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Paramecium jenningsi Species Complex (Ciliophora, Protista) in India: the First Description of New Stands of *P. bijenningsi* and *P. trijenningsi*

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Paramecium jennngsi (Ciliophora, Protista) is a complex of three cryptic species known from 13 sampling points situated around the world, mainly in the tropics. Two strains recently collected in India were identified as *P. bijenningsi* and *P. trijenningsi* from the *P. jenningsi* complex, based on an analysis of 16 (both nuclear and mitochondrial) loci, strain crosses, and cytological analyses. Current results increase the knowledge about the species range of particular members of the *P. jenningsi* complex.

Key words: Ciliates, *Paramecium jenningsi species* complex, multi-loci analysis, strain crosses, cytological analysis.

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Microbial eukaryotes are one the of the main components of tropical food webs in different ecosystems. However, our knowledge of their biodiversity is still significantly underestimated (MEDINGER *et al.* 2010). Even in *Paramecium* (one of the best studied genera of microbial eukaryotes), the problem of under-sampling is still a concern in less frequently studied parts of the world, particularly the southern hemisphere (FOKIN 2010/2011). Similarly, data concerning the occurrence of particular species from the tropics are rare. One of the *Paramecium* morphospecies with preference for a warm climate and therefore poorly studied as concerns its range and biodiversity is *P. jenningsi* (DILLER & EARL 1958). Although up to now it was collected in only 13 sampling sites distributed worldwide but situated mainly in the tropics (PRZYBOŚ *et al.* 1999), it was suggested (PRZYBOŚ *et al.* 1999, 2003; MACIEJEWSKA 2007) and then confirmed by strain crosses, morphological and molecular analyses (PRZYBOŚ & TARCZ 2013) to be a complex of three cryptic species (PRZYBOŚ & TARCZ 2016). Moreover, two of them are known from one (*P. primjenningsi*) or two (*P. bijenningsi*) southern Asian locations in contrast to *P. trijenningsi* collected from the Japanese islands (6 sampling points), Africa (2 sampling points) and Americas (2 sampling points). With reference to the above, every new sampling point of *P. jenningsi* complex should bring key information about its biogeography and cryptic diversity. In the present study, based on strain crosses, cytological analyses as well as a comparison of 11 nuclear and 5 mitochondrial loci, we showed the *Paramecium* species affiliation of two strains collected in India in 2014.

Material and Methods

Material

The two investigated strains (designated KT1 and MVP1) of the *P. jenningsi* complex (Table 1) originated from India, Andhra Pradesh, Visakhapatnam. More precisely, the KT1 strain was collected in the Kottura, a freshwater pond (18° 5′34.76"N,

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Table 1

Strains of the *Paramecium jenningsi* complex used in present studies

No	Species	Strain index	Strain geographical origin	Collector's name	Reference
1.	*Paramecium bijenningsi*	MVPI	India, Andra Pradesh	Valentina SERRA and Charan Kumar BASURI	unpublished
2.	*Paramecium trijenningsi*	KTI	India, Andra Pradesh	Sergei I. FOKIN, Valentina SERRA and Charan Kumar BASURI	SERRA *et al.* 2014

83° 8′10.56"E) on 27/01/2014. The MVP1 strain was sampled in a wastewater stream in MVP Colony, which is an urban neighbourhood of the city of Visakhapatnam (17°44′14.63"N, 83°20′16.79"E) on 24/03/2014. The general morphology and nuclear apparatuses in vegetative and autogamous individuals are characterized by cytological preparations (presented in Fig. 1), and the dimensions of particular strains are given in Table 3.

Strain cultivation

Paramecia were cultured in a medium made of dried lettuce and distilled water inoculated with *Enterobacter aerogenes* according to the methods of SONNEBORN (1950, 1970) and supplemented with 0.8 µg/ml ß sitosterol (Merck, Darmstadt, Germany). Methods used in cytological studies, strain measurements, and crosses, as well as molecular analyses were as in PRZYBOŚ and TARCZ (2016).

Molecular methods and data analysis

Paramecium genomic DNA was isolated from vegetative cells at the end of the exponential phase (approx. 1000 cells were used for DNA extraction). Eleven nuclear and five mitochondrial DNA fragments were amplified, sequenced and analyzed. The above techniques as well as the nucleotide sequences of the primers used for amplification and sequencing were described in detail in PRZYBOŚ and TARCZ (2016). The DNA sequences of both newly identified strains from India are available from the NCBI GenBank database (see Table 2). The studied sequences were aligned using ClustalW (THOMPSON *et al.* 1994) as part of BioEdit software (HALL 1999) and checked manually. All of the obtained sequences were unambiguous and incorporated in analysis. Phylograms were constructed for the studied fragments as in PRZYBOŚ and TARCZ (2016).

