Acta Protozool. (2006) 45: 387 - 393

ACTA
PROTOZOOLOGICA

Sibling Species within *Paramecium jenningsi* Revealed by PCR-RFLP

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Summary. *Paramecium jenningsi* Diller *et* Earl, 1958 is classified in the so-called "aurelia" subgroup. The original assumption of the monomorphic status of *P. jenningsi* (Sonneborn 1970) has been repeatedly challenged over the years with the application of increasingly sophisticated methods and knowledge of the geographic distribution of particular strains of this species. Investigations concerning genetic polymorphism suggested the existence of two sibling species in *P. jenningsi*, comprised of strains inhabiting India, Saudi Arabia, China and Japan. In order to confirm the existence of these sibling species, here we apply the PCR-RFLP method for analysis of a fragment of the *hsp70* gene coding for the Hsp70 cytosol heat shock protein. PCR-RFLP with enzymes *Alu*1I and *Eco*RI did not reveal size polymorphism of restriction fragments. Band patterns obtained with enzyme *Tru*1I showed variation corresponding to genetic polymorphism and were subject to comparative analysis using the BIO1D program which applies Nei and Li's and Jaccard's similarity coefficients. The results of these analyses are homology trees showing the genetic similarity between West-Central Asian and Japanese groups, the intra-group similarity coefficients had a value of 1, i.e. the strains were 100% homologous. The similarity between both of these groups was calculated as 32% and 20% depending on which coefficient was used. West-Central Asian and Japanese groups detected in this study on the basis of PCR-RFLP result can be considered separate species after interpretation of inter-strain crosses, which have shown the existence of reproductive isolation barriers between West-Central Asian and Japanese strains on the basis of the percentage of surviving clones.

Key words: *Paramecium aurelia*, *P. jenningsi*, PCR-RFLP, sibling species.

INTRODUCTION

Protozoans are particularly valuable research subjects for many biological disciplines because their exceptional diversity (morphological as well as physiological) allows for wide-ranging analyses using contemporary methods (electron microscopy, *in vitro* cultures, biochemical and genetic methods) (Kazubski 1988). The genus *Paramecium* has been known for 250 years and its representatives include some of the most commonly studied ciliates. They are model organisms for many investigations concerning questions in protozoology, cytology, genetics, ecology, general biology and many other fields in biology. The cosmopolitan distribution, straightforward culture and relatively large size (80-400 µm) of paramecia have made them universal laboratory organisms (Fokin *et al.* 2001).

Paramecium jenningsi Diller *et* Earl, 1958 is classified in the so-called "aurelia" subgroup including *P. caudatum*, *P. multimicronucleatum*, *P. schewiakoffi* and species of the *P. aurelia* complex (Przyboś 1975, 1978; Fokin *et al.* 2001, 2004). Many studies using

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traditional and molecular methods of analysis have shown that *P. jenningsi* is closely related with species comprising the *P. aurelia* complex (Strüder-Kypke *et al.* 2000a, b; Fokin *et al.* 2001, 2004). It has even been suggested that *P. aurelia* and *P. jenningsi* are sibling species, and that the *P. aurelia* complex evolved from a *P. jenningsi* lineage (Yamauchi *et al.* 1995). Recent comparative analyses of SS rRNA sequences (Fokin *et al.* 2004) have also shown that *P. jenningsi* is the sister species to the newly described *P. schewiakoffi* from the monophyletic "aurelia" subgroup. The original assumption of the monomorphic status of *P. jenningsi* (Sonneborn 1970) has been repeatedly challenged over the years with the application of increasingly sophisticated methods and knowledge of the geographic distribution of particular strains of this species. At first, traditional cytogenetic and karyological studies (Przyboś 1975, 1978, 1980, 1986a, b; Jurand and Przyboś 1984) as well as biochemical analyses (Allen *et al.* 1983) supported the monomorphic status of *P. jenningsi*. Investigations concerning genetic polymorphism in *P. jenningsi* were commenced after the morphological and biochemical stability of paramecia was contrasted with the discovery of substantial variation at the molecular level (Strüder-Kypke *et al.* 2000a, b; Fokin *et al.* 2001). This suggested the existence of genetic polymorphism decoupled with detectable polymorphism at other organizational levels of the cell. Another important determinant of further studies on the genetic polymorphism of *P. jenningsi* was the discovery of new habitat of this species in Japan, China and Saudi Arabia (Przyboś *et al.* 2003b). The results of comparative genomic analyses using RAPD-PCR fingerprinting contested the assumed monomorphic nature of this species because they suggested the existence of two sibling species in *P. jenningsi*, comprised of strains inhabiting India, Saudi Arabia, China and Japan (Przyboś *et al.* 2003b; Skotarczak *et al.* 2004a, b).

