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Molecular Polymorphism of *Paramecium tetraurelia* (Ciliophora, Protozoa) in Strains Originating from Different Continents

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Fragments of LSU rDNA and COI mtDNA genes were sequenced in *Paramecium tetraurelia* strains originating from different continents, i.e. from Australia (Sydney), Europe (Spain, Poland), Asia (Israel, India, Japan) and North America (Indiana) in order to investigate intra-specific polymorphism in this species. Phylogenetic trees (based on analyses using the NJ, MP and BI methods) revealed that *P. tetraurelia* strains from Australia, Europe, North America and Asia (Israel, Japan) belong to one group divided into two main clusters, while a strain from India is separate and belongs to a different group. The Indian strain groups together with strains representing different species of the *P. aurelia* complex: *P. septaurelia*, *P. octaurelia,* and *P. dodecaurelia*. Polymorphism within *P. tetraurelia* was confirmed, however, it seems that the applied markers did not explain the ways of divergence of strains within species (Indian strain and others), and also did not show correlations between geographic origin of strains and their genetic diversity. Some species of the *P. aurelia* complex seem closely related.

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

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Paramecium tetraurelia is a cosmopolitan species of the *P. aurelia* complex occurring in temperate and subtropical climates and is known from North, Central, and South America, Asia, Australia, and Europe (SONNEBORN 1975; PRZYBOŚ & FOKIN 2000; PRZYBOŚ 2008). In Europe, *P. tetraurelia* was reported from several countries, i.e. Finland, Great Britain, France, Holland, Poland, the Czech Republic, Slovakia, Italy, Bulgaria, Spain, and Russia. In Australia the species was recorded in Sydney and Melbourne, whereas in Asia it was found only in Japan and Israel (cf PRZYBOŚ 2008). Certainly, large territories within Asia are still in need of study. The present paper presents a new locality of the species in Delhi, India. As our collection of *P. tetraurelia* strains has been enlarged, we decided to investigate the intra-specific polymorphism by sequencing fragments of the LSU rDNA and COI mtDNA genes in strains originating from different continents, and if possible from distant regions on a continent.

Material and Methods

Material

Three strains (ID1, ID2, ID3) were established from water samples collected by S. Fokin in 2007 in Delhi, India from 2 collecting sites, one in the city (ID1, ID2) and a second (ID3) in the garden of Delhi University. Other stocks of *P. tetraurelia* (S, 51, IT, J, PK, SM) and *P. aurelia* spp. (strain 38 of *P. septaurelia*, strain 138 of *P. octaurelia*, and strain 246 of *P. dodecaurelia*) used for sequencing are listed in Table 1.

Table 1

Paramecium aurelia spp. strains used for sequencing

Species	Strain designation	Origin	Reference	Accession numbers	
				5'LSU rDNA	COI mt DNA
P. tetraurelia	S, standard of the species	Australia, Sydney	SONNEBORN 1974	EU717652	EU717643
P. tetraurelia	51	USA, Indiana	SONNEBORN 1974	EU717647	EU717638
P. tetraurelia	ID	India, Delhi	Present paper	EU717648	EU717639
P. tetraurelia	IT	Israel, Tabga	PRZYBOŚ 1995	EU717649	EU717640
P. tetraurelia	J	Japan, Honshu Island	PRZYBOŚ & FOKIN 2001	EU717650	EU717641
P. tetraurelia	PK	Poland, Kraków	KOMALA & PRZYBOŚ 2000	EU717651	EU717642
P. tetraurelia	SM	Spain, Madrid	PRZYBOŚ 1980	EU717653	EU717644
P. septaurelia	38	USA, Florida	SONNEBORN 1974	EU717646	EU717637
P. octaurelia	138	USA, Florida	SONNEBORN 1974	EU717645	EU717636
P. dodecaurelia	246	USA, southern state	SONNEBORN 1974	DQ207369	EU086108
P. caudatum	Pc	Cyprus, Akamas	unpublished	DQ207375	DQ837977

Table 2

Primers used in this study

Amplified region	Primer	Sequence 5'-3'	References
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

Methods

1. Culture and identification of paramecia as species of the *P. aurelia* complex

Cultivation and identification of paramecia were performed according to SONNEBORN's (1970) methods by conjugation tests and verification of survival of hybrid clones in F1 and F2 generations. The paramecia were cultivated on a lettuce medium inoculated with *Enterobacter aerogenes*.

