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Relationships of new sibling species of *Paramecium jenningsi* based on sequences of the histone *H4* gene fragment

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Abstract

Paramecium jenningsi Diller & Earl, 1958 belongs to the "aurelia" subgroup of the genus, together with *Paramecium caudatum*, *Paramecium multimicronucleatum*, *Paramecium schewiakoffi* and species of the *Paramecium aurelia* complex. The original assumption that the morphospecies *P. jenningsi* was a single genetic species was questioned because a comparison of genome analyses suggested the possibility that this morphospecies contained two sibling species. To refine understanding of relationships between the strains of *P. jenningsi*, a molecular phylogenetic analysis was conducted using *H4* gene sequences. Some polymorphic sites were found among the compared sequences, and specific patterns of single nucleotide polymorphism (SNP) markers characterize two groups of strains of *P. jenningsi*. Phylogenetic trees constructed by different methods identified two clearly different groups (from Japan and mainland Asia) whatever the method used. The sequences of the *H4* gene analyzed in the present study are closely related, and provide a good subject for phylogenetic analysis. The presence of two isolated groups of strains in the *P. jenningsi* group can reveal the evolutionary relationship between them; it confirms the presence of two sibling species among the known strains of *P. jenningsi*, and the close relationships between them and species of the *P. aurelia* complex.

Keywords: *Paramecium jenningsi*; *Paramecium aurelia* complex; Sibling species; Histone *H4* gene

Introduction

Some years ago, it was recognized that the morphological species *Paramecium aurelia* was in fact comprised of 14 sibling species that were almost completely reproductively isolated (see review by Sonneborn 1975). Many well-studied ciliate morphospecies, of other genera as well as *Paramecium*, have now been found to contain two or more sibling species; it is assumed that these ciliates display evolution in progress, and that reproductive isolation has become extensive before morphological differentiation is evident. The presence of sibling species within these morphospecies was first demonstrated by mating studies, and sibling species can also be distinguished by molecular sequencing. While the latter method does not confirm reproductive isolation, it does have the potential to bring better understanding of the phylogenetic relationships between sibling species.

As a group of species, an "aurelia subgroup" has been recognized within the genus *Paramecium*. This subgroup comprises *Paramecium caudatum*, *Paramecium multimicronucleatum*, *Paramecium schewiakoffi* and *Paramecium jenningsi*, as well as *P. aurelia* itself. *P. jenningsi* appears close to *P. aurelia* from nuclear behavior (Fokin

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et al. 2001) and was shown by SSrDNA sequence analysis (Strüder-Kypke et al. 2000a, b) to be closely related to *P. aurelia*. It was even suggested that *P. aurelia* and *P. jenningsi* may be sibling species, the *P. aurelia* species being derived from the *P. jenningsi* evolutionary line (Yamauchi et al. 1995). More recently it has been concluded from SSrDNA sequencing that *P. jenningsi* is a sister species to *P. schewiakoffi* within the monophyletic "aurelia subgroup" (Fokin et al. 2004). Original studies of various isolates concluded that *P. jenningsi* was a single species (Sonneborn 1970), and this was confirmed by many later studies (e.g. Allen et al. 1983; Przyboś 1975, 1978, 1980). However, the existence of two syngens within the morphospecies was shown when crossing studies demonstrated that strains derived from mainland Asia were genetically separate from Japanese strains (Przyboś et al. 2003), and genetic (RAPD) "fingerprinting" studies confirmed this (Skotarczak et al. 2004a, b).

In order to investigate the relationship between Asian and Japanese strains of *P. jenningsi*, we chose to study the patterns of single nucleotide polymorphisms (SNP) in the *H4* histone gene. This gene was chosen because it has high diversity in ciliates (Bernhard and Schlegel 1998), although its highly conserved nature makes it unsuitable for studies on evolution of closely related species in other groups (Bender et al. 1992; Hecker 1993).

