Pakistan J. Zool., vol. 49(5), pp 1767-1774, 2017. DOI: http://dx.doi.org/10.17582/journal.pjz/2017.49.5.1767.1774

Phylogenetic Relationship of Locally Isolated *Paramecium* Species Inferred from Histone H4 Genes

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Article Information
Received 16 February 2017
Revised 28 April 2017
Accepted 03 July 2017
Available online 08 September 2017

Authors' Contribution
FRS and ARS conceived and supervised the study. FT executed the experimental work and wrote the manuscirpt.

Key words
Phylogenetic relationships of ciliates, Ribotyping, 18SrDNA analysis, FT8, *Paramecium*.

ABSTRACT

The histone H4 gene is supposed to be highly conserved among all organisms with the exception of variations found in the ciliate species. Thus a fragment of histone H4 gene (160bp) was sequenced in ten locally isolated strains of *Paramecium* species including a standing-alone on the basis of 18SrDNA gene sequence strain FT8. In order to readdress the relationships of FT8 strain with other strains of *Paramecium* species, a molecular phylogenetic analysis was performed on the basis of H4 gene sequences. A phylogenetic tree was constructed using the aligned sequences of our study with 22 entries of closely related species from GenBank Data. Highly polymorphic sites were observed in FT8 strain sequence as compared to other species. Analysis of H4 gene sequences in our study showed they are closely related and behaved as a good substitute for phylogenetic analysis. Phylogenetic tree constructed by maximum likelihood method placed the FT8 strain very distinctly from other species, giving it a clearly separate position. Phylogeny results based on the H4 gene sequences corresponded to the results obtained by 18SrDNA sequences. Both markers proved the FT8 strain is a highly divergent species based on the phylogenetic relationships, opening new realms for the researchers of this field.

INTRODUCTION

Ciliates are characterized as monophyletic group of the unicellular eukaryote. Their distinctive feature is the presence of cilia and other infraciliature structures including kinetosomes (Lynn and Small, 1981; Bernhard and Schlegel, 1997; Shin, 2005; Hampl *et al*., 2009; Gould *et al*., 2011). All species of ciliates harbor two different kinds of nuclei; micronucleus that is required for sexual reproduction and other one is transcriptionally active macronucleus (Baroin-Tourancheau *et al*., 1992; Forney and Rodkey, 1992; Budin and Philippe, 1998; Katz, 2001; Rautian and Potekhin, 2002; Garnier *et al*., 2004; Shin, 2005; Zufall *et al*., 2006; Nekrasova *et al*., 2010). Among them genus *Paramecium* of the oligohymenophorea ciliates is well studied in terms of its diversity, ultrastructure, physiology and genetics. Especially, the sibling species of the *Paramecium aurelia* complex have been intensively studied because of their complex genomics and as an example of species radiation (Coleman, 2005; Aury *et al*., 2006; Hori *et al*., 2006; Przybos *et al*., 2008; McGrath *et al*., 2014). It, therefore, becomes imperative to study the evolution and phylogenetic relationships of these popularly used eukaryotic model organisms. However, the systematics and phylogeny of *Paramecium* has been well developed in last decade (Fokin *et al*., 2004; Tarcz *et al*., 2012; Boscaro *et al*., 2012; Krenek *et al*., 2015; Lanzoni *et al*., 2016) but at the same time some relations within the genus remain dubious and questionable leaving many described species as invalid (Tarcz *et al*., 2012; Krenek *et al*., 2015; Struder-Kypke *et al*., 2000; Barth *et al*., 2006; Catania *et al*., 2009; Greczek-Stachura *et al*., 2010).

Like many other molecular markers (rDNA, HSP70, COI, COII), genes for the histone proteins (mainly H3 and H4) are being used as phylogenetic markers because of their considerable variation found in ciliates (Wang *et al*., 2016). It would be pertinent to mention that because of the highly conserved nature of these proteins in vast majority of organisms, histones are not considered to be appropriate markers for phylogenetic studies (de Lange and Smith, 1971; Wells and Brown, 1991; Thatcher and Gorovsky, 1994). These proteins are very conservative also in ciliates; however, there were several attempts to use the fragments of their genes as phylogenetic markers to infer the relationships of closely related groups–at species or even within-species level. Recently, investigations on H4 proteins for understanding of the evolutionary relationship of *Paramecium* species have increased tremendously. For example, Przyobs *et al*. (2006) investigated the H4 gene of a number of species of the *P. aurelia* complex (*P. aurelia*:

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0030-9923/2017/0005-1767 $ 9.00/0

P. primaurelia, *P. biaurelia*, *P. tetraurelia*, *P. pentaurelia*, *P. septaurelia*, *P. octaurelia*, *P. decaurelia*, *P. undecurelia*, *P*. *dodecaurelia*, *P. tredecaurelia* and *P. quadecaurelia*) in order to untangle their phylogenetic connections. Similarly, H4 gene sequence have been studied for the phylogentic relationship of *P. jeningsi* strains by Maciejewska (2006).