The strains ID1 and ID2 were identified as *P. tetraurelia* on the basis of strong conjugation (95% pairs) between the mating types of the investigated strains with mating types of the standard strain of the species from Sydney, Australia. The strain ID3 was identified as *P. primaurelia* using standard strain 90 of this species.

2. 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*. (2003). The strains used in molecular studies are listed 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 [1]. The reverse primer LR6 is the universal eukaryotic primer [2]. PCR amplification was carried out in a final volume of 30 μl containing: 2 μl of DNA, 1.5 U Taq-

[1] http://scitools.idtdna.com/analyzer/

[2] http://www.biology.duke.edu/fungi/mycolab/primers.htm.

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 the mitochondrial cytochrome oxidase (COI) gene fragment

To amplify the CO I region (880bp) of mitochondrial DNA, Cox_L and Cox_H primers were used (according to BARTH *et al*. 2006). PCR amplification was carried out in the same volume as in the case of 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 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 (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). Sequenced 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 (THOMPHSON *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 NJ (Neighbor-Joining method) (SAITOU & NEI 1987), MP (Maximum Parsimony) (NEI & KUMAR 2000) and Bayesian analyses (BI) (RONQUIST & HUELSENBECK 2003). The NJ analysis was performed using a Kimura 2-parameter correction model (KIMURA 1980) and Jukes-Cantor method (JUKES & 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. Bayesian analyses (BI), were performed in MrBayes 3.1.2 (RONQUIST & HUELSENBECK 2003). Analysis was run with 5,000,000 generations and trees were sampled every 100 generations. All trees were reconstructed with TreeView 1.6.6 (PAGE 1996).

Results and Discussion

The strains ID1 and ID2 were identified as *P. tetraurelia* on the basis of strong conjugation (95% pairs) between the mating types of the investigated strains with mating types of the standard strain of the species from Sydney, Australia. As strains are from the same habitat, only one (ID1) was used for sequencine (Table 1) designated ID. The strain ID3 was identified as *P. primaurelia* using standard strain 90 of the species.

The presence of *Paramecium tetraurelia* and *P. primaurelia* in Delhi, India was recorded for the first time. *P. tetraurelia* was known before in Asia from Japan and Israel, and *P. primaurelia* from Japan, Turkmenia, Russia (East Siberia), Vietnam, and Israel (cf PRZYBOŚ & FOKIN 2000). In India, only *P. sexaurelia* was known before (strains from Bangalore, SONNEBORN 1974).

The new stand of *P. tetraurelia* in Asia on the Indian subcontinent prompted the molecular investigations of intra-specific polymorphism in this species. Fragments of the LSU rDNA and COI mtDNA genes were sequenced in several *P. tetraurelia* strains originating from different continents, e.g. from Australia (Sydney), Europe (two distant stands Spain and Poland), Asia (stands from Israel, India and Japan) and North America (Indiana) in order to reveal the intra-specific polymorphism of the species as well as other strains representing several species of the *P. aurelia* complex (Table 1) used as outgroups. These species were chosen as mating reactions can occur between their mating type combinations, however, crosses are nonviable, although the species seem related.

Phylogenetic trees (based on NJ, MP, BI analyses) constructed on the basis of the fragment of LSU rDNA showed that *P. tetraurelia* strains belong to two main clusters (Fig. 1). One cluster is composed of two sub-clusters (one with strains from Spain, Israel and USA; the second with strains from Australia, Japan and Poland). The strain from India is placed in the second cluster, together with strains representing different species of the *P. aurelia* complex: *P. septaurelia*, *P. octau-*