In order to confirm the existence of these sibling species, here we apply the PCR-RFLP method, also known as CAPS (Cleaved Amplified Polymorphic Sequence), based on sequential amplification and restriction. This method can be used if the amplified products do not possess length variation and nucleotide variation at sites complementary to the applied primers. Restriction enzymes reveal nucleotide variation at enzyme-specific sites in the amplified fragments (Jerome and Lynn 1996, Burucoa *et al.* 1999). An alternative and more detailed method involves sequencing, however, it considerably increases costs.

MATERIALS AND METHODS

Genetic material isolated from 9 strains of *P. jenningsi* was used as listed in Table 1. Paramecia were raised in a lettuce culture inoculated with *Enterobacter aerogenes* at room temperature in daylight according to the method of Sonneborn (1970).

Paramecia DNA was isolated using the DNeasy™ Tissue Kit (Qiagen, Germany). For PCR-RFLP a fragment of the *hsp70* gene coding for the Hsp70 cytosol heat shock protein of 70 kDa (Budin and Philippe 1998) was used for digestion with restriction enzymes. The primer pair Afor (5'GAG GAG AAG ATT TCG ATA AC3') and Arev (5'GCT TCA TCT GGG TTG ATT GA3'), amplifying a 440 bp fragment of the aforementioned gene (Przyboś *et al.* 2003a), was used in PCR. The 50 µl PCR consisted of 5 µl DNA isolate; 2.5 U DNA recombinant *Taq* polymerase in buffer (20 mM Tris-HCl, 100 mM KCl, 1 mM DTT, 0.1 mM EDTA, 0.5% Nonidet P-40, 0.5% Tween 20, 50% glicerol; pH 8,0) (Qiagen, Germany); 1x PCR buffer (Tris-HCl, KCl, $(NH_4)SO_4$, 15 mM $MgCl_2$; pH 8.7) (Qiagen, Germany); 87.5 mM $MgCl_2$; 25 pM primer Afor and Arev (Biomers, Germany) in TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8); 5 µM of each dNTP. Amplification of the *hsp70* gene in the 9 strains of *P. jenningsi* was performed in a Peltier Thermal Cycler PTC-200 MJ Research (USA) and a T-Gradient Biometra (Germany). The thermal-time PCR profile of Przyboś *et al.* (2003a) was used: initial denaturation at 94°C for 2 min, 30 cycles (denaturation at 94°C for 45 s, primer annealing at 50°C for 60 s, chain extension at 72°C for 60 s) and final extension at 72°C for 5 min. The reaction was performed in 18 samples, i.e. 2 samples per isolate from 9 cultured strains of *P. jenningsi*.

PCR products were purified and precipitated using a 96% and 70% ethanol/sodium acetate protocol. Purified PCR products were subsequently digested with three restriction enzymes: *Alu*I, *Eco*RI, *Tru*1I (*Mse*I) (Fermentas, Lithuania), using 3U in 20 µl reactions.

RESULTS

Amplification with primers Afor and Arev in 18 samples (2 samples per isolate from 9 cultured strains of *P. jenningsi*) gave a 440 bp product representing a fragment of the *hsp70* gene (Fig. 1).

No size polymorphism of restriction fragments was detected after restriction of the purified PCR products with *Alu*I and *Eco*RI. For each sample the same number of products of identical size was obtained: 5 and 4 fragments for *Alu*I and *Eco*RI restrictases, respectively (Figs 2, 3). The application of *Tru*1I revealed restriction site polymorphism in the sequence of the *hsp70* gene in *P. jenningsi*. The number of obtained restriction fragments was the same for all studied strains however, their lengths were different for West-Central Asian and Japanese strains. No intra-strain polymorphism was detected (Fig. 4). Approximate sizes of all fragments are compared in Table 2.