Independent evolution of histone proteins and their prominent sequence variation in ciliates as compared to other animals have made them potential candidate for cross check analysis. Bernhard and Schlegel (1998) made a comparative analysis of H4 and H3 genes including the amino acid sequences of corresponding proteins, and an intergenic DNA fragment between them. According to them, phylogenetic relationships of *P. tetraurelia* and *P. bursaria* based on H4 and H3 sequences were similar to the rDNA phylogeny, thus making it valid for the studies on evolutionary relationships. Katz *et al*. (2006) compared thirteen ciliates including *Paramecium* with other eukaryotes. They found large variations among ciliates as compared to homologous fragments of other animals based on parsimony and maximum likelihood methods. This gives an idea of functional constraints of these proteins and their adaptive evolution probably due to nuclear dualism and peculiar organization of chromatin in macronucleus of ciliates. So the aim of the present study was to resolve the phylogenetic relationship of *Paramecium* species on the basis of analysis of H4 gene sequences. FT8 strain (*Paramecium caudatum pakistanicus*) being a unique species on the basis of 18SrDNA sequences already published by Shakoori *et al*. (2014) was also included in our study. This strain has been included in this study to make evolutionary comparison of this species on the basis of 18S rDNA and H4 gene sequences and to find its position in phylogenetic tree with reference to other *Paramecium* species.

MATERIALS AND METHODS

Sampling and maintenance of Paramecium *strains*

Nine isolates of *Paramecium* sp. were collected from different regions of Punjab Province, Pakistan: two strains (FT2.1 and F3.1) from Kasur, three (FT4.1, FT10.1 and FT11.1) from Lahore, two (FT5.1, FT6.1) from Sheikhupura and three (FT7.1, FT9.1) from Mansehra. All of them were purified and acclimatized according to Shakoori *et al*. (2004).

Genomic DNA extraction

Genomic DNA extraction was followed by incubation of *Paramecium* cells with 10mM Tris-HCl (pH 7.5) for 10-12h at 27°C. Incubated cells were harvested at low speed centrifugation of 6741x g at 7°C for 10 min. The cells were lysed in the lysis buffer (42% urea, 0.30M NaCl, 10Mm Tris-HCl pH 7.5, 10mM EDTA, 10% Nonidet P40 and 1% SDS). The lysis mixture was extracted two times with phenol:chloroform (1:1) following Sambrook *et al*. (1989).

Amplification of H4 genes

A 160bp fragment of the H4 gene was amplified by 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') as used by Maciejewska (2006), who applied H4 histone gene for phylogenetic analysis of *P. jenningsi*. PCR was performed in a final volume of 50µl containing 10mM dNTPs, 5µl MgCl, 3µl of 10x buffer (Thermo Fisher Scientific), 1µl 10mM of each primer and 1µl of 2.5 U Taq-polymerase (Thermo Fisher Scientific). PCR amplification protocol composed of 1 cycle at 94°C for 5 min, followed by 35 cycles, each of 2 min denaturation at 94°C, 1 min annealing at 54°C, 2 min extension at 72°C and final extension at 72°C for 20 min. Amplification was performed in Gene Amp PCR System 9700 (Applied Biosystem).

Sequencing of amplified products

PCR products, after their appropriate size was confirmed on 0.8% agarose gel, were cut and purified using Nucleospin Extract 11 (Macherey-Nagel Germany). Purified products were sequenced by Macrogen (South Korea).

