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A New Ciliate Species, *Tetrahymena farahensis,* Isolated from the Industrial Wastewater and Its Phylogenetic Relationship with Other Members of the Genus *Tetrahymena*

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Abstract.- Anthropogenic activities are dumping heavy metals into the environment as waste effluents or integral part of some compounds. This has resulted in an increase in the metal concentration, beyond the permissible threshold, leading to metal toxicity for all forms of life. Metal resistant ciliates remove metal ions from contaminated water, mainly by the process of bioaccumulation. This bioaccumulation is due to low molecular weight, metal ions chelating proteins known as metallothioneins. In the present study, a new species of *Tetrahymena* (*Tetrahymena*1.7) is being reported from the local industrial wastewater. Analysis of *Tetrahymena* 1.7 SS rDNA showed 99% homology to seven different species of the genus *Tetrahymena*. SS rRNA secondary structure appeared in 40 helices with 18 variations, including 17 substitutions and one deletion. All the variations are present in 6 variable lengths, namely, V2, V3, V4, V7, V8 and V9. Cytochrome c oxidase subunit 1 (COX1) gene sequence was quite variable, with 91% homology to its closest relative *T. thermophila*. Since this value was higher than the intraspecific variations (≥99% homology), *Tetrahyemna*1.7 has been considered as a new species *i.e., Tetrahymena farahensis*. Phylogenetic analysis based on both SS rDNA and COX1 using maximum likelihood and neighbor joining methods showed that *Tetrahymena farahensis,* new species was related to *T. thermophila* and *T. malaccensis*. Thus, it appeared to be a new member of riboset A1 and coxiset A1 on the basis of SS rRNA and COX1 gene, respectively.

Key words: *Tetrahymena farahensis* new species, *SS rRNA* gene, *COX1* gene, cytochrome c oxidase subunit 1, rRNA secondary structure, ribotyping.

INTRODUCTION

Among protozoa, ciliates are found in almost all types of aquatic habitats including marine, freshwater, soil and within animal bodies (Small and Gross, 1985). Ciliates are thought to have evolved during early Cambrian period, well before the evolution of other eukaryotes (Finlay *et al.,* 2000). They are considered as grazers of bacteria and other microbes (Fenchel, 1987; Finlay *et al.,* 2000) thus improving the effluents and increasing turnover rate (Nicolau *et al.,* 2001). Ciliates lack cell wall and give a quick response to environmental changes, which indicates that ciliates are better pollution detectors, compared to bacteria and fungi (Gutierrez *et al.,* 2003).

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Tetrahymena is a genus of small ciliates which were previously studied by the names of Leucophyres and Glaucoma (Furgason, 1940). Most of the *Tetrahymena* species are cosmopolitan in distribution. They have eight ciliated membranous structures including four oral, one undulating and three adoral membranelles. Macronuclei of all the members of genus *Tetrahymena* are transcriptionally active (Simon *et al.,* 2008). *Tetrahymena* species rapidly grow in axenic medium and thus become a model organism for research in physiology and biochemistry (Hill, 1972; Elliott, 1973). *Tetrahymena* show closer genetic resemblance to human as compared to yeast model and share a higher degree of functional conservation to human genes (Eisen *et al.,* 2006). This is a valid point to use *Tetrahymena* instead of other organisms for ecotoxicological studies (Martin-Gonzalez *et al.,* 1999).

Although *Tetrahymena* is the best studied organism among ciliates, yet it is ambiguous to discriminate among species of *Tetrahymena* on the

basis of physical and morphological characteristics (Kher *et al.,* 2011; Gruchy, 1955), particularly when grown in different culture media (Corliss, 1973; Struder-Kypke *et al.,* 2001). Use of silver staining for morphological identification has been used as primary method for identification (Corliss, 1973). Mating technique has also remained an effective tool to identify the cryptic species within genus *Tetrahymena* as they are reproductively isolated (Doerder *et al.,* 1995; Nanney *et al.,* 1998). This technique, however, is not only laborious and impractical (Sonneborn, 1959), but is also useless for amicronucleate strains of *Tetrahymena* which do not adopt sexual reproduction (Nanney and McCoy, 1976).

Several of molecular biology techniques have also been used to discriminate among *Tetrahymena* species. Initially these approaches included isozyme mobility and RFLP (Chantangsi *et al.,* 2007). Later, it was found that several species of *Tetrahymena* have identical RFLP pattern (Jerome and Lynn, 1996), while in the case of isozyme mobilities, similar polymorphism was observed (Nanney *et al.,* 1998). Nucleotide sequences of histones and SSrRNA genes have also been used to discriminate among different species of *Tetrahymena*, but there are several species which have identical SSrRNA genes (Jerome and Lynn, 1996; Struder-Kypke *et al.,* 2001). So, SSrRNA subunit is considered as too conserved for species identification (Boenigk *et al.,* 2012).

Analysis of cytochrome c oxidase subunit 1 (COX1) technique is an effective approach to identify different species of invertebrates (Folmer *et al.,* 1994). Using 980bp fragment of COX1 as DNA barcode, Lynn and Struder-kypke (2006) could successfully identify the reproductively isolated species of *Tetrahymena*. Intraspecific sequence divergence for *T. thermophila* was less than 1%, while interspecific distance divergence was up to 12%. Similar results from other experiments suggest that sequence divergence of <1% might be used to identify different isolates of a species, while a divergence higher than this threshold particularly near to 10% can be used to discriminate different species (Chantangsi *et al.,* 2007) and to identify new species.