Materials and methods

The nine strains of *P. jenningsi* listed in Table 1 were grown at room temperature and in daylight on lettuce medium inoculated with *Enterobacter aerogenes* following the method of Sonneborn (1970). *Paramecium* DNA was isolated using the DNeasy™ Tissue Kit (Qiagen, Germany) as described by Przyboś et al. (2003).

A 160 bp fragment of the *H4* gene was amplified using the primers H4-F02 (5′GGT ATT ACT AAG CCC GCT ATC AGA AGA3′) and H4-R02 (5′GGT CTT TCT TCT GGC GTG TTC AGT GTA3′) following Bernhard and Schlegel (1998). PCR amplification was carried out in a final volume of 50 µl containing: 25 pM of each primer (Biomers, Niemcy); 10 mM Tris, pH 8.7; 75 mM $MgCl_2$; 5 nM dNTPs; 2.5 U Taq-Polymerase (Qiagen, Germany). It was performed with 35 cycles of 1-min denaturation at 93 °C, 2-min primer annealing at 54 °C and 2-min primer extension at 72 °C. The amplified fragment was sequenced in the Institute of Biochemistry and Biophysics, Polish Academy of Science.

The *H4* sequences determined in this study were aligned by the DNAMAN program (Lynnon BioSoft, Canada) with the following homologous sequences from GenBank: *P. primaurelia* (DQ067620), *P. biaurelia* (DQ067621), *P. tetraurelia* (DQ067622), *P. pentaurelia* (DQ067623), *P. septaurelia* (DQ067624), *P. octaurelia* (DQ067625), *P. decaurelia* (DQ067626), *P. undecaurelia* (DQ067627), *P. dodecaurelia* (DQ067628), *P. tredecaurelia* (DQ067629) and *P. quadecaurelia* (DQ067630).

Sequences were compared for the construction of homology and distance matrices which group the divergence coefficients as well as coefficients calculated using Jukes and Cantor (Jukes and Cantor 1969) or the Kimura two-parameter model (Kimura 1980) and the maximum likelihood algorithm (Tamura and Nei 1993). Graphic transformation by the Unweighted Pair Group Method with Arithmetic Averages (UPGMA) was used to illustrate the genetic similarity between strains and methods of neighbor-joining (Saitou and Nei 1987) were used to construct phylogenetic trees describing the relationships of the examined strains. Stability or accuracy of inferred topologies were assessed via bootstrap analysis (Felsenstein 1988) of 1000 replicates.

Results

Nucleotide sequences derived from the *H4* genes of the nine analyzed strains of *P. jenningsi* have been deposited with GenBank, and their accession numbers are given in Table 1. Comparison of the nine sequences revealed five polymorphic sites involving substitutions of the A↔G and C↔T transition and A↔T and T↔G transversion types (Fig. 1, asterisks).

These nine *P. jenningsi* strain sequences were also compared to homologous *H4* gene sequences of *P. aurelia* species obtained from GenBank. This comparison revealed genetic diversity with 23 polymorphic sites involving the same types of mutations (Fig. 1, gray areas).

The extent of homology between the studied isolates can be seen from the similarity matrix in Table 2. This clearly displays the differences among Japanese strains of *P. jenningsi* and the separation of Japanese strains of this species from those of mainland Asia. At the same time, it reveals that some species of the *P. aurelia* complex show more similarity in this part of the *H4* gene to the used strains of *P. jenningsi* than to their *P. aurelia* siblings.

Phylogenetic trees were constructed from nucleotide sequences in the *H4* genes of *P. jenningsi* strains and from species of the *P. aurelia* complex available in GenBank using the methods mentioned above. Similar topologies were revealed by different methods, and phylogenetic trees inferred by the maximum likelihood method are shown in Figs 2 and 3 as examples. These trees again show the separation of Japanese strains of *P. jenningsi* from those for mainland Asia, as well as similar interrelationships with species of the *P. aurelia* complex to those seen in Table 2.