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Relationships of Species of the *Paramecium aurelia* Complex (Protozoa, Ph. Ciliophora, Cl. Oligohymenophorea) Based on Sequences of the Histone H4 Gene Fragment*

Ewa PRZYBOŚ, Agnieszka MACIEJEWSKA and Bogumiła SKOTARCZAK

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A fragment of histone H4 gene (160 bp long) was sequenced in the standard strains of *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). The tree constructed according to the Kimura model presents two main species clusters, one comprising *P. undecaurelia*, *P. octaurelia*, *P. septaurelia*, and the second cluster with *P. pentaurelia*, *P. tredecaurelia*, *P. quadecaurelia*, *P. tetraurelia*, *P. decaurelia*, *P. primaurelia*, *P. biaurelia. P. dodecaurelia* was recovered as a separate branch. The tree constructed on the basis of the maximum likelihood method also presents two species clusters, one with *P. undecaurelia*, *P. octaurelia*, *P. septaurelia*, and the second with *P. primaurelia*, *P. decaurelia*, *P. pentaurelia*, *P. tredecaurelia*, *P. quadecaurelia*, *P. tetraurelia. P. biaurelia* forms a basal clade to the latter cluster, and *P. dodecaurelia* was recovered as a clearly distinct branch from the clusters.

Key words: *Paramecium aurelia* species complex, species relationships, histone H4 sequencing.

Ewa PRZYBOŚ, Department of Experimental Zoology, Institute of Systematics and Evolution of Animals, Polish Academy of Sciences, Sławkowska 17, 31-016 Krakow, Poland.
E-mail: przybos@isez.pan.krakow.pl
Agnieszka MACIEJEWSKA, Bogumiła SKOTARCZAK, Department of Genetics, Faculty of Biology, Szczecin University, Piastów 40B, 71-065 Szczecin, Poland.
E-mail: boskot@univ.szczecin.pl

The phylogenies of ciliate groups, particular genera or species may be explored using molecular data based on analysis of different rRNA components (NANNEY *et al*. 1989; PREPARATA *et al*. 1989; NANNEY *et al*. 1998; COLEMAN 2005). Several genes (or their products) have also recently been used in phylogenetic analysis, e.g. conservative genes coding tubulins, actins, histones, Hsp 70 protein, and DNA polymerase α .The histones H4 are known as highly conserved proteins but in ciliates some degree of variation was found between species, e.g. BERNHARD and SCHLEGEL (1998) in which sequence analysis of PCR-amplified internal H4 gene fragments from 12 species representing seven ciliate classes were used. The obtained histone gene variation was used for reconstruction of phylogenetic relationships among ciliates, and the obtained tree is congruent with the ribosomal data.

Histone H4 analysis was also used for estimation of silent site polymorphism levels in some species of the *Paramecium aurelia* complex (SPOKE unpublished, personal communication).

In the present paper, a fragment of the histone H4 gene was analysed in the standard strains of 11 species of the *P. aurelia* complex (*P. primaurelia*, *P. biaurelia*, *P. tetraurelia*, *P. pentaurelia*, *P. septaurelia*, *P. octaurelia*, *P. decaurelia*, *P. undecaurelia*, *P. dodecaurelia*, *P. tredecaurelia*, *P. quadecaurelia*) with the aim to recover the relationships of species in the complex.

Material and Methods

Material

The strains of 11 species of the *P. aurelia* complex used in the studies are listed in Table 1. They are the standard strains of the particular species which have been kept in the collection of the Institute of

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Systematics and Evolution of Animals, Polish Academy of Sciences, Kraków (ISEZ in Polish).

Table 1

Strains used for sequencing of the fragment of the H4 histone gene and their Gene Bank accession numbers

Code Gen Bank	Species and designation of strain	Geographical origin of the strain
DQ067620	*P. primaurelia*, 90	USA, Pennsylvania
DQ067621	*P. biaurelia*, Rieff	Scotland, Rieff
DQ067622	*P. tetraurelia*, S	Australia, Sydney
DQ067623	*P. pentaurelia*, 87	USA, Pennsylvania
DQ067624	*P. septaurelia*, 38	USA, Florida
DQ067625	*P. octaurelia*, 138	USA, Florida
DQ067626	*P. decaurelia*, 223	USA, Florida
DQ067627	*P. undecaurelia*, 219	USA, Texas
DQ067628	*P. dodecaurelia* , 246	USA, Southern state
DQ067629	*P. tredecaurelia*, 209	France, Paris
DQ067630	*P. quadecaurelia*, 328	Australia, Emily Gap

Methods

Cell culture

The methods of SONNEBORN (1970) were applied for the cultivation of strains on a lettuce medium inoculated with *Enterobacter earogenes.*

Molecular methods

1. Isolation of DNA

Paramecium genomic DNA was isolated from vegetative cells at the end of the exponential phase using QIAamp DNA Mini Kit (Qiagen, Germany) as described in PRZYBOŚ *et al*. (2003). DNA was stored at –20°C until analysis.