Phylogenetic relationships among different species have also been established on the basis of DNA-DNA hybridization (Allen and Li, 1974), and analysis of SSrRNA gene (Sogin *et al.,* 1986; Jerome and Lynn, 1996; Chantangsi and Lynn, 2008). There is a need, however, for a sequence which is variable enough but not up to the extant where homologues are difficult to arrange (Pace *et al*., 1989). Hebert *et al.* (2003) have suggested the use of a 650bp long fragment of COX 1 as universal barcode, since its variable domains are useful in establishing phylogenetic relationships among *Tetrahymena*.

In this study we have used the analysis of SS rRNA and COX1 genes for identification of a ciliate protozoan.

MATERIALS AND METHODS

Samples of industrial wastewater collected from Preliminary Tanneries Wastewater Treatment Plant, Kasur were brought to the lab for microscopic observation. On the basis of size, shape, pattern of ciliary lining and pattern of locomotion of the organisms (Edmondson, 1966; Curds *et al*., 1983; APHA, 1989), they were identified as *Tetrahymena* 1.7. Wastewater samples, besides ciliates also contained algae, fungi and bacteria. Algae were eliminated by keeping the cultures in dark. Fungizone/Amphotericin B (1µg/ml) was also added to remove fungal contamination. Ampicillin (100µg/ml), kanamycin (50µg/ml) and chloramphenicol (20µg/ml) were used to inhibit the growth of bacteria when required.

Primarily, this culture was maintained in Bold-basal salt medium (Shakoori *et al.,* 2004). Considering that identification of *Tetrahymena* was not reliable, exclusively on the morphological characteristics, molecular tools were used to identify and discriminate among different species. SS rRNA and mitochondrial COX 1 genes were used as markers genes for identification up to species level and for further phylogenetic analysis.

DNA barcoding

For this, isolated DNA was amplified for SS rDNA and COX1 genes which were afterwards cloned, sequenced and analyzed for nucleotide homologies and analyzed for variations.

PCR amplification and nucleotide sequencing of SS rRNA gene

Genomic DNA was isolated from rapidly growing log phase *Tetrahymena* cells. SS rRNA gene was PCR amplified using the following primers:

EukF (5'-AATATGGTTGATCCTGCCAGT-3')
EukR (5'-TGATCCTTCTGCAGGTTCACCTAC-3').

The reaction mixture (50 µl) contained 1X $NH_4(SO_4)_2$ buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs (Fermentas #RO181), 0.4 pmol each primer, 2.5 U Taq DNA polymerase (Fermentas #EP0402) and 1µg genomic DNA. PCR was performed in GeneAmp thermocycler with initial denaturation of 5 min at 94°C, followed by 35 cycles, each of denaturation at 95°C for 50 sec, annealing at 51°C for 45 sec and elongation at 72°C for 50 sec. It was followed by a final elongation step at 72°C for 7 min. PCR product was loaded on 1% agarose gel and electrophoresis was performed at 80V for 45min.

Amplified PCR product of SS rRNA gene was ligated in pTZ57R/T cloning vector using InstaClone PCR cloning kit (#K1214). *E.coli* DH5α competent cells were transformed with these recombinant plasmid (Sambrook and Russel, 2001).

Purified recombinant plasmid (about 300ng) containing SS RNA gene was sent to Macrogen Korea for nucleotide sequencing. Sequence homology was performed by Basic Local Alignment Search Tool (Altschul *et al*., 1990).

PCR amplification and nucleotide sequencing of COX1 gene

A fragment (986nt long) of COX1 gene was PCR amplified using the following primers (Lynn and Strüder-Kypke, 2006; Folmer *et al.*, 1994):

CoX-F (5'- TCAGGTGCTGCACTAGC-3')
Cox-R (5'-TAAACTTCAGGGTGACCAAAAAATCA-3')

The composition of the PCR mixture was the same as for SS RNA gene amplification.

PCR was performed in GeneAmp thermocycler with initial denaturation at 94°C for 5min followed by 35 cycles, each of denaturation at 94°C for 45sec, annealing at 53°C for 35 sec and elongation at 72°C for 40sec; final extension was given for 7 min at 72°C. Amplified PCR product of COX1 gene was ligated in pTZ57R/T cloning vector using InstaClone PCR cloning kit (#K1214). *E. coli* DH5α competent cells were transformed with recombinant plasmids. This cloned gene was sent to Macrogen Korea for automated sequencing by ABI sequencer.

Phylogenetic analysis

Phylogeny of *Tstrahymena* 1.7 was studied by drawing phylogenetic tree based on maximum likelihood and neighbour joining methods using SSrRNA gene and COX1 gene sequences. The phylogenetic tree was constructed through Mega 5.2 software (Tamura *et al.,* 2011).

RESULTS AND DISCUSSION

Tetrahymena *isolates*

Tetrahymena 1.7 was found in two water samples (pH 6.8-7.9, temperature 33-37°C) of Preliminary Tanneries Wastewater Treatment Plant. Ciliates are integral part of land and water ecosystems (Finlay *et al.,* 2000). They have been found in a number of heavy metal polluted wastewater ponds. This shows they have capability to survive in metal stressed environment (Rehman *et al.,* 2006).

Tetrahymena 1.7 cells were recognized by one pointed and the other broader end, centrally positioned nucleus, ciliated oral groove near the pointed end and the characteristic longitudinal pattern of ciliary linings (Fig. 1). Average size of *Tetrahymena* varied from 26.5µm - 43µm in length and 21µm–35.5µm in width. Different stages of asexual reproduction were also clearly visible under the microscope (Fig. 2).

