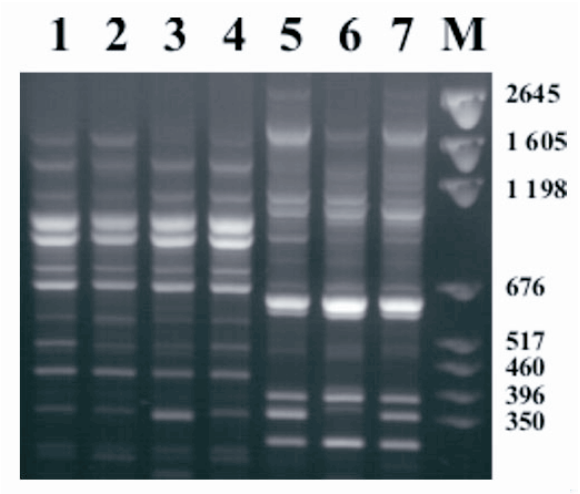

Fig. 1. RAPD fingerprints of *P. pentaurelia* strains: 1 – 87, 2 – ISN, 3 – ALT, 4 – HB and *P. septaurelia* strains: 5 – 38, 6 – F, 7 – RA. M – pGEM marker. Molecular weight of the marker DNA bands is given in bp.

The strain TR (Trento, Italy) was identified as *P. dodecaurelia* on the basis of strong conjugation with the standard strain of the species (Table 2). A high percentage of surviving clones was observed in both generations (Table 3) of inter-strain crosses of the TR strain with the standard strain (246 from USA) and the known European strain of the species, IE from Italy. The other European strain of *P. dodecaurelia*, G from Germany, is characterized by one mating type, the same situation appears in TR strain, so crossing them was not possible.

RAPD-PCR analysis

Fingerprints (band patterns), revealed by amplification with primer (Ro 460-04), of the new strains ISN2 and ISN8 (characterized by identical band patterns) of *P. pentaurelia*, strains GF of *P. septaurelia*, and strain TR of *P. dodecaurelia* as well as the other strains of the studied species are presented (Figs 1 and 2). Strains of *P. pentaurelia* and *P. septaurelia* (Fig. 1) show no polymorphism within species, while *P. dodecaurelia* revealed high polymorphism of band patterns (Fig. 2a,b) of the particular studied strains. The band pattern of the TR strain is different from patterns of the other European strains, strain IE (Elbe Island, Italy) and strain G (Germany, Münster) as well as strain 246 from USA (Fig. 2 a,b).