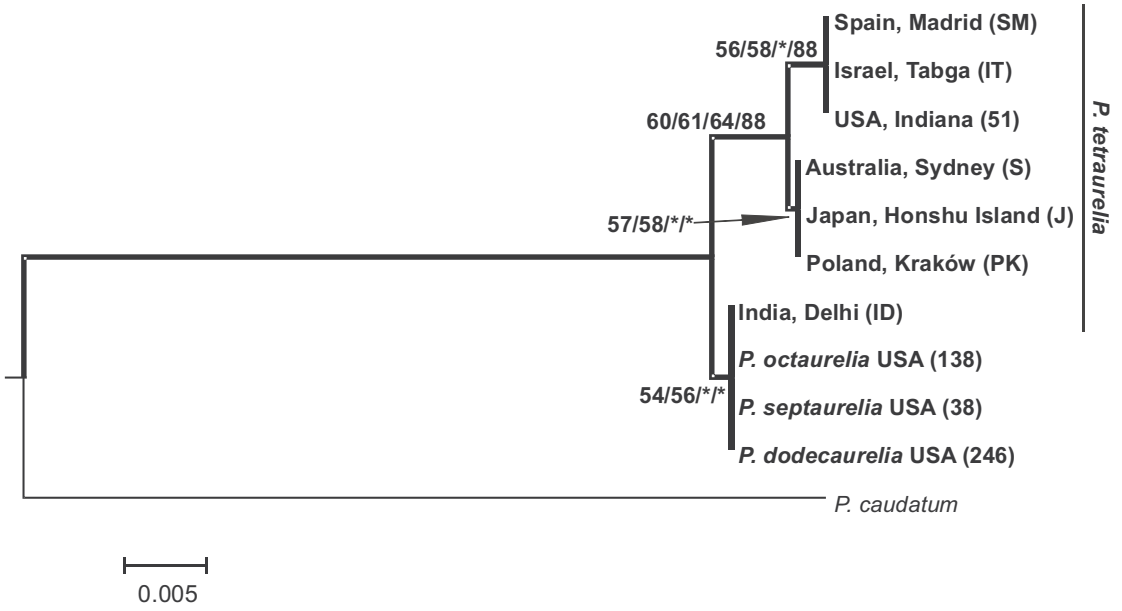

Fig. 1. Phylogenetic tree constructed for 7 strains of *P. tetraurelia, P. septaurelia, P. octaurelia P. dodecaurelia* strains and *P. caudatum* strain as outgroup, based on a comparison of sequences from the 5' LSU rDNA fragment using the NJ (neighbor joining) method (with the application of the Jukes-Cantor correction and Kimura two-parameter model), MP (maximum parsimony) analysis and Bayesian Interference (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.

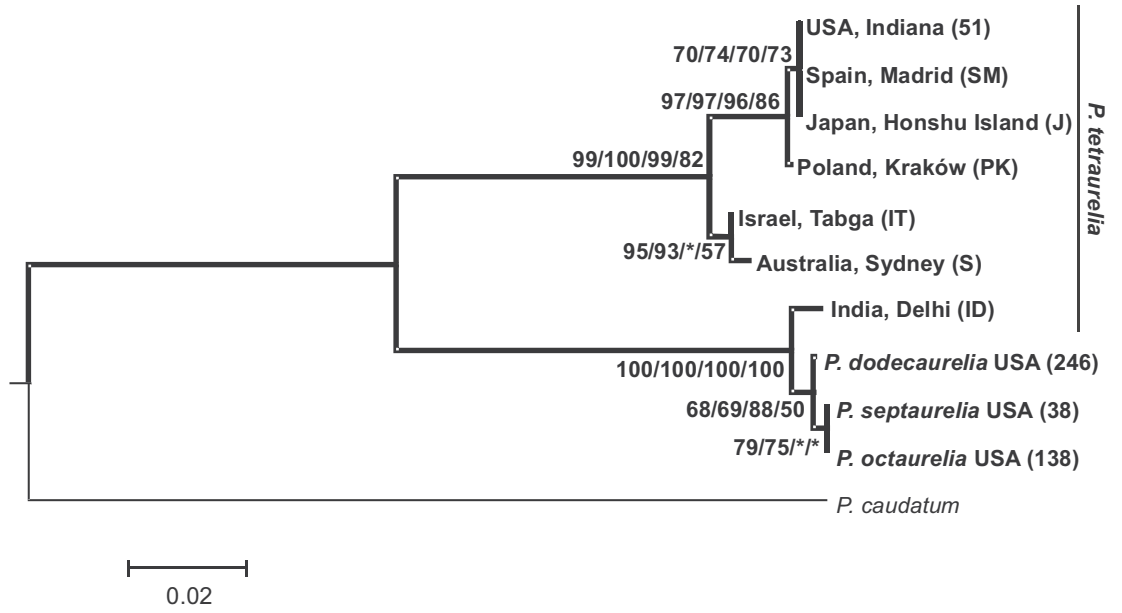

Fig. 2. Phylogenetic tree constructed for 7 strains of *P. tetraurelia, P. septaurelia, P. octaurelia P. dodecaurelia* strains and *P. caudatum* strain as outgroup, based on a comparison of sequences from the COI mtDNA fragment using the NJ (neighbor joining) method (with the application of the Jukes-Cantor correction and Kimura two-parameter model), MP (maximum parsimony) analysis and Bayesian Interference (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.