2. Amplification of the H4 gene region

To isolate the fragment of the H4 gene, the following specific nucleotide primers were used: H4/F02 (5' GGTATTACTAAGCCCGCTATCAGAAGA 3') and H4/R02 (5' GTTCTTTCTTCTGGCGTGTTCAGTGTA 3') (BERNHARD & SCHLEGEL 1998), which yielded a PCR product of 160 bp. PCR amplification was carried out in a final volume of 50 μl containing: 20 pM of each primer; 10 mM Tris pH 8,7; 1.5 mM MgCl2; 5 μM dNTPs; 2.5 U Taq-Polymerase (Qiagen, Germany).The amplification was performed with 35 cycles consisting of 1-min denaturation at 93°C, 2-min primer annealing at 54°C and 2-min primer extension at 72°C.

3. Sequencing

A fragment of 160 bp of the histone H4 gene was sequenced in the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw.

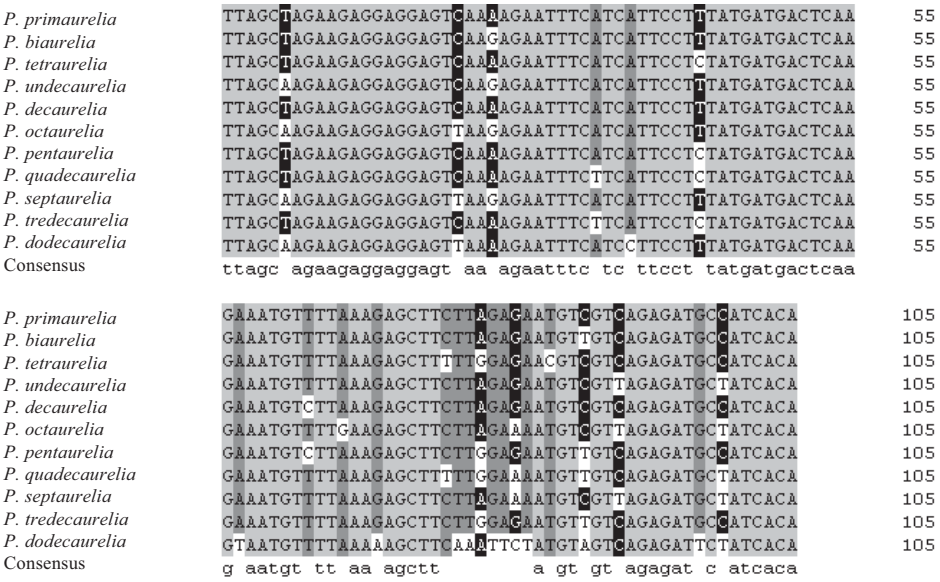

Fig. 1. Alignment of sequences of H4 gene fragment (160 pb) in the standard strains of 11 species of the *P. aurelia* complex.

4. Data analysis

The H4 sequences determined in this study were aligned using the DNAMAN program (Lynnon BioSoft, Canada). Phylogenetic trees were generated using the neighbor-joining method (SAITOU & NEI 1987) with application of the Kimura two--parameter correction model or maximum-likelihood method. Data were bootstrap resampled 1000 times (FELSENSTEIN 1985).

Results

Amplification of the coding fragment of the histone H4 gene in strains representing different species of the *P. aurelia* species complex produced a product of 160 bp. This fragment was sequenced in the standard strains of *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). Alignment of these sequences is available online in the NCBI PopSet data base (gi:67772313).

Sequence alignment of the histone H4 gene in strains representing 11 species of the *P. aurelia* complex was carried out using the DNAMAN program (Fig. 1). Phylogenetic trees were constructed with the application of different models and methods usually used in phylogenetic analysis. Both trees are similar, which is an important feature of the probability of the applied topology (Figs 2 & 3).