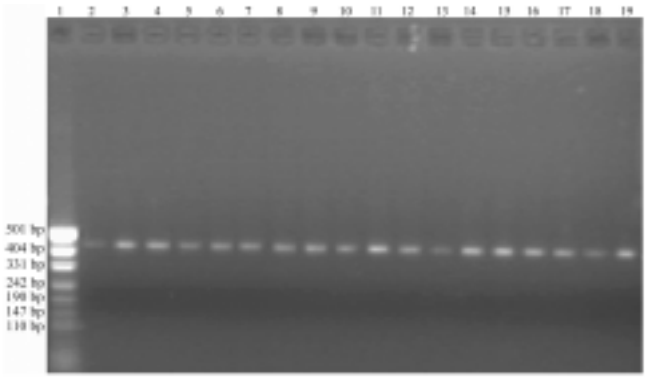

Fig. 1. Amplification products of *hsp70* from 9 strains of *P. jenningsi*. Lanes: 1 - molecular mass marker MW 501, 2 and 3 - *P. jenningsi* strain from India (Bangalore), 4 and 5 - strain from Saudi Arabia, 6 and 7 - strain from China (Shanghai), 8 and 9 - strain from Japan (Yamaguchi), 10 and 11 - strain from Japan (Hagi), 12 and 13 - strain from Japan (Ube), 14 and 15 - strain from Japan (Nagato), 16 and 17 - strain from Japan (Shinnamyou), 18 and 19 - strain from Japan (Okinawa, Hujigawa).

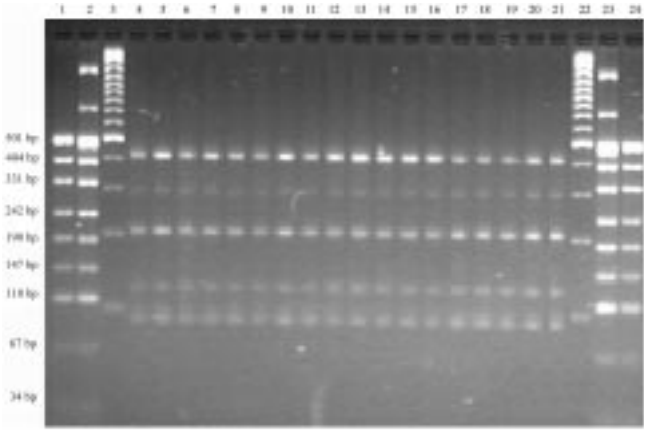

Fig. 2. Restriction products of the *hsp70* gene with enzyme *Alu*I in 9 strains of *P. jenningsi*. Lanes: 1 and 24 - mass marker MW 501, 2 and 23 - mass marker MW 1444, 3 and 22 - mass marker GeneRuler, 4 and 5 - strain of *P. jenningsi* from India (Bangalore), 6 and 7 - strain from Saudi Arabia, 8 and 9 - strain from China (Shanghai), 10 and 11 - strain from Japan (Yamaguchi), 12 and 13 - strain from Japan (Hagi), 14 and 15 - strain from Japan (Ube), 16 and 17 - strain from Japan (Nagato), 18 and 19 - strain from Japan (Shinnamyou), 20 and 21 - strain from Japan (Okinawa, Hujigawa).

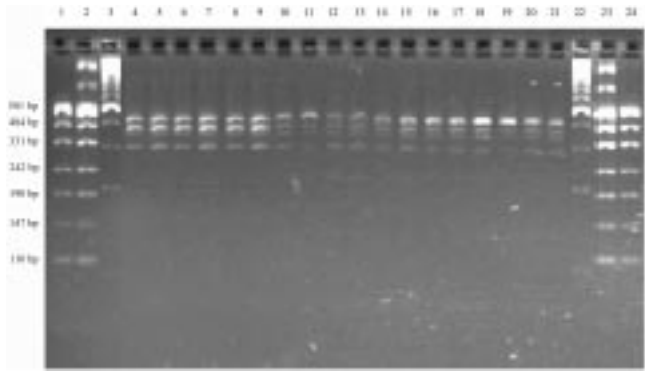

Fig. 3. Restriction products of the *hsp70* gene with enzyme *Eco*RI in 9 strains of *P. jenningsi.* Lanes as in Fig. 2.