Phylogenetic analysis

The nucleotide sequences of all analyzed strains were compared with the sequences present in database using online website program (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). All the sequences were aligned by online Muscle alignment program (http://www.ebi.ac.uk/Tools/msa/muscle/) with the available sequences from GenBank/EMBL databases under the following accession numbers: *P. tetraurelia* (XM001425872), *P. tetraurelia* (XM001455606), *P. tetraurelia* (XM001452606), *P. tetraurelia* (XM001452073), *P. tetraurelia* (XM001459068). *P. decaurelia* (DQ067622), *P.tetraurelia* (AJ004699), *P. tetraurelia* (XM001442554), 19 *P. tredecaurelia* (DQ067629), *P. pentaurelia* (DQ067623), *P. primaurelia* (DQ067620), *P. quadecaurelia* (DQ0676630), *P. decaurelia* (DQ067626). *P. jenningsi* (DQ001056), *P. jenningsi* (DQ001064), *P. jenningsi* (DQ001062), *P. jenningsi* (DQ001059), *P. jenningsi* (DQ001061), *P. jenningsi* (DQ001057). *P. undecaurelia* (DQ067627), *P. septaurelia* (DQ067624), *P. tertraurelia* (AJ004700), *P. caudatum* (AB670962) and *P. bursaria* (AJ004702). Percentage and distance matrix of *Paramecium* species of present study with the above mentioned *Paramecium* species from GenBank were compared. The phylogenetic tree was constructed to get the final position of all isolated *Paramecium* species along with FT8 strain by using online website program (http://www.phylogeny.fr/) by Dereeper *et al*. (2008). Maximum likelihood (neighbor joining) method was used to construct phylogenetic trees describing the relationships of the examined strains. Accuracy of inferred topologies was assessed via bootstrap analysis of 1000 replicates.