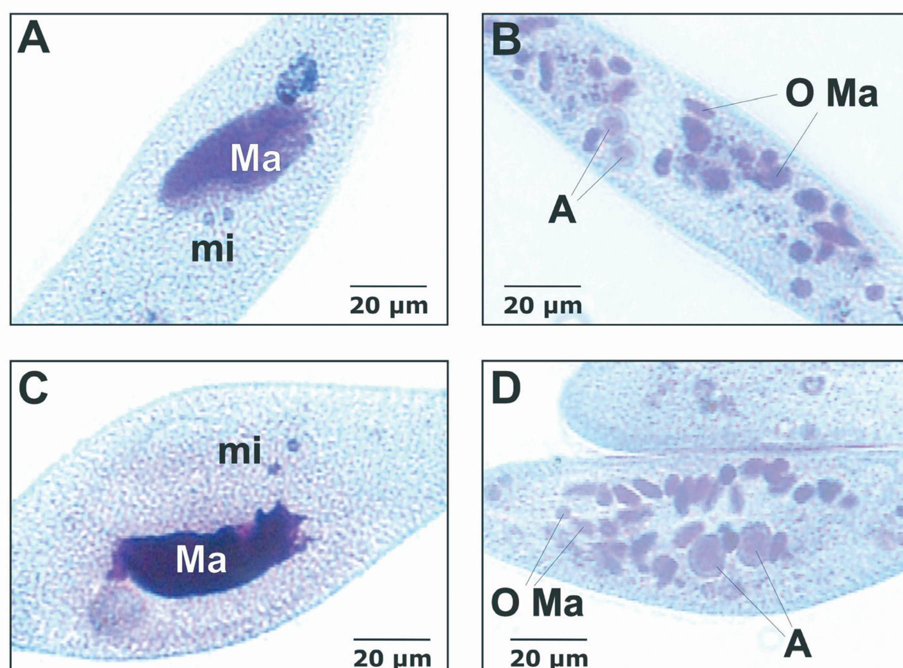

Fig. 1. A – strain MVPI of *P. bijenningsi*. Vegetative individual, macronucleus (Ma), two micronuclei (mi). B – strain MVPI of *P. bijenningsi*. Autogamous individual, fragmented old macronucleus (OMa), two macronuclear anlagen (A) with chromatin centre. C – strain KTI of *P. trijenningsi*. Vegetative individual, macronucleus (Ma), two micronuclei (mi). D – strain KTI of *P. trijenningsi*. Autogamous individual, fragmented old macronucleus (OMa), two macronuclear anlagen (A) with chromatin centre.

Table 2

GenBank accession numbers to studied DNA fragments

		nucDNA 1	nucDNA 2	nucDNA 3	nucDNA 4	nucDNA 5	nucDNA 6	nucDNA 7	nucDNA 8	nucDNA 9	nucDNA 10	nucDNA 11	mtDNA 1	mtDNA 2	mtDNA 3	mtDNA 4	mtDNA 5
		rDNA	Msh2	tRNAmet	Ubiq	PTMB.21c	PTMB.42	PTMB.159	PTMB.376	PTMB.377	PTMB.412	PTMB.418	COI	CytB	COII	3'COI-inter genic region	rns a
1.	*Paramecium bijenningsi*, strain MVP1	KY635365	KY635367	KY635369	KY635371	KY635373	KY635375	KY635377	KY635379	KY635381	KY635383	KY635385	KY635387	KY635389	KY635391	KY635393	KY635395
2.	*Paramecium trijenningsi*, strain KTI	KY635364	KY635366	KY635368	KY635370	KY635372	KY635374	KY635376	KY635378	KY635380	KY635382	KY635384	KY635386	KY635388	KY635390	KY635392	KY635394

Results and Discussion

Cytological characteristics of strains from the *P. jenningsi* complex

The strains are characterized by two micronuclei (Figs 1A,C) of chromosomal type with a reticular structure (Fokin 1997; Przyboś *et al.* 2015) and two macronuclear anlagen with chromatin centres (Diller & Earl 1958; Mitchell 1963; Przyboś 1975, 1986; Przyboś *et al.* 2003; Przyboś & Tarcz 2013) seen at a later stage of nuclear reorganization in sexual processes (autogamy, conjugation) (Figs 1B,D). Paramecia have the shape of a cigar, with two contractive vacuoles on the dorsal side, usually with 8 long radial canals.