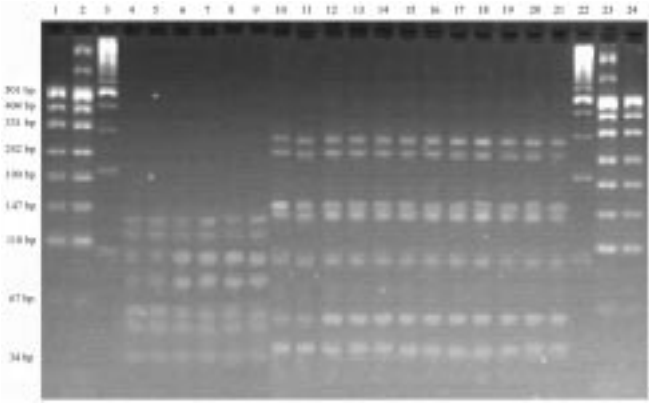

Fig. 4. Restriction products of the *hsp70* gene with enzyme *Tru*1I in 9 strains of *P. jenningsi.* Lanes as in Fig. 2.

PCR-RFLP with enzymes *Alu*1I and *Eco*RI did not reveal size polymorphism of restriction fragments, because all fragments were identical. As so, the patterns were not analyzed further. The results of comparative analysis of band patterns obtained by PCR-RFLP with enzyme *Tru*1I using the BIO1D program which applies Nei and Li's and Jaccard's similarity coefficients are homology trees showing the genetic similarity in percentage values for each strain of *P. jenningsi* from different geographical localities (Figs 5, 6).

DISCUSSION

The taxonomic structure of ciliates is characteristically complicated. At present the systematics of this

Table 1. Strains of *Paramecium jenningsi* and their origin.

Strain Symbol	Collection site	Collection date of sample for first culture	Collector
B	India, Bangalore	1955	P. B. Padmavathi
SA	Saudi Arabia, vicinity of Riyadh	July 1999	K. A. S. AL Rasheid
C2	China, Shanghai	November 1999	M. Fujishima
JAP	Japan, Honshu Island, Yamaguchi	November 1999	S. Fokin
JYR	Japan, Honshu Island, Hagi	September 1997	S. Fokin
J1	Japan, Honshu Island, Ube	October 2000	M. Fujishima
J2	Japan, Honshu Island, Nagato	September 2000	M. Fujishima
J3	Japan, Honshu Island, Shinnamyou	October 2000	M. Fujishima
JOH	Japan, Okinawa Island, Hujigawa	2000	M. Fujishima

Table 2. Approximate lengths of restriction fragments after digestion of gene *hsp70* with enzymes *Alu*I, *Eco*RI and *Tru*1I. Lengths in base pairs.

*Alu*I	*Eco*RI	*Tru*1I	
All - *P. jenningsi* strains		West-Central Asian strains of *P. jenningsi*	Japanese strains of *P. jenningsi*
200	171	59	120
90	91	54	110
80	81	40	63
61	79	34	59
43		29	40
		24	25
		13	16

group is rapidly changing because of an immense increase of information, including the discovery of new, oftentimes substantial differences between groups of ciliates which leads to the description of new taxa (Kazubski 2000). Representatives of many genera, including *Paramecium*, show incredible species diversity. We now have described many more species of paramecia than had been estimated beforehand (Sonneborn 1957). In evolutionary studies, sibling species seem to be particularly important, such as those detected in *Paramecium*. Sibling species are considered to be real biological species in which reproductive isolation is fully developed, but are morphologically similar or even identical (Sonneborn 1975), due to recent common ancestry. In evolutionary terms, not much time has past since sibling species have diverged, thus these organisms are relatively young and useful for the study of evolutionary processes. The anatomy and functioning of *Paramecium* cells, and especially their life strategy guaranteeing a fast rate of evolution (Fokin *et al*. 2001), allows for an in-depth look into these processes at many levels of cell organization, which particularly predestines these organisms to evolutionary and phylogenetic study.