Molecular identification of Tetrahymena *1.7 on the basis of SS DNA analysis*

Genomic DNA of *Tetrahymena* 1.7 was isolated (Fig. 3a) and amplified for small subunit ribosomal RNA gene (see 1.8kb bands in Fig. 3b) and cloned in pTZ57R/T.

Nucleotide sequencing of the gene showed 1753 nucleotides (accession No. HE820726). nBLAST of 1753 nucleotides of SS rRNA gene of

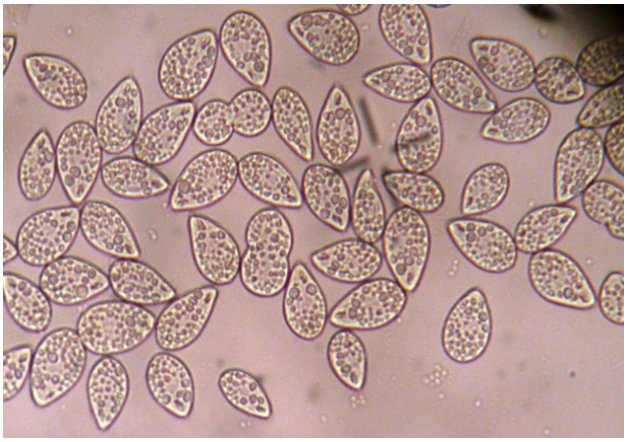


Fig. 1. Microscopoic observation of *Tetrahymena farahensis*, new species at 100X. Different cellular structures including nucleus, oral groove, contractile vacuoles are easily visible.

Tetrahymena 1.7 showed 99% homology with seven different species of genus *Tetrahymena*. Comparative analysis of SSrRNA gene sequence with GenBank derived sequences of other *Tetrahymena* spp. revealed 98.3-99.1% homology of *Tetrahymena* 1.7 with other members of the genus (Table I). Through multiple alignment of theswe sequences four regions within the SSRNA gene *i.e.*, 268-277, 485-488, 1329-1343 and 1660-1672 were identified as more variable (Fig. 4). Only one insertion (C) was observed at nucleotide 488. Both transition and transversion mutations were observed, however transversions were of relatively higher frequency. Transition T→A was observed at 268, 277, 285, 486 and 519 nucleotide positions, while transition A→T was observed at 487, 661 and 1037. There was no C→G or G→C transition observed in the sequence.

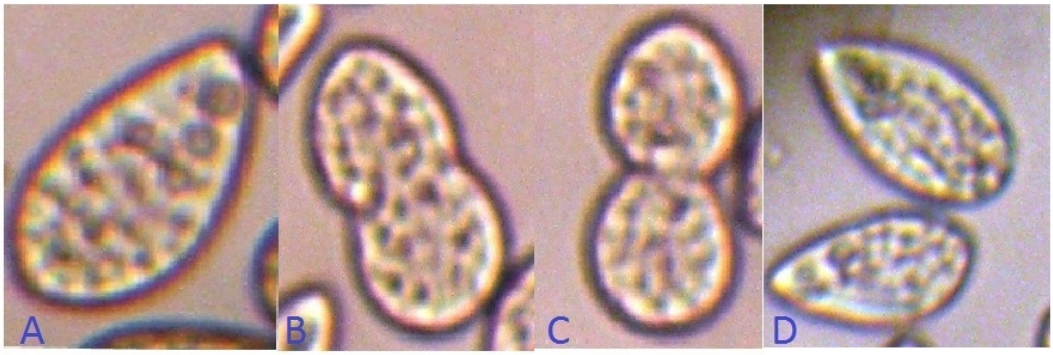


Fig. 2. Different stages of asexual reproduction in *Tetrahymena farahensis*, new species. A, interphase cell; B, nuclear division proceeds and cytokinesis also starts; C, nuclear division has completed while cytokinesis is at its final stage; D, birth of two daughter cells.