Dimensions of strains

The dimensions (length and width of cells and macronuclei) of the strains (fixed, Giemsa-stained) and diameter of micronuclei are given in Table 3. The average length of the cells in the strain MVPI equals 248.03 μm, and 228.15 μm in the strain KTI. The diameter of micronuclei did not show much variation between the strains (3.12 - 3.85 μm in both strains).

Biology – the appearance of sexual processes

Autogamy (leading to homozygosity of all genes) was observed in strains that were starved for 16-20 fissions (as an inter-autogamous period between successive autogamies) in daily isolated lines (d.i.l.) cultivated at 27°C in a medium enabling three fissions daily. In turn, conjugation was obtained on the sixth or seventh day of clone culture at 27°C at a rate of three fissions daily. The cytoplasmic type of mating type inheritance is characteristic for the species.

Identification of species and results of strain crosses

Strain MVPI was identified as *P. bijenningsi* and strain KTI as *P. trijenningsi*, based on conjugation between the studied strains and the reference strains of the particular species, i.e. the strain CS from China, Shanghai, reference for *P. bijenningsi*, and the strain JH from Japan, Honshu Island, Hagi, reference for *P. trijenningsi*. The percentage of surviving hybrid clones in F1 and F2 was high (Table 4). The results of the strain crosses are in concordance with the results of multi-locus analyses of the studied strains of the *P. jenningsi* complex (Figs 2,3).

Table 3

Dimensions of studied strains of the *P. jenningsi* complex

No	Strain, species	Average length of cells (µm)	SD	Average cell width (µm)	SD	Average length of MAC (µm)	SD	Average width of MAC (µm)	SD	Average diameter of MIC (µm)	SD
1.	*P. bijenningsi*, MVPI	248.03	26.14	88.22	9.61	56.40	7.17	23.64	4.46	3.12	0.41
2.	*P. trijenningsi*, KTI	228.15	22.78	80.66	11.32	58.46	8.76	17.40	3.85	3.01	0.53

Table 4

Mean percentage of surviving clones (F_1/F_2 generations) in strain crosses within the species of the *Paramecium jenningsi* complex

Species	Strain designation	F1 obtained by crosses	F2 obtained by autogamy
P. bijenningsi	MVPI (India) x CS (China)	91	82
P. trijenningsi	KTI (India) x JH (Japan)	98	94