PCR-RFLP allows for the detection of genetic polymorphism due to nucleotide variation at enzyme-specific sites in amplified DNA fragments. Various band patterns can be used as RFLP markers for identification purpose or for further phylogenetic analyses describing the evolutionary relationships between these organisms (Jerome and Lynn 1996, Burucoa *et al.* 1999). In this paper, we applied PCR-RFLP to a fragment of the *hsp70* gene coding for the Hsp70 cytosol heat shock protein. Recently, *hsp70* genes have been shown to be reliable as a phylogenetic marker in protozoa and to be suitable for separating sibling species among *Paramecium* genus (Hori *et al.* 2006). These genes has also been previously used in phylogenetic analyses of diverse organisms, including ciliates and other protozoans, by many indepen-

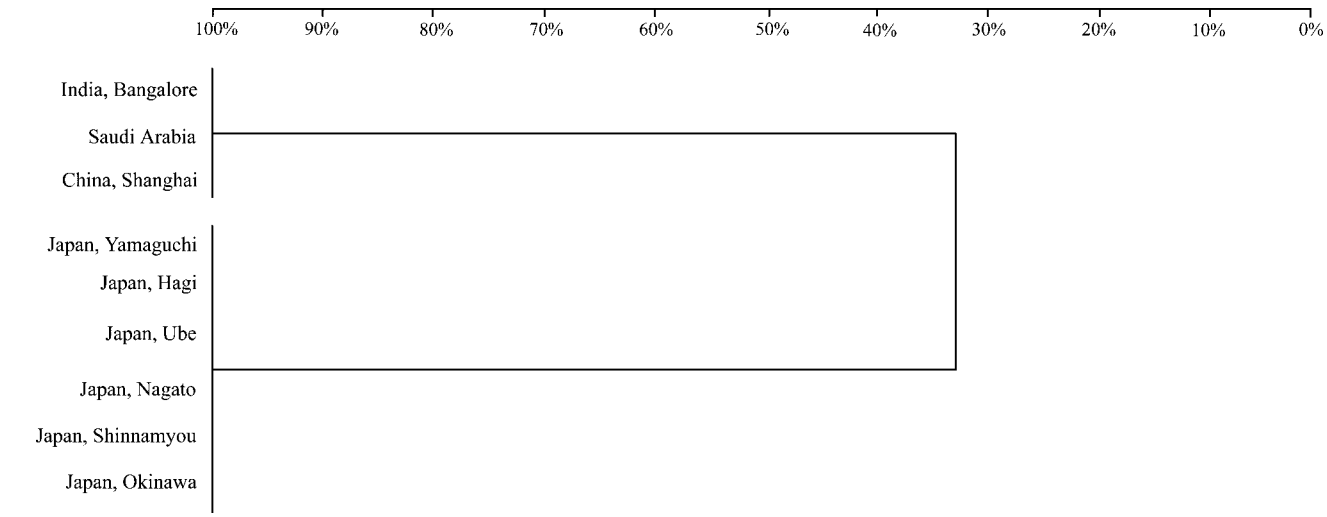

Fig. 5. Homology diagram calculated on the basis of Nei -Li coefficients for *P. jenningsi* strains through PCR-RFLP with *Tru*1I on the basis of a fragment of the *hsp*70 gene.