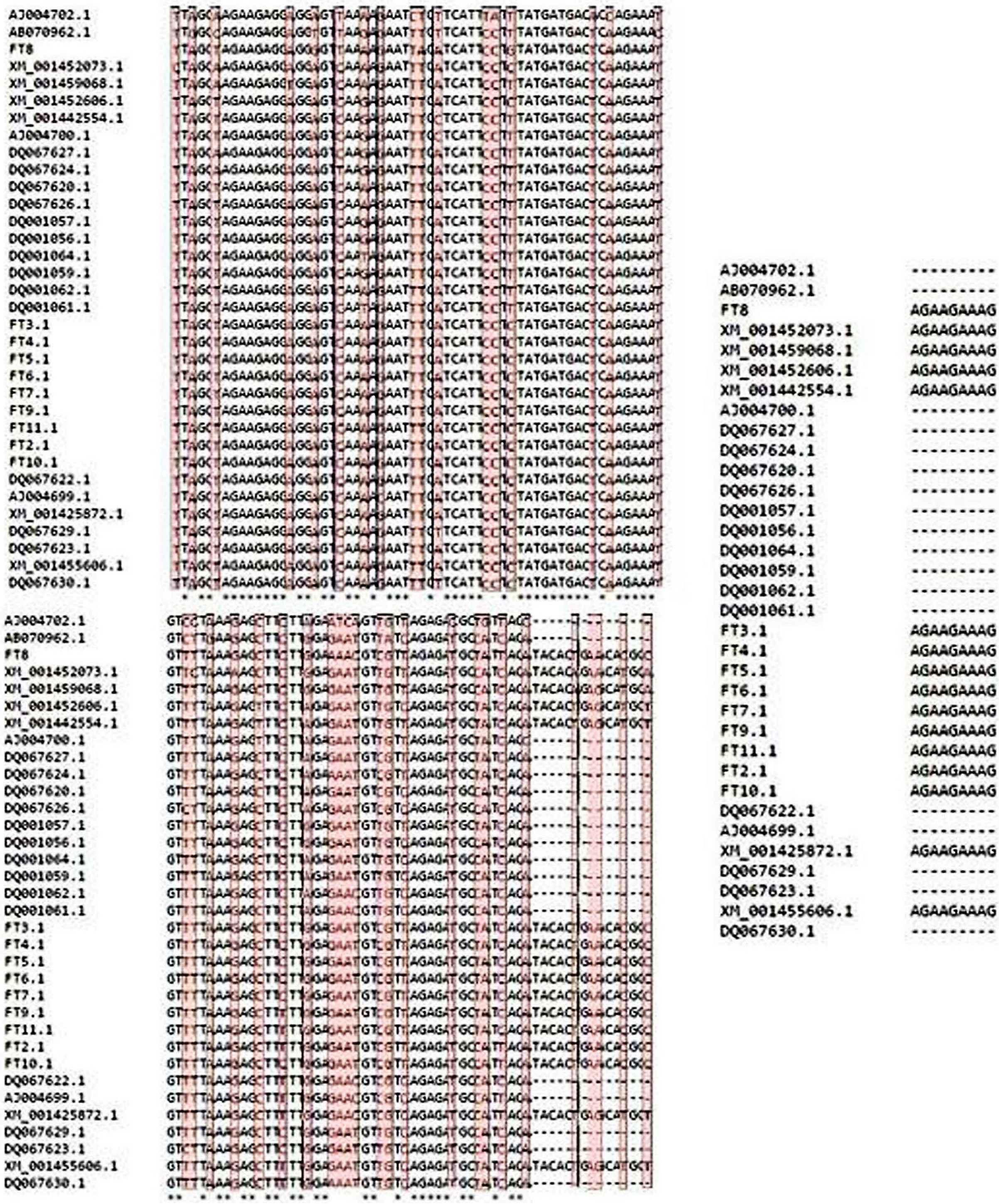

Fig. 1. Multiple alignment of sequences of the studied histone H4 gene in 10 strains along with 24 species from the GenBank Data. Differences in nucleotides of sequences are highlighted in pink.

RESULTS

PCR amplification reactions of all nine strains yielded a fragment of approximately 160bp in size. Sequences of the histone H4 gene fragment of all strains were obtained. Sequencing was based on both the forward and reverse primers used for amplification. Sequences were verified for identity by comparing with the H4 gene sequences of *Paramecium* species already available in GenBank.

Phylogenetic analysis

Careful alignment of the nucleotide sequences derived from the H4 genes of the ten analyzed strains of *Paramecium* species revealed sixteen polymorphic sites. These sites are indicating both the transition (A to G, C to T) and transversion (A to T, T to G) substitutions, out of which four transitions revealed by only FT8 strain. All of these strain sequences were also aligned to homologous H4 gene sequences of *Paramecium* species obtained from GenBank. This comparison with other species displayed genetic diversity with 25 polymorphic sites involving both types of mutations (Fig. 1). The extents of divergence between the studied isolates are displayed in the distance