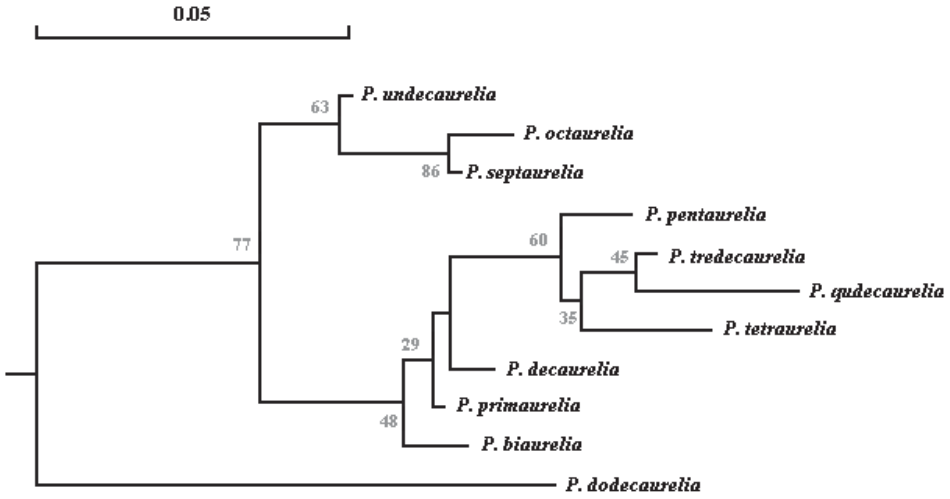

Fig. 2. Phylogenetic tree constructed for 11 species of the *P. aurelia* complex based on comparison of sequences of the histone H4 gene fragment with application of the Kimura two-parameter correction model. Bootstrap values are presented as percentages for 1000 comparisons. Upper scale calibrates the length of branches showing the number of different nucleotides for 100 compared nucleotides.

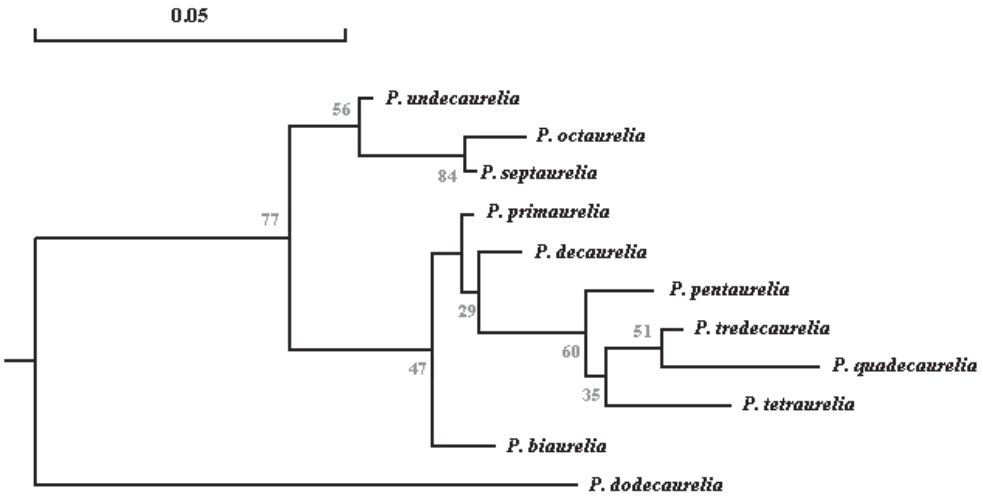

Fig. 3. Phylogenetic tree constructed for 11 species of the *P. aurelia* complex based on comparison of sequences of the histone H4 gene fragment with application of the maximum likelihood method. Bootstrap values are presented as percentages for 1000 comparisons. Upper scale calibrates the length of branches showing the number of different nucleotides for 100 compared nucleotides.

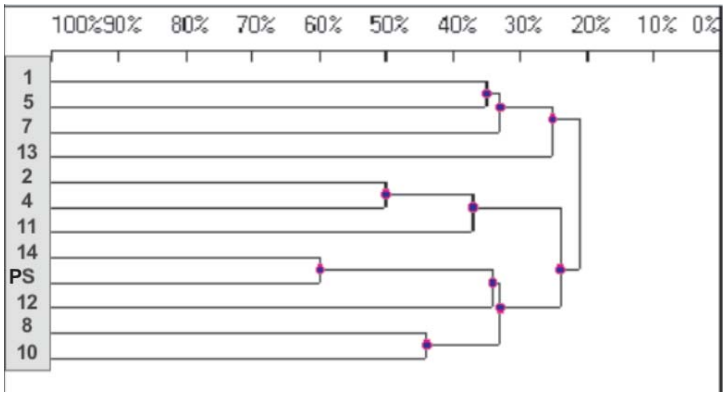

Fig. 4. Dendrogram presenting the relationships of the studied species of the *P. aurelia* complex constructed on the basis of RAPD fingerprints of the standard strains representing the particular species. Numbers designate particular species, i.e. 1 – *P. primaurelia,* 2 – *P. biaurelia*, etc; PS – *P. sonneborni* (from PRZYBOŚ *et al.* 2006).