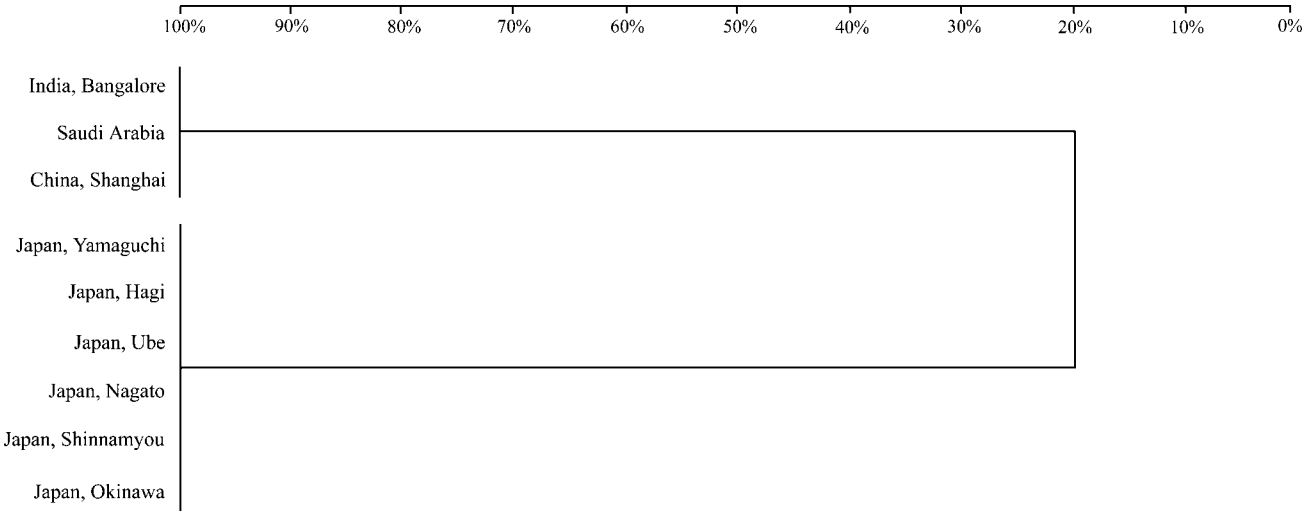

Fig. 6. Homology diagram calculated on the basis of Jackard's coefficients for *P. jenningsi* strains through PCR-RFLP with *Tru*1I on the basis of a fragment of the *hsp*70 gene.

dent research groups (Gupta and Golding 1993, Boorstein *et al.* 1994, Rensing and Maier 1994, Budin and Philippe 1998, Przyboś *et al.* 2003a).

Very recently one more molecular marker for testing genetic variation in ciliates, which has very good resolution, the mitochondrial cytochrome *c* oxidase I (Barth *et al.* 2006) was successfully tested.

PCR-RFLP using *Alu*I and *Eco*RI revealed a lack of size polymorphism of restriction fragments in all studied strains of *P. jenningsi*, which did not permit their

differentiation. Band patterns obtained by PCR-RFLP with enzyme *Tru*1I showed genetic polymorphism within the 9 *P. jenningsi* strains and were subject to comparative analysis using Nei and Li's and Jaccard's similarity coefficients. Both diagrams differentiated the West-Central Asian and Japanese strains into two groups on the basis of different electrophoretic band patterns of RFLP products obtained with the *Tru*1I restrictase. Probably because of PCR products used for this experiment included two or three isoforms of hsp70 the amount of length of restriction fragments was not ca 440bp. Because of the identical band patterns within the West-Central Asian and Japanese groups, the intra-group similarity coefficients had a value of 1, i.e. the strains were 100% homologous. The similarity between both of these groups was calculated as 32% and 20% depending on which coefficient was used. The difference is attributable to different mathematical formulas used to calculate each coefficient, thus these values must be considered as relative values and not absolute genetic distances.