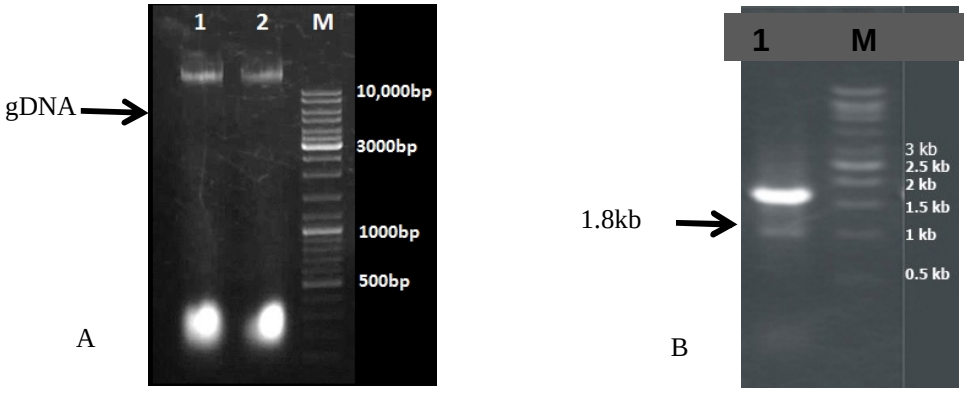
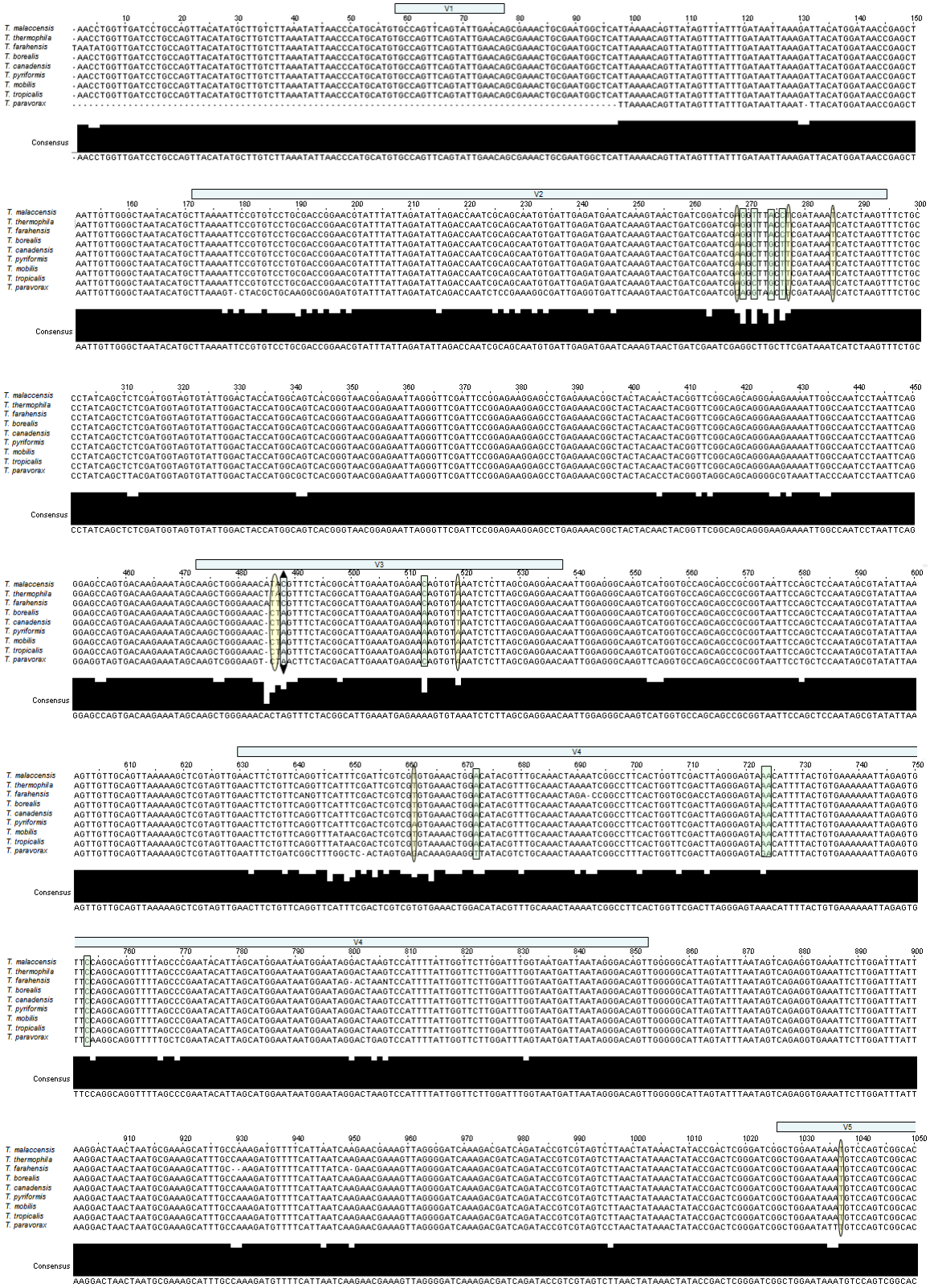


Fig. 3. **A,** Agarose gel electrophoresis of genomic DNA of *Tetrahymena farahensis*, new species. Lanes 1 and 2, genomic DNA; M, DNA marker. **B,** PCR amplification of SS rRNA gene. Lane 1, amplified SS rRNA gene; M, DNA Marker.


V1
V2
V3
V4
V4
V5
T. malaccensis
T. thermophila
T. farahensis
T. borealis
T. canadensis
T. pyriformis
T. mobilis
T. tropicalis
T. paravorax
Consensus