relia, and *P. dodecaurelia. P. caudatum*, representing a different *Paramecium* morphotype, is clearly separated. The genetic distance between the studied *P. tetraurelia* strains varies from 0% to 0.9%, the highest distance being between the strain from India (Table 3) and other strains of *P. tetraurelia*.

Phylogenetic trees based on analyses of the fragment of COI mtDNA also showed that *P. tetraurelia* strains belong to different clusters (Fig. 2). One cluster was composed of strains from USA, Spain, Japan, Poland on one branch, and strains from Israel and Australia on a second branch. The strain from India, similarly as in the case of the LSU

Table 3

Distance matrix presenting the number of base substitutions in *Paramecium tetraurelia, P. septaurelia, P. octaurelia, P. dodecaurelia,* 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)

5'LSU	*P. tetr.* 51	*P. tetr.* ID	*P. tetr.* IT	*P. tetr.* J	*P. tetr.* PK	*P. tetr.* S	*P. tetr.* SM	*P. sept.* 38	*P. oct.* 138	*P. dodec.* 246	*P. caud.*
P. tetraurelia, 51		0.009	0.000	0.003	0.003	0.003	0.000	0.009	0.009	0.009	0.096
P. tetraurelia, ID	0.009		0.009	0.006	0.006	0.006	0.009	0.000	0.000	0.000	0.092
P. tetraurelia, IT	0.000	0.009		0.003	0.003	0.003	0.000	0.009	0.009	0.009	0.096
P. tetraurelia, J	0.003	0.006	0.003		0.000	0.000	0.003	0.006	0.006	0.006	0.099
P. tetraurelia, PK	0.003	0.006	0.003	0.000		0.000	0.003	0.006	0.006	0.006	0.099
P. tetraurelia, S	0.003	0.006	0.003	0.000	0.000		0.003	0.006	0.006	0.006	0.099
P. tetraurelia, SM	0.000	0.009	0.000	0.003	0.003	0.003		0.009	0.009	0.009	0.096
P. septaurelia, 38	0.009	0.000	0.009	0.006	0.006	0.006	0.009		0.000	0.000	0.092
P. octaurelia, 138	0.009	0.000	0.009	0.006	0.006	0.006	0.009	0.000		0.000	0.092
P. dodecaurelia, 246	0.009	0.000	0.009	0.006	0.006	0.006	0.009	0.000	0.000		0.092
P. caudatum	0.095	0.091	0.095	0.098	0.098	0.098	0.095	0.091	0.091	0.091	

Table 4

Distance matrix presenting the number of base substitutions in *Paramecium tetraurelia, P. septaurelia, P. octaurelia, P. dodecaurelia,* 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)

CO I	*P. tetr.* 51	*P. tetr.* ID	*P. tetr.* IT	*P. tetr.* J	*P. tetr.* PK	*P. tetr.* S	*P. tetr.* SM	*P. sept.* 38	*P. oct.* 138	*P. dodec.* 246	*P. caud.*
P. tetraurelia, 51		0.151	0.020	0.000	0.003	0.023	0.000	0.146	0.146	0.142	0.279
P. tetraurelia, ID	0.147		0.130	0.151	0.151	0.134	0.151	0.010	0.010	0.013	0.270
P. tetraurelia, IT	0.020	0.127		0.020	0.017	0.003	0.020	0.134	0.134	0.130	0.273
P. tetraurelia, J	0.000	0.147	0.020		0.003	0.023	0.000	0.146	0.146	0.142	0.279
P. tetraurelia, PK	0.003	0.147	0.016	0.003		0.020	0.003	0.146	0.146	0.142	0.279
P. tetraurelia, S	0.023	0.131	0.003	0.023	0.020		0.023	0.138	0.138	0.134	0.279
P. tetraurelia, SM	0.000	0.147	0.020	0.000	0.003	0.023		0.146	0.146	0.142	0.279
P. septaurelia, 38	0.143	0.010	0.131	0.143	0.143	0.135	0.143		0.000	0.003	0.286
P. octaurelia, 138	0.143	0.010	0.131	0.143	0.143	0.135	0.143	0.000		0.003	0.286
P. dodecaurelia, 246	0.139	0.013	0.127	0.139	0.139	0.131	0.139	0.003	0.003		0.292
P. caudatum	0.266	0.262	0.262	0.266	0.266	0.266	0.266	0.275	0.275	0.280	