These results are compatible with earlier studies using the RAPD method in which these two groups were also detected (Przyboś *et al.* 2003b; Skotarczak *et al.* 2004a, b). However, in contrast to the restriction analyses, RAPD showed a higher level of variation, determining lower level divisions within the West-Central Asian and Japanese groups. Congruent results from independent phylogenetic analyses strongly support the likelihood of the tree topology and the conclusion that the described phylogenetic relationships between the 9 *P. jenningsi* strains are biologically real. The results of the phylogenetic analyses performed on data from various molecular markers and mathematical models unambiguously verify the existence of two sibling species in *P. jenningsi*. However, it should be underlined that the application of molecular methods does not allow for the description of new species. The use of genetic variation only, however detailed, is not sufficient for the evaluation of new taxa. The detection of genetic polymorphism is not sufficient for the description of the basic systematic unit, i.e. the species. The only uncontested means of establishing a new species is the reproductive isolation criterion which is the essence of the biological species concept (Burma and Mayr 1949). For this reason, molecularly differentiated West-Central Asian and Japanese strains detected in this study can be considered separate species only after interpretation of inter-strain crosses carried out by Przyboś *et al.* (2003b). The investigations carried out have shown the existence of reproductive isolation barriers between West-Central Asian and Japanese strains on the basis of the percentage of surviving clones, which confidently establishes these strains as separate, sibling species. The crosses prove that these sibling species are true biological species and therefore binomial species names can be proposed for them. According to the priority statute, the first name for these ciliates (*P. jenningsi*) should be maintained by the species with the West-Central Asian strains, including the Indian strain from Bangalore, a model strain for the species. The name for the second sibling species encompass the Japanese strains could be created following Sonneborn through analogy with sibling species from the *P. aurelia* complex by adding a latin numerical prefix before the *aurelia* root. However in order to establish a new species name some more detailed research (i.e. morphometric and ecological description) should be done.

Despite the fact that the molecular methods applied in this study cannot be used as the only means of establishing a new species, they are necessary for all research of this type because of their usefulness in detecting genetic polymorphism, critical for the formation of reproductive isolation. According to the contemporary synthetic theory of evolution, mechanisms maintaining isolation between species are by-products of genome divergence through natural selection (Gavrilets 2003), therefore the detection of these changes is important for determining the nature of reproductive isolation and should be the aim of studies of the processes of speciation and the discovery of new species.

Acknowledgements. I would like to thank Prof. Ewa Przyboś from the Institute of Systematics and Evolution of Animals, Polish Academy of Sciences, Kraków, Poland for providing *P. jenningsi* strains. This research was supported financially by the Grant No. 2P04C 01126 of the State Committee for Scientific Research, Warsaw Poland.

REFERENCES

Allen S.L., Rushford C.L., Nerad T.A., Lau E.T. (1983) Intraspecies variability in the esterases and acid phosphatases of *Paramecium jenningsi* and *Paramecium multimicronucleatum*: assignment of unidentified paramecia: comparison with *P. aurelia* complex. *J. Protozool.* **30:** 155-163

Barth D., Krenek S., Fokin S.I., Berendonk T. U. (2006) Intraspecific genetic variation in *Paramecium* revealed by mitochondrial cytochrome *c* oxidase I sequences. *J. Eukaryot. Microbiol.* **53:** 20-25

Boorstein W. R., Ziegelhoffer W. R., Craig E. A. (1994) Molecular evolution of Hsp70 multigene family. *J. Mol. Evol.* **38:** 1-17

Budin K., Philippe H. (1998) New insights into the phylogeny of Eukaryotes based on Ciliate Hsp70 sequences. *Mol. Biol. Evol.* **15:** 943-956

Burma B. H., Mayr E. (1949) The species concept. *Evolution Int. J. Org. Evol.* **3:** 369-373

Burucoa Ch., Lhomme V., Fauchere J. L. (1999) Performance criteria of DNA fingerprinting methods for typing of *Helicobacter pylori* isolates: experimental results and meta-analysis. *J. Clin. Microbiol.* **37:** 4071-4080

Fokin S. I., Przyboś E., Chivilev M. (2001) Nuclear reorganization variety in *Paramecium* (Ciliophora: Peniculida) and its possible evolution. *Acta Protozool.* **40:** 249-261

Fokin S. I., Przyboś E., Chivilev M., Beyer C. L., Horn M., Skotarczak B., Wodecka B., Fujishima M. (2004) Morphological and molecular investigations of *Paramecium schewiakoffi* sp. nov. (Ciliophora, Oligohymenophorea) and current status of distribution and taxonomy of *Paramecium* species. *Europ. J. Protistol.* **40:** 225-243

Gavrilets S. (2003) Perspective: models of speciation. What we have learned in 40 years? *Evolution* **57:** 2197-2215

Gupta R. S., Golding G. B. (1993) Evoluytion of Hsp70 gene and its implications regarding relationships between Archaebacteria, Eubacteria and Eucaryotes. *J. Mol. Evol.* **37:** 573-582