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	
1		97.7	97.7	97.7	97.7	97.7	93.8	98.4	99.2	98.4	96.1	94.6	91.5	92.2	92.2	97.1	97.1	90.7	95.2	95.2	95.2	94.3	94.3	94.3	93.3	93.3	93.3	92.4	94.3	93.3	91.4	89.6	85.7	79.0	1
2	2.4		100.0	100.0	100.0	100.0	94.6	99.2	98.4	99.2	93.8	95.3	93.8	93.0	93.0	96.2	94.3	93.0	96.2	96.2	96.2	95.2	95.2	95.2	94.3	94.3	94.3	93.3	97.1	96.2	94.3	92.5	86.7	80.0	2
3	2.4	0.0		100.0	100.0	100.0	94.6	99.2	98.4	99.2	93.8	95.3	93.8	93.0	93.0	96.2	94.3	93.0	96.2	96.2	96.2	95.2	95.2	95.2	94.3	94.3	94.3	93.3	97.1	96.2	94.3	92.5	86.7	80.0	3
4	2.4	0.0	0.0		100.0	100.0	94.6	99.2	98.4	99.2	93.8	95.3	93.8	93.0	93.0	96.2	94.3	93.0	96.2	96.2	96.2	95.2	95.2	95.2	94.3	94.3	94.3	93.3	97.1	96.2	94.3	92.5	86.7	80.0	4
5	2.4	0.0	0.0	0.0		100.0	94.6	99.2	98.4	99.2	93.8	95.3	93.8	93.0	93.0	96.2	94.3	93.0	96.2	96.2	96.2	95.2	95.2	95.2	94.3	94.3	94.3	93.3	97.1	96.2	94.3	92.5	86.7	80.0	5
6	2.4	0.0	0.0	0.0	0.0		94.6	99.2	98.4	99.2	93.8	95.3	93.8	93.0	93.0	96.2	94.3	93.0	96.2	96.2	96.2	95.2	95.2	95.2	94.3	94.3	94.3	93.3	97.1	96.2	94.3	92.5	86.7	80.0	6
7	6.5	5.7	5.7	5.7	5.7	5.7		93.8	94.6	93.8	91.5	89.9	88.4	87.6	88.4	91.4	91.4	88.4	89.5	89.5	90.5	90.5	89.5	89.5	88.6	90.5	88.6	89.5	91.4	90.5	92.4	86.8	83.8	81.0	7
8	1.6	0.8	0.8	0.8	0.8	0.8	6.5		97.7	100.0	94.6	96.1	93.0	92.2	92.2	97.1	95.2	92.2	95.2	95.2	95.2	96.2	94.3	94.3	93.3	93.3	93.3	92.4	96.2	95.2	93.3	91.5	85.7	79.0	8
9	0.8	1.6	1.6	1.6	1.6	1.6	5.7	2.4		97.7	95.3	93.8	92.2	93.0	93.0	96.2	96.2	91.5	96.2	96.2	96.2	93.3	95.2	95.2	94.3	94.3	94.3	93.3	95.2	94.3	92.4	90.6	86.7	80.0	9
10	1.6	0.8	0.8	0.8	0.8	0.8	6.5	0.0	2.4		94.6	96.1	93.0	92.2	92.2	97.1	95.2	92.2	95.2	95.2	95.2	96.2	94.3	94.3	93.3	93.3	93.3	92.4	96.2	95.2	93.3	91.5	85.7	79.0	10
11	4.0	6.6	6.6	6.6	6.6	6.6	9.2	5.7	4.9	5.7		96.9	93.8	90.7	92.2	99.0	99.0	91.5	95.2	95.2	95.2	94.3	94.3	94.3	93.3	95.2	93.3	94.3	92.4	91.4	89.5	87.7	85.7	78.1	11
12	5.7	4.9	4.9	4.9	4.9	4.9	11.1	4.0	6.6	4.0	3.2		96.9	92.2	93.8	97.1	95.2	94.6	97.1	97.1	95.2	98.1	94.3	96.2	95.2	95.2	95.2	94.3	96.2	93.3	91.4	91.5	87.6	79.0	12
13	9.3	6.6	6.6	6.6	6.6	6.6	13.0	7.5	8.4	7.5	6.6	3.2		90.7	92.2	93.3	91.4	97.7	95.2	95.2	95.2	94.3	94.3	96.2	96.2	95.2	97.1	95.2	96.2	95.2	93.3	95.3	87.6	79.0	13
14	8.3	7.4	7.4	7.4	7.4	7.4	13.8	8.3	7.4	8.3	10.1	8.3	10.1		95.4	92.4	90.5	89.9	94.3	94.3	92.4	91.4	91.4	93.3	92.4	92.4	92.4	91.4	93.3	92.4	90.5	88.7	85.7	80.0	14
15	8.2	7.3	7.3	7.3	7.3	7.3	12.7	8.2	7.3	8.2	8.2	6.5	8.2	4.8		93.3	91.4	93.0	95.2	95.2	95.2	92.4	94.3	96.2	95.2	95.2	95.2	94.3	96.2	95.2	93.3	91.5	88.6	81.0	15
16	2.9	4.0	4.0	4.0	4.0	4.0	9.2	2.9	4.0	2.9	1.0	2.9	7.2	8.2	7.0		98.1	90.5	96.2	96.2	96.2	95.2	95.2	95.2	94.3	96.2	94.3	95.2	93.3	92.4	90.5	89.5	86.7	77.1	16