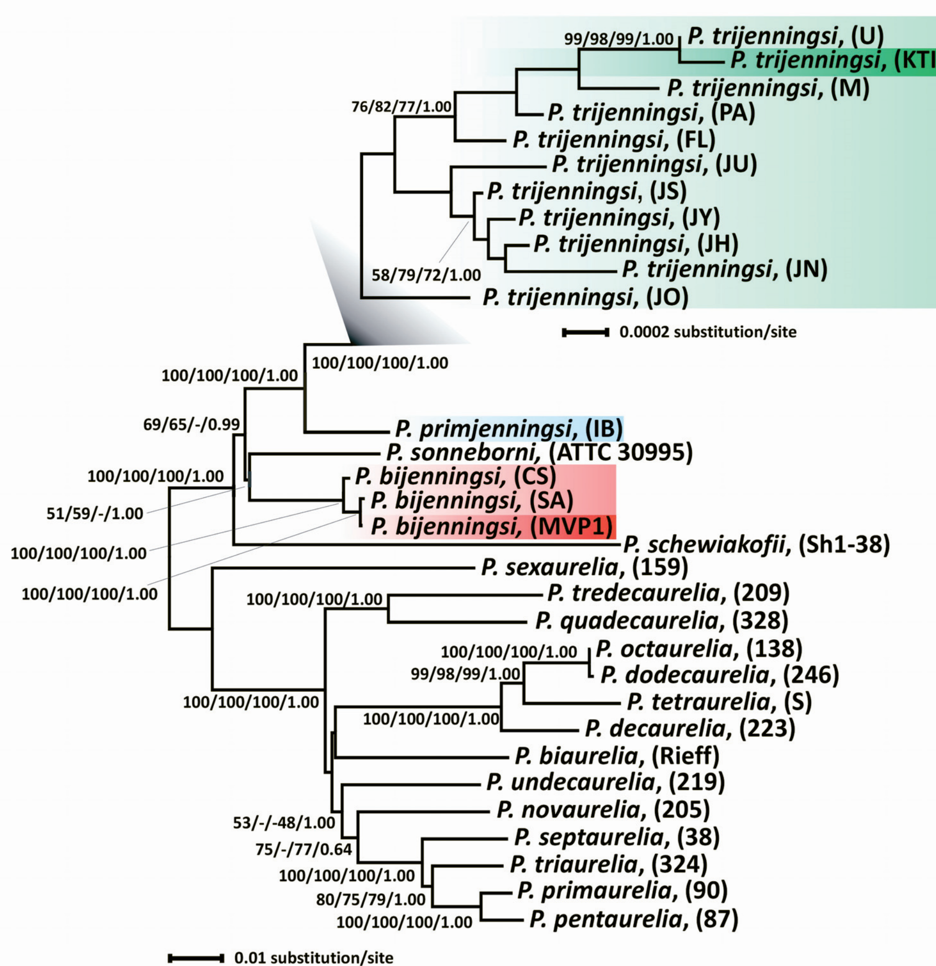

Fig. 2. Unrooted phylogram *for 15 P. jenningsi* complex strains and 16 other strains of the subgenus *Paramecium* constructed on the basis of a comparison of eleven concatenated nuclear genome fragments using the neighbor joining method. Bootstrap values for neighbor joining, maximum parsimony, and maximum likelihood analysis, as well as posterior probabilities for Bayesian inference are presented. Bootstrap values of less than 50% (posterior probabilities of less than 0.50) are not shown. All positions containing gaps and missing data were eliminated. There were a total of 3922 positions in the final dataset. Phylogenetic analyses were conducted in MEGA 6.0 (NJ/MP/ML) and MrBayes 3.1.2 (BI).