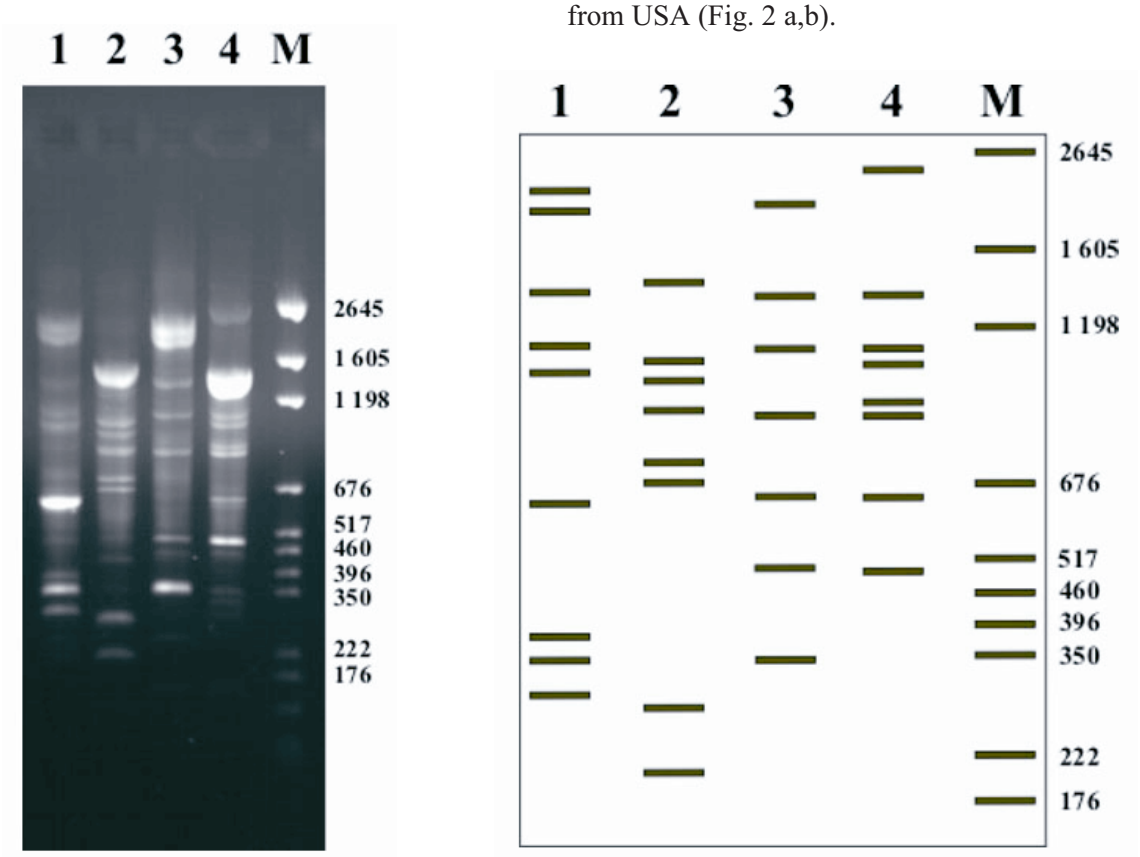

Fig. 2. RAPD fingerprints of *P. dodecaurelia* strains: 1 – 246, 2 – TR, 3 – IE, 4 – G. M – pGEM marker. Molecular weight of the marker DNA bands is given in bp. 2a – gel, 2b – diagram presenting band patterns of the particular strains.

Discussion

The presence of *P. tetraurelia* in Italy was confirmed by finding new habitat in Naxos, Sicily. This is a cosmopolitan species (SONNEBORN 1975) known from North, Central, South America, Australia, Asia, Europe. It was found earlier in Italy (SONNEBORN *et al*. 1959) but the authors did not give information concerning the origin of the strain.

Two other strains collected in the same site in Sicily and designated ISN2 and ISN8 were identified as *P. pentaurelia.* This is the first information about this species in Italy. *P. pentaurelia* seems to be rather rare in Europe, as until now it was known only from Balatonfüzfo, Hungary (KOŚCIUSZKO 1964), Oradea, Romania (PRZYBOŚ 1968), Cuenca, Spain (PRZYBOŚ 1993), Astrahan Nature Reserve in Russia (PRZYBOŚ *et al*. 2004, 2005), and the Belgorodsky region (FOKIN & OSSIPOV 1986). The occurrence of this species seems to be limited to the southern zone. The new strains of *P. pentaurelia* from Sicily, Italy confirm to this. The intra-species relationships of strains and possible differentiation within *P. pentaurelia* (crosses between strains from Hungary, Spain and USA) has been studied previously (STOECK *et al*. 2000). A high percentage of surviving clones of F1 and F2 generations was observed at that time as well as at present (Table 3). The problem of possible species polymorphism (intra-species differentiation) was also studied by RAPD-PCR analysis by the same authors (STOECK *et al*. 2000), and only a single genotype was found in all studied strains. The authors suggested that this is associated with a weak degree of inbreeding characteristic for the species. The range of *P. pentaurelia* was enlarged by finding the new strain in Russia (Astrahan Nature Reserve, Caspian Sea, PRZYBOŚ *et al.* 2004, 2005). RAPD fingerprinting was carried (PRZYBOŚ *et al*. 2006) and again no polymorphism was found in the species. At present RAPD fingerprints of the new strains from Italy (ISN) were compared with fingerprints of strains from Hungary (HB) , Russia (ALT) and USA (87) (Fig. 1) and again no polymorphism was observed. All band patterns are similar.