17	2.9	6.0	6.0	6.0	6.0	6.0	9.2	5.0	3.9	5.0	1.0	5.0	9.3	10.4	9.2	1.9		88.6	94.3	94.3	94.3	93.3	93.3	93.3	92.4	94.3	92.4	93.3	91.4	90.5	88.6	87.6	84.8	77.1	17
18	10.2	7.5	7.5	7.5	7.5	7.5	12.9	8.4	9.3	8.4	9.3	5.7	2.4	10.9	7.3	10.5	12.7		93.3	92.4	94.3	92.4	93.3	95.2	95.2	94.3	96.2	94.3	97.1	96.2	94.3	98.1	87.6	81.0	18
19	5.0	3.9	3.9	3.9	3.9	3.9	11.5	5.0	3.9	5.0	5.0	2.9	5.0	6.0	4.9	3.9	6.0	7.2		98.1	96.2	97.1	95.2	97.1	96.2	96.2	96.2	95.2	95.2	92.4	90.5	92.4	90.5	80.0	19
20	5.0	4.0	4.0	4.0	4.0	4.0	11.5	5.0	4.0	5.0	5.0	2.9	5.0	6.0	4.9	4.0	6.0	8.2	1.9		96.2	95.2	97.1	97.1	96.2	96.2	96.2	95.2	95.2	92.4	90.5	91.4	90.5	80.0	20
21	5.0	4.0	4.0	4.0	4.0	4.0	10.4	5.0	4.0	5.0	5.0	5.0	5.0	8.2	4.9	4.0	6.0	6.0	3.9	4.0		93.3	99.0	97.1	98.1	98.1	98.1	97.1	95.2	96.2	94.3	93.3	88.6	80.0	21
22	6.0	5.0	5.0	5.0	5.0	5.0	10.3	3.9	7.1	3.9	6.0	1.9	6.0	9.2	8.1	5.0	7.0	8.3	2.9	5.0	7.1		92.4	94.3	93.3	93.3	93.3	92.4	94.3	91.4	91.4	91.4	87.6	81.0	22
23	6.1	5.0	5.0	5.0	5.0	5.0	11.5	6.1	5.0	6.1	6.1	6.1	6.1	9.3	6.0	5.0	7.1	7.1	5.0	2.9	1.0	8.2		96.2	97.1	97.1	97.1	96.2	94.3	95.2	93.3	92.4	89.5	81.0	23
24	6.1	5.0	5.0	5.0	5.0	5.0	11.5	6.1	5.0	6.1	6.1	4.0	4.0	7.1	3.9	5.0	7.1	5.0	2.9	2.9	2.9	6.0	4.0		98.1	97.1	99.0	97.1	98.1	95.2	93.3	94.3	91.4	79.0	24
25	7.1	6.0	6.0	6.0	6.0	6.0	12.6	7.1	6.0	7.1	7.1	5.0	3.9	8.1	4.9	6.0	8.1	4.9	3.9	3.9	1.9	7.0	2.9	1.9		98.1	99.0	99.0	96.2	95.2	93.3	94.3	89.5	80.0	25
26	7.2	6.1	6.1	6.1	6.1	6.1	10.4	7.2	6.1	7.2	5.0	5.0	5.0	8.2	4.9	4.0	6.0	6.0	3.9	4.0	1.9	7.1	2.9	2.9	1.9		98.1	99.0	95.2	94.3	92.4	93.3	88.6	81.0	26
27	7.2	6.1	6.1	6.1	6.1	6.1	12.7	7.2	6.1	7.2	7.2	5.0	2.9	8.2	4.9	6.1	8.2	3.9	3.9	4.0	1.9	7.1	2.9	1.0	1.0	1.9		98.1	97.1	96.2	94.3	95.2	90.5	80.0	27
28	8.2	7.1	7.1	7.1	7.1	7.1	11.5	8.2	7.1	8.2	6.0	6.0	5.0	9.2	6.0	5.0	7.0	6.0	4.9	5.0	2.9	8.1	3.9	2.9	1.0	1.0	1.9		95.2	94.3	92.4	93.3	88.6	80.0	28
29	6.1	2.9	2.9	2.9	2.9	2.9	9.2	4.0	5.0	4.0	8.3	4.0	4.0	7.1	3.9	7.2	9.3	2.9	5.0	5.0	5.0	6.0	6.1	1.9	3.9	5.0	2.9	5.0		97.1	95.2	96.2	89.5	81.0	29
30	7.1	3.9	3.9	3.9	3.9	3.9	10.3	5.0	6.0	5.0	9.3	7.1	5.0	8.3	5.0	8.2	10.4	3.9	8.1	8.2	3.9	9.2	5.0	5.0	4.9	6.0	3.9	6.0	2.9		98.1	95.2	87.6	81.9	30
31	9.3	6.0	6.0	6.0	6.0	6.0	8.1	7.1	8.2	7.1	11.6	9.3	7.1	10.6	7.1	10.5	12.7	6.0	10.4	10.5	6.0	9.2	7.1	7.1	7.0	8.2	6.0	8.1	5.0	1.9		93.3	87.6	83.8	31
32	10.4	7.0	7.0	7.0	7.0	7.0	13.7	8.1	9.2	8.1	12.7	8.1	3.9	11.5	8.0	11.5	13.8	1.0	8.2	9.2	7.0	9.3	8.1	6.0	6.0	7.0	4.9	7.0	3.9	4.9	7.0		86.7	81.9	32
33	16.5	15.2	15.2	15.2	15.2	15.2	18.8	16.5	15.2	16.5	16.5	13.9	13.9	16.3	12.5	15.2	17.6	14.1	10.5	10.4	12.7	14.1	11.5	9.2	11.5	12.7	10.4	12.6	11.5	13.8	13.8	15.2		77.1	33
34	25.0	23.6	23.6	23.6	23.6	23.6	22.0	25.0	23.6	25.0	26.4	25.0	25.0	23.7	22.2	27.9	27.7	22.3	23.7	23.6	23.6	22.3	22.2	25.0	23.5	22.2	23.6	23.5	22.2	21.0	18.4	21.1	27.9		34
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	