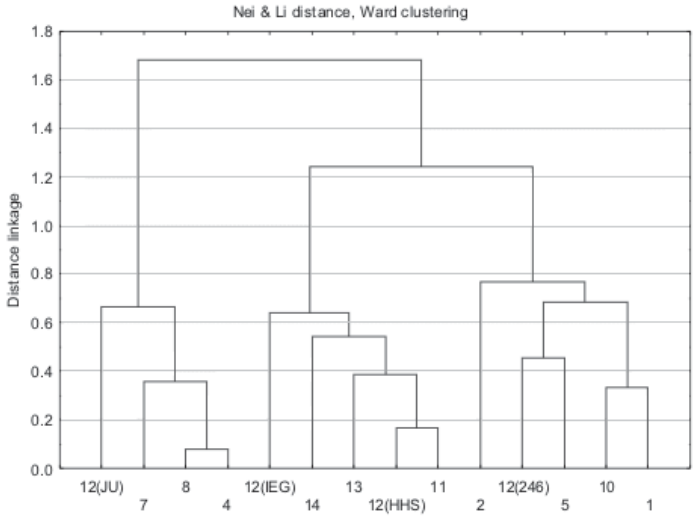

Fig. 5. Dendrogram of band pattern similarity (NEI & LI distance, Ward clustering) of the studied species of the *P. aurelia* complex based on RFLP analysis. Numbers designate particular species, i.e. 1 – *P. primaurelia,* 2 – *P. biaurelia*, 4 – *P. tetraurelia,* 5 – *P. pentaurelia,* 7 – *P. septaurelia,* 8 – *P. octaurelia,* 10 – *P. decaurelia*, 11 – *P. undecaurelia*, 12 – *P. dodecaurelia* (JU, IE, G, HHS, 246 particular strains), 13 – *P. tredecaurelia,* 14 – *P. quadecaurelia* (from PRZYBOŚ *et al.* 2006).

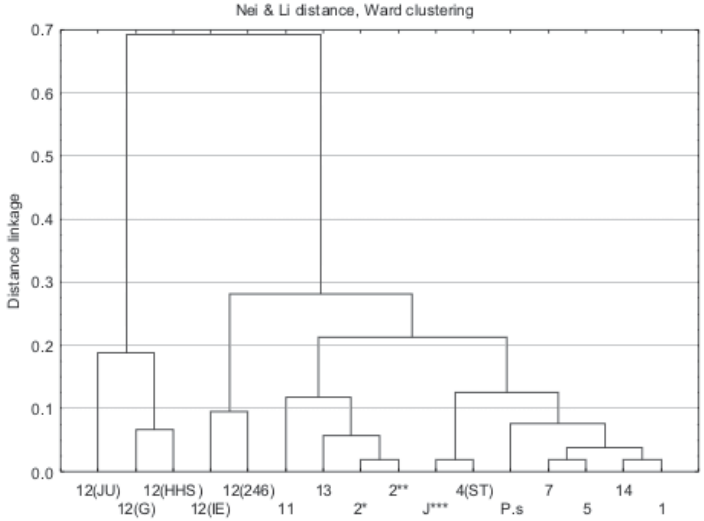

Fig. 6. Dendrogram of band pattern similarity (NEI & LI distance, Ward clustering) of the studied species of the *P. aurelia* complex based on ARDRA riboprinting (*AluI, HhaI, HinfI, TaqI* enzymes). Designations: 1 – *P. primaurelia*, 2* – strains RC and RI of *P. biaurelia* with identical band patterns but different from other strains by one extra band, 2** – the other strains of *P. biaurelia* ; J***- strains of *P. tetraurelia* (excluding strain ST), *P. octaurelia*, *P. decaurelia*, all with identical band patterns; 4(ST) – strain ST of *P. tetraurelia*, 5 – *P. pentaurelia,* 7 – *P. septaurelia*, 11 – *P. undecaurelia*, 12 – *P. dodecaurelia* (JU strain, G strain, HHS strain, IE strain, 246 strain), 13 – *P. tredecaurelia*, 14 – *P. quadecaurelia*, P.s. – *P. sonneborni* (from PRZYBOŚ *et al.* 2006).