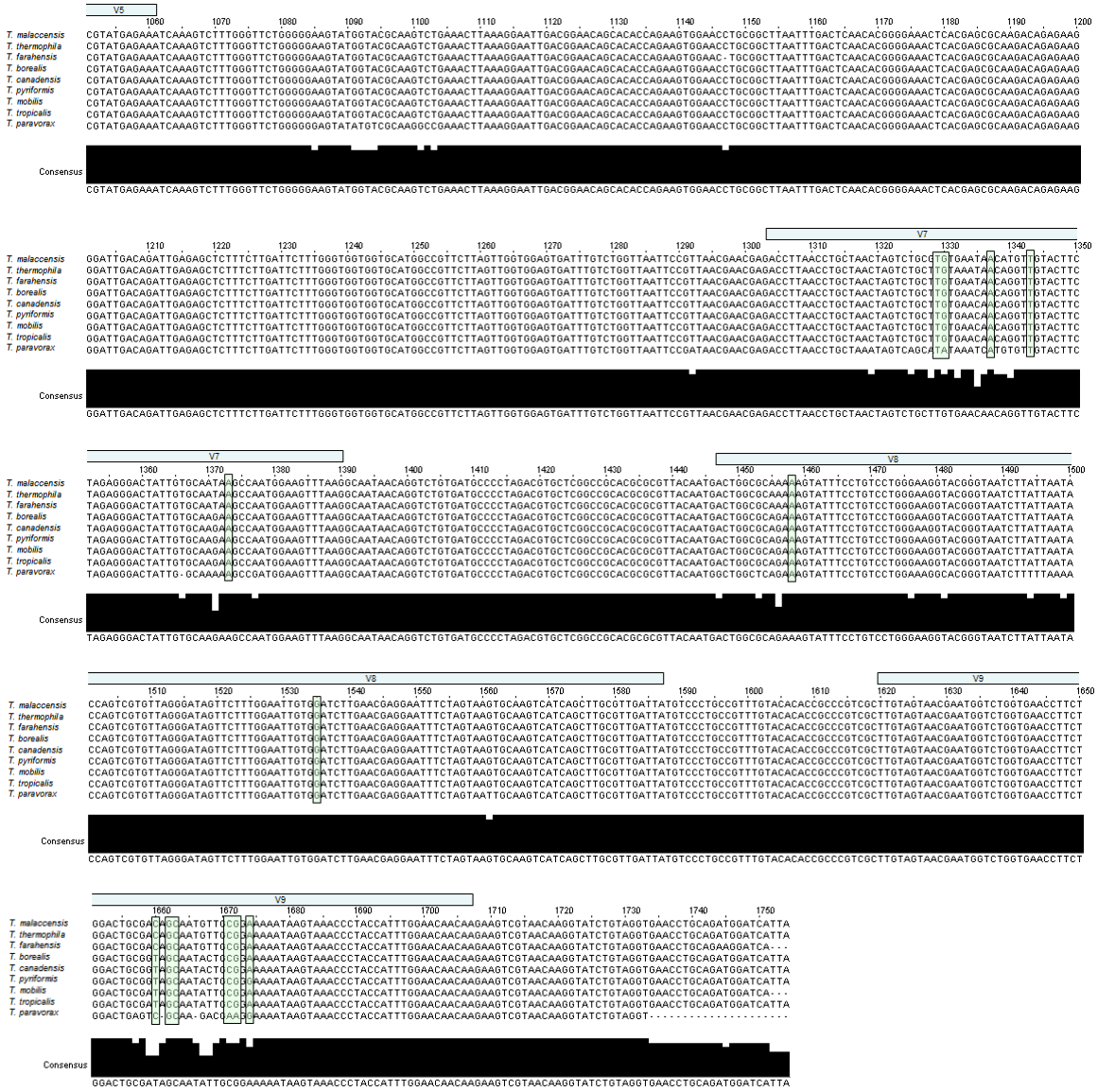


Fig. 4. Multiple alignment for SS rRNA gene of *T. farahensis* along with closely related *Tetrahymena* species shows variable regions V1-V9. Transversions and transitions are enclosed by rectangle and oval shapes, respectively. Insertion is indicated by a symbol ◊.

In case of transverions, A↔G mutations were observed at 269, 274, 672, 723, 724, 1343, 1458, 1662, 1671 and 1674. Similarly, C↔T transversions were observed at 271, 276, 753, 1329, 1330, 1337, 1660, 1663 and 1672 positions. While there was only one A→C transversion at 513 nucleotide position and two G↔T transversions at 1373 and 1535 nucleotide positions.

nBLAST results of SS rRNA gene of *Tetrahymena* isolate showed 1% divergence with more than seven different species of *Tetrahymena* including *T. malaccensis* and *T. thermophila.* This shows SS rRNA gene is too conserved to resolve the closely related species. The mean sequence divergence of SS rRNA gene for 45 species of *Tetrahymena* is 1.56±0.16. Most of the *Tetrahymena* species showed sequence divergence of 0-2% (Chantangsi *et al.*, 2007). This