Fig. 2. Percentage identity and divergance matrix of 10 strains of locally isolated *Paramecium* species from 1 to 10 (FT2.1-FT11.1 respectively) and 24 sequences from GenBank data based on the histone H4 gene fragment. From 11-15 are *P. tetraurelia* species (XM001425872, XM001455606, XM001452606, XM001452073, XM001459068 respectively). 16 is *P. decaurelia* (DQ067622), 17 and 18 again representing *P.tetraurelia* (AJ004699, XM001442554), 19 *P.tredecaurelia* (DQ067629), 20 *P. pentaurelia* (DQ067623), 21 is *P. primaurelia* (DQ067620), 22 *P. quadecuarelia* (DQ0676630), 23 *P. decaurelia* (DQ067626). 24 to 29 are *P. jenningsi* (DQ001056, DQ001064, DQ001062, DQ001059, DQ001061, DQ001057 respectively). 30 *P. undecaurelia* (DQ067627), 31 *P. septaurelia* (DQ067624), 32 *P. tertraurelia* (AJ004700), 33 *P. caudatum* (AB670962) and 34 *P. bursaria* (AJ004702).

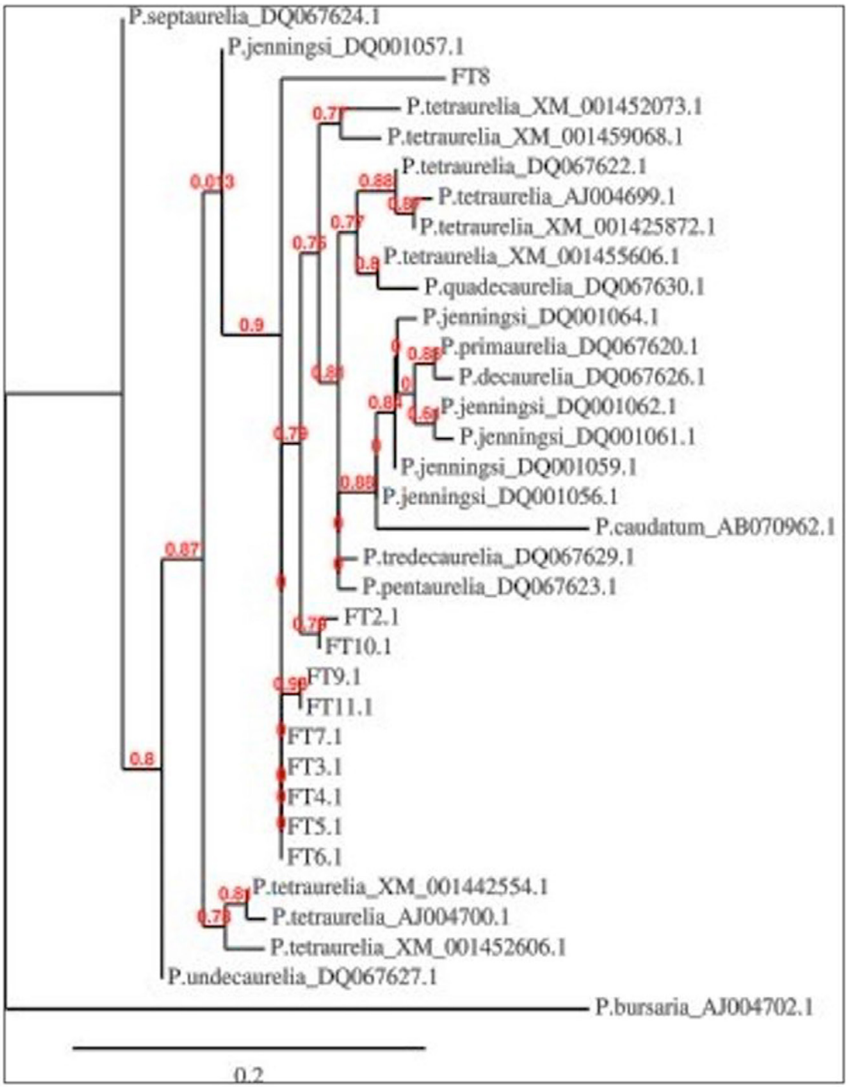

Fig. 3. Phylogenetic tree of 10 strains of locally isolated *Paramecium* species (FT2.1-FT11.1) based on comparison of sequences of the histone H4 gene fragment with the application of maximum likelihood correction method. Bootstrap values are presented as percentages from 1000 comparisons. Sequence of *Paramecium bursaria* is representing as an out group.

matrix in Figure 2 and 3. This clearly indicates the differences of FT8 strain as compared to all other strains. P. bursaria is used as an out group for the construction of phylogenetic tree. Phylogeny constructed by bootstrap analysis revealed a similar pattern of species emplacement within the tree. Most of the species collected from GenBank as a result of blast analysis of our strain sequences were belonging to the species of the *P. aurelia* complex. The tree represents nine entries of Paramecium tetraurelia under variety of accession numbers submitted by different authors along with the sequence of fifteen other species. All of the species in phylogenetic tree are not making any definite group or clades, rather are dispersed throughout the tree. However, most of the strains under our study did not show close relationship with any of the species. Rather all of them fell into same group by making their own clade. Whereas, FT8 was the only strain found to be at very distinct position from the rests of the species.

DISCUSSION

With the early studies on protein it was found out that histone H4 was strongly conserved throughout the plants and animal kingdom. Despite the fact that H4 protein sequences are invariant among vertebrates, considerable differences have been observed within ciliates allowing them to systematize their position in phylogenetic tree. H4 sequence variation was first time described in *Tetrahymena pyriformis* by Hayashi *et al*. (1984). In spite of the fact that variations in the H4 gene sequences of different species of ciliates do not show the direct effect of the reproductive affinity, they can contribute the differences at genetic level and play an alternative role in the evolution of whole genome duplication that may lead to complete reproductive isolation Aury *et al*. (2006).