The tree constructed according to the Kimura model (Fig. 2) presents two main species clusters, one comprising *P. undecaurelia*, *P. octaurelia*, *P. septaurelia*, the second cluster with *P. pentaurelia*, *P. tredecaurelia*, *P. quadecaurelia*, *P. tetraurelia*, *P. decaurelia*, *P. primaurelia*, *P. biaurelia. P. dodecaurelia* was recovered as a separate branch. The tree constructed on the basis of the maximum likelihood method (Fig. 3) also presents two species clusters, one with *P. undecaurelia*, *P. octaurelia*, *P. septaurelia*, and the second cluster with *P. primaurelia*, *P. decaurelia*, *P. pentaurelia*, *P. tredecaurelia*, *P. quadecaurelia*, *P. tetraurelia. P. biaurelia* forms a basal clade to the latter cluster, and *P. dodecaurelia* was recovered as a clearly distinct branch from the clusters. Both trees are very similar.

Discussion

Previous studies (PRZYBOŚ *et al*. 2006) concerning the relationships of species comprising the *P. aurelia* complex as well as their characteristics by RAPD analysis showed that all species of the *P. aurelia* complex possessed characteristic band patterns and the majority of species were also polymorphic intraspecifically. Intraspecific polymorphism was revealed as different genotypes (band patterns) by primer Ro 460-04, Roth, Karsruhe, Germany in *P. triaurelia* and *P. sexaurelia* (STOECK *et al*. 1998), *P. novaurelia* (STOECK *et al*. 2000), *P. septaurelia* (PRZYBOŚ & TARCZ 2005), as well as in *P. biaurelia*, *P. tetraurelia*, *P. octaurelia*, *P. dodecaurelia* (PRZYBOŚ *et al*. 2006). The other species (*P. pentaurelia*, *P. decaurelia*, *P. tredecaurelia*, *P. quadecaurelia*) showed highly similar band patterns of strains within species (PRZYBOŚ *et al*. 2006). Other molecular analyses (RFLP and ARDRA with the application of restriction enzymes) distinguished among clusters of species, species and intraspecific polymorphism as in the case of *P. dodecaurelia.* All applied analyses, i.e. RAPD, ARDRA, and RFLP distinguished the following clusters of species of the *P. aurelia* complex: *P. primaurelia* and *P. pentaurelia* in one group, *P. tetraurelia* and *P. octaurelia* in another group, other species appear in different clusters depending on the applied method (cf Figs 4, 5, 6). Intraspecific differentiation within *P. dodecaurelia* is remarkable and was obtained by all implemented methods (PRZYBOŚ *et al*. 2006). It seemed interesting to find out if such polymorphism is based on differences at the nucleotide level. Preliminary results of sequencing of ITS1 and a fragment of LSU rRNA in geographically distant strains of *P. dodecaurelia* showed differences at the nucleotide level within species (unpublished TARCZ *et al*.).

The present results based on sequences of the coding fragment of the histone H4 gene in the standard strains representing 11 species of the *P. aurelia* complex grouped together *P. primaurelia, P. pentaurelia* in one cluster and *P. octaurelia* in a separate cluster, similarly as clusters of species obtained by RAPD and RFLP analyses (PRZYBOŚ *et al*. 2006), (Figs 4 & 5). In the *P. aurelia* complex only one gene of H4 exists because only one product was obtained by PCR. NANNEY *et al.* (1998) compared sequence differences in a variable D2 domain of 23S rRNA (190 bp long) among several species of the *P. aurelia* complex: *P. biaurelia*, *P. triaurelia*, *P. tetraurelia*, *P. sexaurelia*, *P. octaurelia*, *P. tredecaurelia*, and *P. sonneborni.* They found that the genetic species in the *P. aurelia* complex are separated from each other by one or more site changes "but constitute a dense evolutionary cluster". COLEMAN (2005), in turn, sequenced the ITS region of the nuclear ribosomal cistron in 13 among 15 known (SONNEBORN 1975; AUFDERHEIDE *et al.* 1983) species of the *P. aurelia* complex and found that "The ITS1 and ITS2 sequences of all the *P. aurelia* species complex are nearly identical". "…syngens 1,3,5,7,8,10,12 and 14 are identical for all 110-111 of these positions, and the major split is between syngens 2,6, and 13, and the remaining syngens".

More detailed relationships of species of the *P. aurelia* complex may be obtained in the future on the basis of sequencing of several strains representing particular species of the complex.

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