rDNA fragment, is situated together with the studied species of the *P. aurelia* complex. The variability within the studied *P. tetraurelia* strains varies from 0% to 15.1% and the highest is between the strain from India and other strains (Table 4).

The 5'LSU rDNA fragment presents less resolution (only three haplotypes within the studied *P. tetraurelia* strains) than the COI mtDNA fragment (seven haplotypes found), which is probably caused by the faster mutation rate of mitochondrial DNA.

It is very interesting that the strain from India is less related to other strains of *P. tetraurelia* from Europe, Asia, North America and Australia. At the same time, this strain is clearly related to strains representing different species of the *P. aurelia* complex, belonging to a closely related group of species with a characteristic clonal type of mating type inheritance (SONNEBORN 1975). Paramecia from some species, i.e. *P. tetraurelia, P. octaurelia* and *P. decaurelia*, can produce interspecific mating reactions, however, without true conjugation (SONNEBORN 1975). Perhaps this may explain the close clustering we found among strains from different species of the *P. aurelia* complex. Similarly BARTH *et al*. (2008) also observed that strains repre-

senting *P. tetraurelia*, *P. septaurelia*, *P. octaurelia*, and *P. decaurelia* possessed similar cytochrome b (cob) sequences. As recombination is rare in the mitochondrial genome, similar results can be obtained independently of the sequenced fragment. Previously, COLEMAN (2005) showed that *P. tetraurelia* and *P. octaurelia* grouped closely on the phylogram based on the ITS region of the nuclear ribosomal cistron. Our previous studies (PRZYBOŚ *et al*. 2007a) concerning 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.

Molecular studies of *P. tetraurelia* strains carried out previously (PRZYBOŚ *et al*. 2007 a,b) with application of RAPD-PCR revealed the existence of intraspecific polymorphism within the species, correlated with characteristic, extreme inbreeding. Six strains originated from remote stands were used for analysis (cf. PRZYBOŚ *et al*. 2007a, i.e. from Sydney, Australia; Honshu Island, Japan; Madrid, Spain; Strbske Pleso in the Tatra Mountains of Slovakia; Kraków, Poland; Tabga, Israel). A further strain recorded from the Black Sea region was also added in a following paper (PRZY- BOŚ *et al*. 2007b). Different "genotypes" based on similarity of strain band patterns were distinguished within *P. tetraurelia* on the basis of RAPD analyses (PRZYBOŚ *et al.* 2007a,b). ARDRA analysis with application of the *Taq*I restriction enzyme revealed a different band pattern in the strain from Slovakia (PRZYBOŚ *et al*. 2007a).

The molecular results obtained at present may recall the problem of origin of species of the *P. aurelia* complex. AURY *et al*. (2006) supposed that most of *P. tetraurelia* genes "arose through…. three successive whole-genome duplications". They were followed by reciprocal silencing of duplicated genes in different populations which caused later isolation of them and explosive radiation of species. According to AURY *et al*. (2006) "the most recent duplication coincides with an explosion of speciation events that gave rise to the *P. aurelia* complex of 15 sibling species".

An important aspect is also the distribution and dispersal of species of the *P. aurelia* complex as "microorganisms have distribution patterns similar to those known from higher plants and animals, and these patterns reflect historical events (split of Pangea etc.), and ecological conditions" … "and in their dispersal human activities … played important role" (FOISSNER 2006; FOISSNER 2008; FOISSNER *et al*. 2008). The spread of paramecia may be connected with animals, as they can be transferred by migrating birds (SONNEBORN 1975) for long distances, or by insects (water beetles, RAZOWSKI 1996) for shorter distances, always with some drops of water, as cysts are unknown in *Paramecium* (LANDIS 1988, GUTIERREZ *et al*. 1998).

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