Recently, the trend is shifting to confirm the phylogenetic results based on mitochondrial and rRNA genes with histone H4 sequences. Likewise, many things left undecided by mtDNA and rRNA genome sequences are needed to be confirmed with some other data. Histone H4 gene has been selected as a reliable marker for the testing of phylogenies based on rRNA or mtDNA sequence. Mostly it has been observed that variations in the nucleotide sequences often occur as synonymous substitutions that tend to arrange the remote species close to each other in the phylogenetic tree (Pineau *et al*., 2005). So in order to confirm the exact position of FT8 strain (Shakoori *et al*., 2014) in phylogenetic tree, H4 gene sequence was selected as a better substitute along with some other strains. This has also been reported that species of *Paramecium aurelia* complex contains a single gene of H4 (Przybos *et al*., 2006). This single gene variations have been extensively studied which is the source of genetic isolation of the *P. aurelia* sibling species, exists from one or more than one site changes "but constitute a dense evolutionary cluster" Coleman (2005).

Species of our study mostly fall under the complex of *Paramecium aurelia* species except FT8 strain that has already been reported as a new species based on the 18SrRNA phylogeny. Present results based on Histone H4 gene sequences confirmed the FT8 strain has a peculiar phylogenetic position in *Paramecium*. This species is showing its distinct position representing it as a highly divergent from other species. Rests of the strains belonging to *Paramecium aurelia* species are showing their positions a little different from each other. The reason could be the isolation of strains from different regions that caused them to make the geographical groups. All of the strains are falling together in same position making their own clade.

According to the tree, FT8 as well as *P. caudatum* sequence, are interspersed in the mass of the *P. aurelia* entries that is revealing not a very good picture. This is the weak point of this study which has a risk to create biases in the phylogeny. Although, it can be true with this set of data, as the fragment of the sequence analyzed was very short. However, this drawback need to be resolved by amplifying the bigger portion of H4 gene. The overall picture of the strains under present study in the tree is representing their close relationship with *P. aurelia* species except FT8 strain which is showing its completely different position as compared to others, indicating it as a new species.

Statement of conflict of interest

Authors have declared no conflict of interest.

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