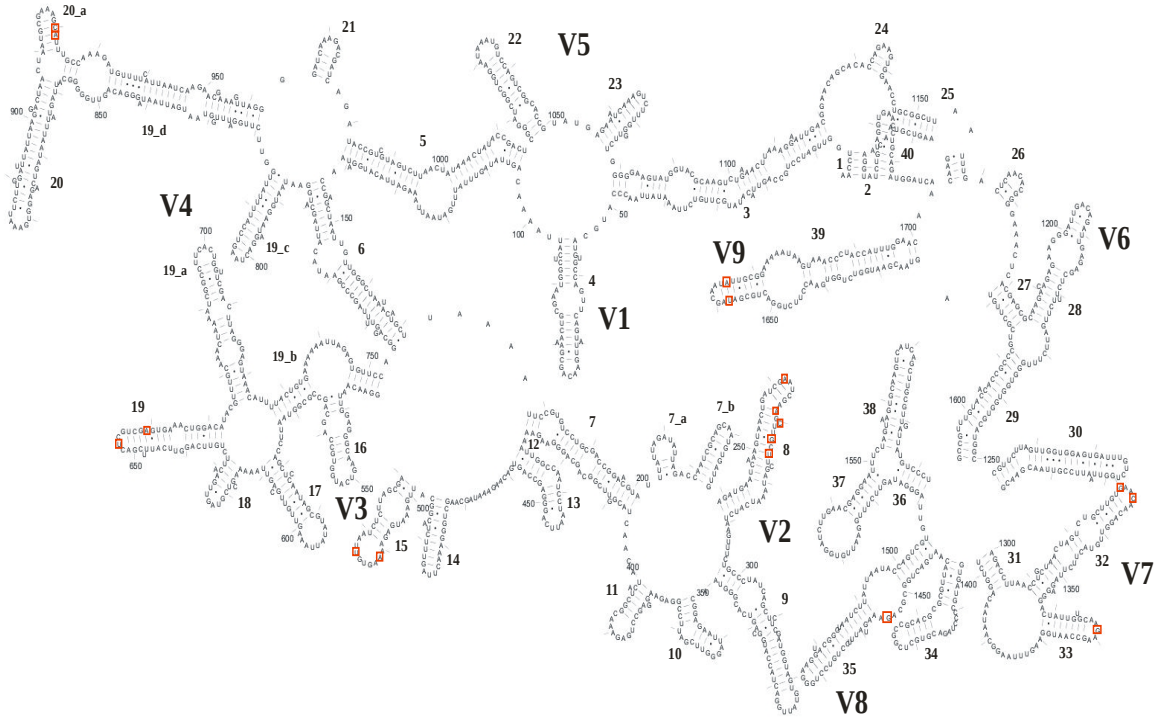


Fig. 5. Secondary structure of SS rRNA of locally isolated *T. farahensis*, new species, variations are highlighted as red marks. These variations (red marks) are observed in V2, V3, V4, V7 and V9 variable regions.

Table I.- Distance matrix for SS rRNA gene of *T. farahensis*, new species shows its percent identity and divergence with other closely related Tetrahymena species.

Percent Identity

Divergence	1	2	3	4	5	6	7	8		
1		99.1	98.5	98.5	98.5	98.4	99.1	98.5	1	T.farahensis
2	0.7		99.0	99.0	99.0	98.9	99.8	99.0	2	T.malaccensis
3	1.3	1.0		99.5	99.5	99.4	99.0	100.0	3	T.mobilis
4	1.3	1.0	0.5		100.0	99.7	99.0	99.5	4	T.borealis
5	1.3	1.0	0.5	0.0		99.7	99.0	99.5	5	T.canadensis
6	1.4	1.2	0.6	0.3	0.3		98.9	99.4	6	T.pyriformis
7	0.7	0.2	1.0	1.0	1.0	1.0		99.0	7	T.thermophila
8	1.3	1.0	0.0	0.5	0.5	0.6	1.0		8	T.tropicalis
	1	2	3	4	5	6	7	8		

is insufficient to discriminate among closely related species (Chantangsi and Lynn, 2008). The comparison of SS rRNA gene sequence of *Tetrahymena* 1.7 with other species of genus *Tetrahymena* showed that the gene was highly conserved with the exception of few intragenic semi-conserved regions which are helpful in establishing the phylogenetic relationships among

different members of genus *Tetrahymena*.

Secondary structure of SS rRNA

The secondary structure models of SS rRNA gene are being used as topographical markers for the analysis of phylogenetic affiliations. These indispensable marker systems have proven to be helpful in exploring potentially useful information among closely and remotely related organisms. Secondary structure models for SS rRNA genes have been documented by various authors (Hirt *et al*., 1994; Struder-Kypke *et al*., 2001; Wuyts *et al*., 2001; Xia *et al*., 2011; Lee and Gutell, 2012). Due to the slowly evolving property of SS rRNA, the basic molecular structure of all eukaryotes is conserved throughout evolution except the nine expansion segments (V1-V9). Among these major lengths variable regions, V2, V4 and V7 have been reported as fast evolving segments (Hwang *et al*., 2000; Alkemar and Nygard, 2003, 2004; Alvares *et al*., 2004). V6 is thought to be the least variable region in all eukaryotes (Gonzalez and Schmickel, 1986). Secondary structure model of SS rRNAs of *Tetraymena* 1.7 shown in Figure 5 is folded into 40 helices. Helices numbering system was followed by Nelles *et al*. (1984). Comparison with other members of genus *Tetrahymena* showed that all of the variability is contributed by 6 major variable lengths, namely, V2, V3, V4, V7, V8 and V9, while V1, V5 and V6 regions appear as more conserved with compound single helices. From the conserved arrangement of these helices it can be deduced that all of the mutations are found either in internal or terminal bulges which retained the stable structural configuration in species.

Phylogenetic analysis of Tetraymena *1.7 on the basis of SS rRNA gene*

Phylogenetic analysis of *Tetraymena* 1.7 with other species was performed by drawing phylogenetic tree using neighbour joining method (Fig. 6) on the basis of their SSRNA gene. All different species of *Tetrahymena* are clustered in different groups, termed as ribosets by Nanney *et al.* (1989). *Tetraymena* 1.7 appears along with *T. malaccensis* and *T. thermophila* which are members of ribose A1. While considering the major grouping *T. farahensis* appears to be a member of *borealis* group instead of *australis* group.