The strain from Freiburg, Germany was identified as *P. septaurelia*. This is the first information about that species from Germany and the second known habitat in Europe. Previously the species was known only from the USA (SONNEBORN 1975) and recently it was found in the Lower Volga Basin of Russia (PRZYBOŚ *et al*. 2004, 2005). In the inter-strain crosses of the German strain and Russian strains, a high percentage of surviving clones was observed in F1 and F2 generations (Table 3). The German strain is limited to the same mating type as the standard strain of the species (strain 38 from USA, Florida), so they could not conjugate. RAPD-fingerprints of the strain from Germany were compared with the band pattern of strain 38, standard for *P. septaurelia.* Their band pattern showed high similarity (Fig. 1).Curiously, rare European species such as *P. pentaurelia, P. sexaurelia* and *P. septaurelia* are common in the recently studied delta of the Volga River (PRZYBOŚ *et al.* 2005) appearing in 6, 3 and 10 populations, respectively, among 19 studied. It seems that the territory might be characterized by certain ecological features allowing the existence of rare species not present in other places in Europe.

The strain from northern Italy, Trento, was identified as *P. dodecaurelia.* A high percentage of surviving clones was observed in F1 and F2 generations (Table 3) of inter-strain cross between strains 246 (USA) and TR. The occurrence of *P. dodecaurelia* in northern Italy is very interesting. Previously the species was known only from the territory of the southern USA bordering on the Gulf of Mexico (SONNEBORN 1975), recently the species has also been recorded in Japan (PRZYBOŚ *et al*. 2003a), Hawaii (PRZYBOŚ & FOKIN 2003a), and in Europe in Germany (Münster) and Italy (Elbe Island) (PRZYBOŚ & FOKIN 2003b). The relationships of the European strains of *P. dodecaurelia* was studied by inter-strain crosses. Strain TR did not conjugate with strain from Germany as they represent the same mating type but could conjugate with the other Italian strain from Elbe Island, showing a high percentage of surviving clones in F1 and F2 generations (Table 3). DINI *et al*. (1995) in the "Checklist della species della fauna Italiana" noted the presence of the *P. aurelia* spp. in northern Italy, without specific information. The RAPD fingerprint of the TR strain is presented (Fig. 2 a,b), and compared with the band pattern of the standard strain (246, USA) and other European strains G (Germany) and IE (Italy, Elbe Island). The substantial polymorphism was revealed in *P. dodecaurelia*, probably connected with extreme inbreeding characteristic for the species.

RAPD-PCR analysis has been applied before (STOECK & SCHMIDT 1998) for characterization of European *P. aurelia* species and as well as a possible method of species identification on the basis of species specific band patterns. DNA fingerprints also revealed four genotypes in an other ciliate, *Stentor coeruleus* (KUSCH 1998) and is frequently applied in studies concerning polymorphism within species in animals and plants. The technique is based on the amplification of random fragments of the genome by PCR using single short (maximum 10mer nucleotide) primers, polymorphism may be found in any region of the genome.

The correlation between the degree of polymorphism revealed by RAPD analysis and degree of inbreeding characteristic for the species, proposed by STOECK *et al.* (1998) when *P. triaurelia* and *P. sexaurelia* were studied and later (STOECK *et al*. 2000) when *P. pentaurelia* and *P. novaurelia* were investigated, was confirmed at present. We came to the same conclusion in the studies carried out on several strains of other species of the *P.aurelia* complex (PRZYBOŚ *et al*. 2006). Species characterized by extreme inbreeding (e.g. *P. tetraurelia*, *P. sexaurelia, P. dodecaurelia)* showed greater intraspecific polymorphism in band pattern than did species characterized by weak inbreeding such as *P. pentaurelia.*

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