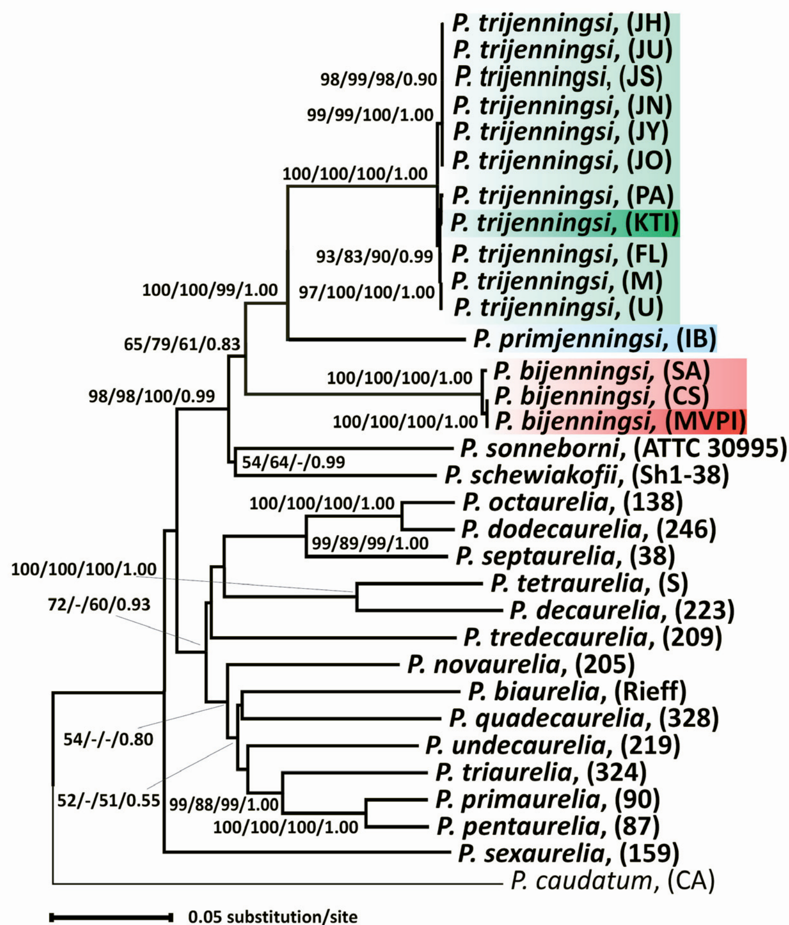

Fig. 3. Phylogram *or 15 P. jenningsi* complex strains and 17 other strains of the subgenus *Paramecium* (the CA strain of *P. caudatum* was used as an outgroup) constructed on the basis of a comparison of five concatenated mitochondrial genome fragments using the neighbor joining method. Bootstrap values for neighbor joining, maximum parsimony, and maximum likelihood analysis, as well as posterior probabilities for Bayesian inference are presented. Bootstrap values of less than 50% (posterior probabilities of less than 0.50) are not shown. All positions containing gaps and missing data were eliminated. There were a total of 2553 positions in the final dataset. Phylogenetic analyses were conducted in MEGA 6.0 (NJ/MP/ML) and MrBayes 3.1.2 (BI).

Phylogenetic analysis

All applied methods (NJ – neighbour-joining, MP – maximum parsimony, ML – maximum likelihood, BI – Bayesian inference) of tree reconstruction gave an almost identical topology, so in the current study only neighbour-joining phylograms (Figs 2, 3) providing bootstrap/posterior probability values for the other methods are presented. Due to the lack of nuclear sequences for *P. caudatum* (outgroup) it was impossible to obtain a rooted tree for the nucDNA dataset.

Based on the two phylogenetic trees (Figs 2, 3) constructed on the basis of two datasets (nucDNA and mtDNA) of 16 sequenced genome fragments, the division of *P. jenningsi* into two clades (*P. bijenningsi* and *P. trijenningsi*) and one branch (*P. primjenningsi*) can be seen. The strains from India identified by cytological characteristics as *P. jenningsi* appear with strong bootstrap support in both trees in two different clades: strain MVPI in the *P. bijenningsi* cluster and strain KTI in the *P. trijenningsi* cluster. The positions of MVPI and KTI strains (Figs 2,3) are in accordance with the results of strain crosses (Table 4). It is worth noting that strain MVPI based on a comparison of the nucDNA dataset (Fig. 2) is most closely related to the *P. bijenningsi* strain from Saudi Arabia (SA) but in the case of the mtDNA dataset (Fig. 3) it appears in one subclade with the *P. bijenningsi* strain from China (CS). A similar situation was observed in the case of the second Indian strain (KTI) which appears in one subclade with the Uganda strain (U) (Fig. 2 – nucDNA tree) and with the Panama strain (PA) (Fig. 3 – mtDNA tree). However, the observed positions did not influence species identification of either of the strains.

Species identification of new *Paramecium* strains

Due to the absence of a uniform species definition, the abundance of cryptic diversity, and the occurrence of convergent morphology (Boenigk

et al. 2012), different approaches to demarcating boundaries between protist species should be applied (CARON 2013). Likewise in the current survey, except existing cryptic differentiation in *P. jenningsi* morphospecies, the occurrence of paraphyly between *P. aurelia* and *P. jenningsi* complexes (PRZYBOŚ *et al.* 2015; PRZYBOŚ & TARCZ 2016; this study Figs 2, 3) forced application of morphological studies, strain crosses and multilocus analysis for proper species identification. Based on the obtained results both strains from Andra Pradesh, India are identified as *P. bijenningsi* (strain MVPI) and *P. trijenningsi* (strain KTI) – members of the *P. jenningsi* species complex (cf PRZYBOŚ & TARCZ 2016). What is more, the first description of *P. jenningsi* (DILLER & EARL 1958) was also based on a strain from Bangalore, India. At present, it is recognized as *P. primjenningsi* (PRZYBOŚ & TARCZ 2016) of the *P. jenningsi* complex. The studied herein *Paramecium* species meet the criteria of a species complex (according to BICKFORD *et al.* 2007) because they can be differentiated based on strain crosses and molecular characteristics but they cannot be differentiated based on morphological features alone. The strains identified in this work as *P. bijenningsi* and *P. trijenningsi* were collected and described as *P. jenningsi* (SERRA *et al.* 2014) but without detailed identification of cryptic species. In 2014 another Indian strain (designated BJ1) was collected in Chilka Lake, Odisha, presenting a macronuclear endosymbiont "*Candidatus* Gortzia infectiva" (SERRA *et al.* 2016), a recently described bacterial species originally retrieved in *P. jenningsi* strain TS-j1-8 from Thailand (BOSCARO *et al.* 2013). Based on the results presented here, the future sampling of tropical regions such as India, which are in general poorly investigated in terms of *Paramecium* diversity, may reveal new data on biodiversity and biogeography of known or even undiscovered morpho-species.

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