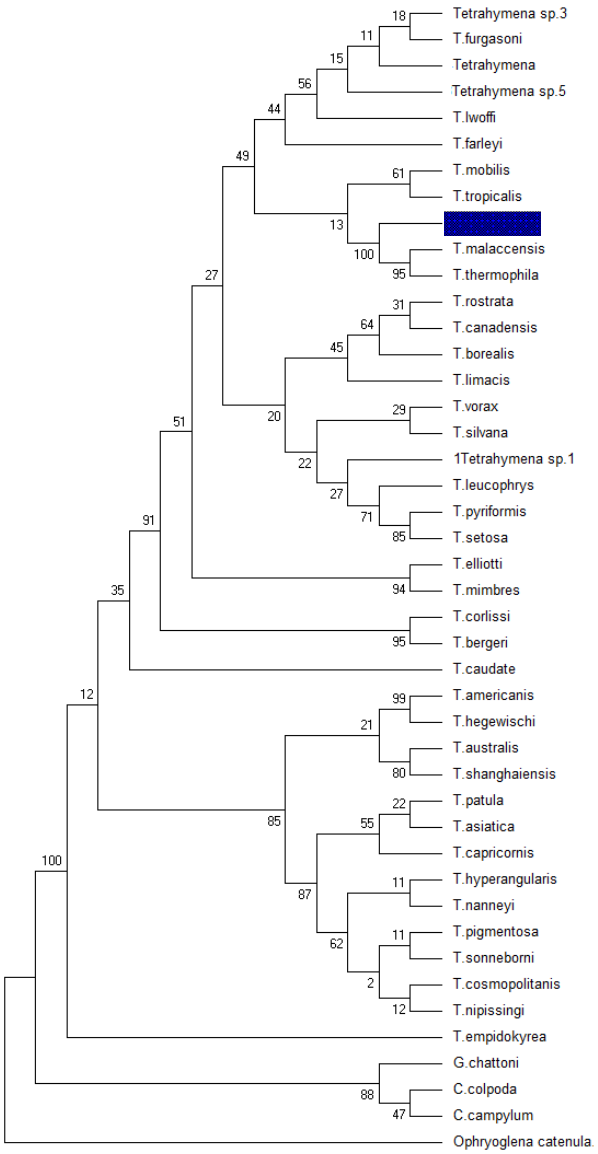


Fig. 6. Phylogenetic tree of locally isolated *T. farahensis* using SS rRNA gene sequence based on neighbour joining method. The organism showed a close evolutionary relationship with *T. thermophila* and *T. malaccensis*.

Sogin *et al.* (1986), using molecular markers, worked on the phylogenetic relationships among members of genus *Tetrahymena.* They are separated into two major groups *i.e.*, *australis* and *borealis* (Struder-Kypke *et al.*, 2001). The *australis* group

consists of riboset C which is homogenous while *borealis* group with riboset A and B is quite heterogeneous (Preparata *et al.*, 1989). Because of very little genetic differences, the branches are not strongly supported by high boot strap value (Struder-Kypke *et al.*, 2001). Some species with similar SS rRNA gene can be distinguished on the basis of their morphology (Roque *et al.*, 1970).

Molecular identification of T. farahensis *on the basis of COX1 gene*

COX1 is more variable and used by the protozologists to discriminate different species of *Tetrahymena* and other ciliates. A 986 nucleotide fragment of COX1 gene was amplified (Fig. 7), cloned in pTZ57R/T.

Cloned fragment of COX1 gene was sequenced from Macrogen Korea using M13 forward and reverse primers. Peaks read through Chromas Lite 2.1 showed a 986 nucleotide fragment of COX1. NCBI nucleotide BLAST showed its 91% homology with *T. thermophila* with 99% query coverage.

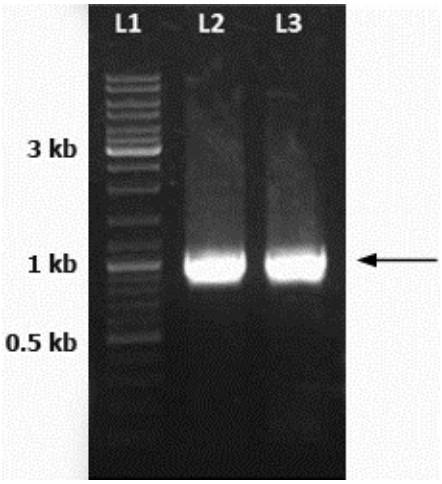


Fig. 7. *In vitro* amplification of a selected fragment of cytochrome c oxidase. L1, DNA ladder mix; L2 and L3, amplified fragment of cytochrome c oxidase appearing ≈ 1 kb size.

The amplified sequence was submitted to EMBL nucleotide sequence database under accession No. HG710169. Multiple sequence alignment of *Tetrahymena* 1.7 COX1 with already reported COX1 of different *Tetrahymena* species showed that the gene is highly variable as compared to SS rRNA (Fig. 8). Homology of COX1 gene of *Tetraymena* 1.7 with most closely related species of *Tetrahymena* falls in the range of 89.1-91.1% (Table II).

Table II- Distance matrix for COX1of *T. farahensis*, new species shows its percent identity and divergence with other closely related *Tetrahymena* species.

Percent Identity

Divergence	1	2	3	4	5	6	7	8	9		
1		96.6	90.3	89.6	92.1	88.1	91.0	90.0	90.7	1	T.borealis
2	3.5		90.7	89.7	92.1	88.3	90.9	90.7	90.3	2	T.canadensis
3	10.4	10.0		91.1	90.6	89.1	90.4	90.6	89.4	3	T.farahensis
4	11.2	11.1	9.5		90.8	87.9	90.7	91.5	89.8	4	T.malaccensis
5	8.4	8.4	10.1	9.8		88.1	91.6	90.5	95.4	5	T.mobilis
6	12.9	12.7	11.7	13.2	12.9		87.5	88.5	87.5	6	T.paravorxx
7	9.6	9.7	10.3	10.0	8.9	13.6		90.2	90.8	7	T.pyriformis
8	10.8	10.0	10.1	9.0	10.2	12.4	10.5		89.2	8	T.thermophila
9	10.0	10.4	11.5	11.0	4.7	13.6	9.9	11.6		9	T.tropicalis
	1	2	3	4	5	6	7	8	9		