Hori M., Tomikawa I., Przyboś E., Fujishima M. (2006) Comparison of the evolutionary distances among syngens and sibling species of *Paramecium*. *Mol. Phylogenet. Evol.* **38:** 697-704

Jerome Ch. J., Lynn D. H. (1996) Identifying and distinguishong sibling species in the *Tetrahymena pyriformis* complex (Ciliophora, Oligohymenophorea) using PCR-RFLP analysis of nuclear ribosomal DNA. *J. Euk. Microbiol.* **43:** 492-497

Jurand A., Przyboś E. (1984) A new method of study of chromosome numbers in *Paramecium*. *Folia biol. (Kraków)* **32:** 295-300

Kazubski S. L. (1988) Ecologic and geographical variability of Protozoa in the light of the faunal studies. *Wiad. Parazytol.* **34:** 579-589

Kazubski S. L. (2000) Pierwotniaki pasożytnicze. *Kosmos* **49:** 627-657 (in Polish)

Przyboś E. (1975) Genetic studies of *Paramecium jenningsi* strains (Diller *et* Earl, 1958). *Folia biol. (Kraków)* **23:** 425-471

Przyboś E. (1978) Cytological and karyological studies of *Paramecium jenningsi*. *Folia biol. (Kraków)* **26:** 25-29

Przyboś E. (1980) The African strain of *Paramecium jenningsi*. Cytological and karyological investigations. *Folia biol. (Kraków)* **28:** 391-397

Przyboś E. (1986a) Species structure in Ciliates. *Folia biol. (Kraków)* **34:** 103-132

Przyboś E. (1986b) Chromosomes in *Paramecium jenningsi* (Diller & Earl, 1958): A serial section study. *Folia biol. (Kraków)* **34:** 133-160

Przyboś E., Hori M., Fokin S. I. (2003a) Strains of *Paramecium quadecaurelia* from Namibia, Africa; genetic and molecular studies. *Acta Protozool.* **42:** 357-360

Przyboś E., Skotarczak. B., Wodecka B. (2003b) Phylogenetic relationships of *Paramecium jenningsi* strains (classical analysis and RAPD studies). *Folia biol. (Kraków)* **51:** 130-145

Rensing S. A., Maier U. G. (1994) Phylogenetic analysis of the stress-70 protein family. *J. Mol. Evol.* **39:** 80-86

Skotarczak B., Przyboś E., Wodecka B., Maciejewska A. (2004a) Sibling species within *Paramecium jenningsi* revealed by RAPD. *Acta Protozool.* **43:** 29-35

Skotarczak B., Przyboś E., Wodecka B., Maciejewska A. (2004b) Random amplified polymorphic DNA fingerprinting as a marker for *Paramecium jenningsi* strains. *Folia biol. (Kraków)* **52:** 117-124

Sonneborn T. M. (1957) Breeding systems, reproductive methods, and species problems in Protozoa. In: The Species Problem, (Ed. E. Mayr). AAAS, Washington, 155-324

Sonneborn T. M. (1970) Methods in *Paramecium* research. In: Methods in Cell Physiology, (Ed. D. M. Prescott). Academic Press, New York, 242-339

Sonneborn T. M. (1975) The *Paramecium aurelia* complex of 14 sibling species. *Trans. Am. Microsc. Soc.* **94:** 155-178

Strüder-Kypke M. C., Wright A. G., Fokin S. I., Lynn D. H. (2000a) Phylogenetic reletionships of the genus *Paramecium* inferred from Small Subunit rRNA gene sequences. *Mol. Phylogenet. Evol.* **14:** 122-130

Strüder-Kypke M. C., Wright A. G., Fokin S. I., Lynn D. H. (2000b) Phylogenetic reletionships of the subclass Peniculia (Oligohymenophorea, Ciliophora) inferred from Small Subunit rRNA gene sequences. *J. Eukaryot. Microbiol.* **47:** 419-429

Yamauchi K., Tada H., Usuki I. (1995) Structure and evolution of *Paramecium* hemoglobin genes. *Biochim. Biophys. Acta* **1264:** 53-62

Received on 9th May, 2006; revised version on 21st June, 2006; accepted on 23rd August, 2006