Tetrahymena isolates having <1% variation in COX1 sequence are considered as same species, while sequence divergence between 1 to 12% indicates different species (Lynn and Struder-kypke, 2006). Kher *et al.* (2011) also used COX1 gene as identification marker for *Tetrahymena* species. However, according to the intraspecific divergence in *Tetrahymena* is likely to increase with spreading geography, so it would be safer to say that strains with higher than 5% divergence are separate species. The GC content of the *Tetraymena* 1.7 COX1 was 27.7% which was slightly higher than *T. pigmentosa*, *T. mobilis* (25% in both) and *T. hyperangularis* (26%) (Lynn and Struder-kype, 2006). Chantangsi *et al.* (2007) has also elaborated the role of COX1 in species identification and calculated the value for mean intraspecific divergence for COX1 as 0.95%, while interspecific divergence as 10.47%. Thus interspecific divergence is 11 to 58.2 folds higher as compared to intraspecific divergences. Keeping in view the above parameters *Tetrahymena* 1.7 can be considered as a new species, which is named here as *Tetrahymena farahensis*, after my supervisor Dr. Farah Shakoori.

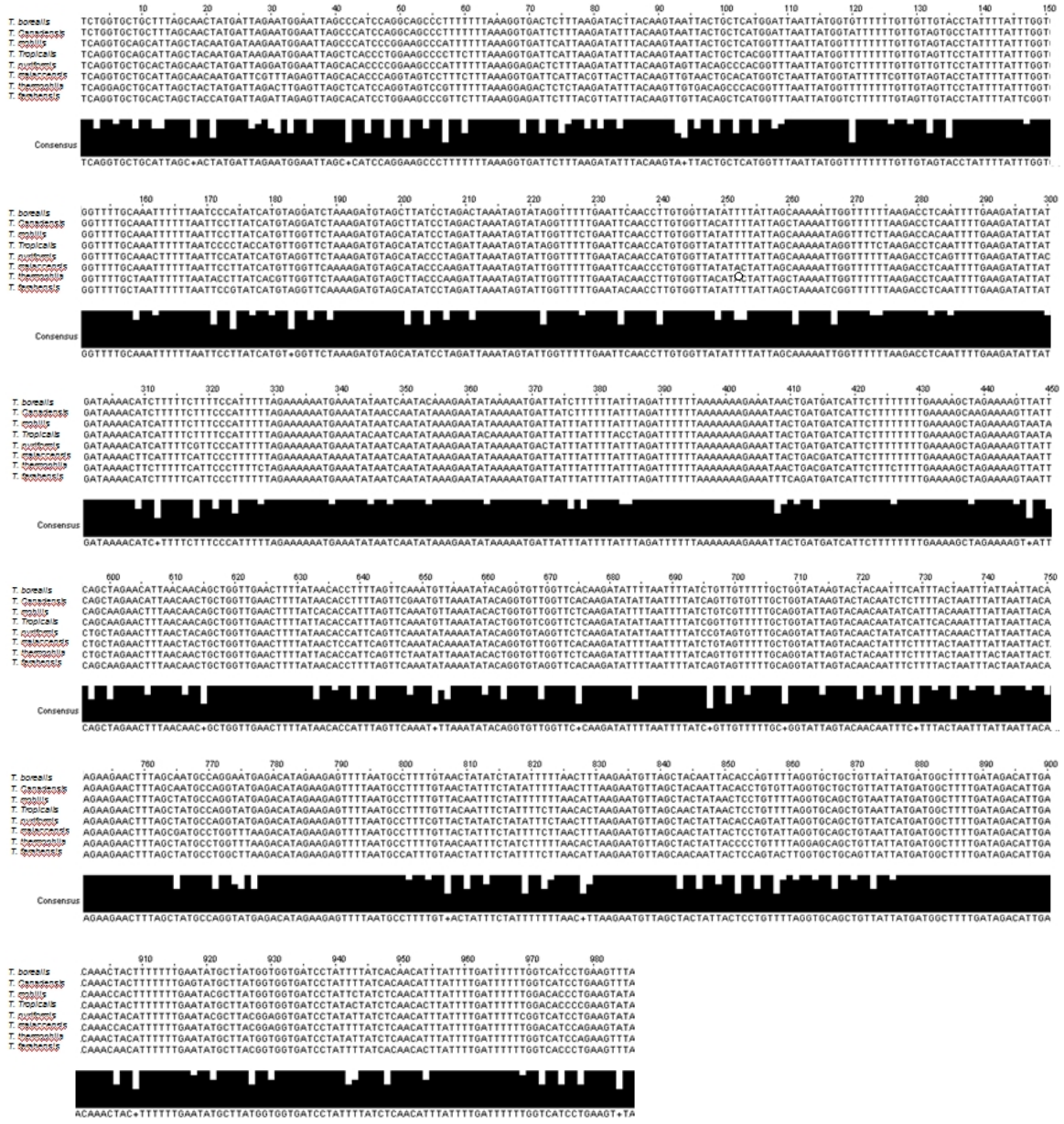


Fig. 8. Multiple alignment for Cytocrome c oxidase subunit 1 (1.7 cox) of *T. farahensis* along with closely related *Tetrahymena* species shows frequent variations as compared to SS rRNA gene.

Phylogenetic analysis of T. farahensis *on the basis of COX 1 gene sequence*

COX1 appears to be more variable phylogenetic marker as compared to SS rRNA gene. Figure 7 shows phylogenetic tree inferred on the basis of neighbour joining method using COX1 nucleotide sequences. *T. farahensis* retains its homology with the same species as in case of SSr RNA gene. The branching pattern is supported by a high boot strap value.

Phylogenetic tree drawn on the basis of *T. farahensis* COX1 nucleotide sequences clearly shows its close homology with *T. malaccensis, T. thermophila and T. paravorax.*

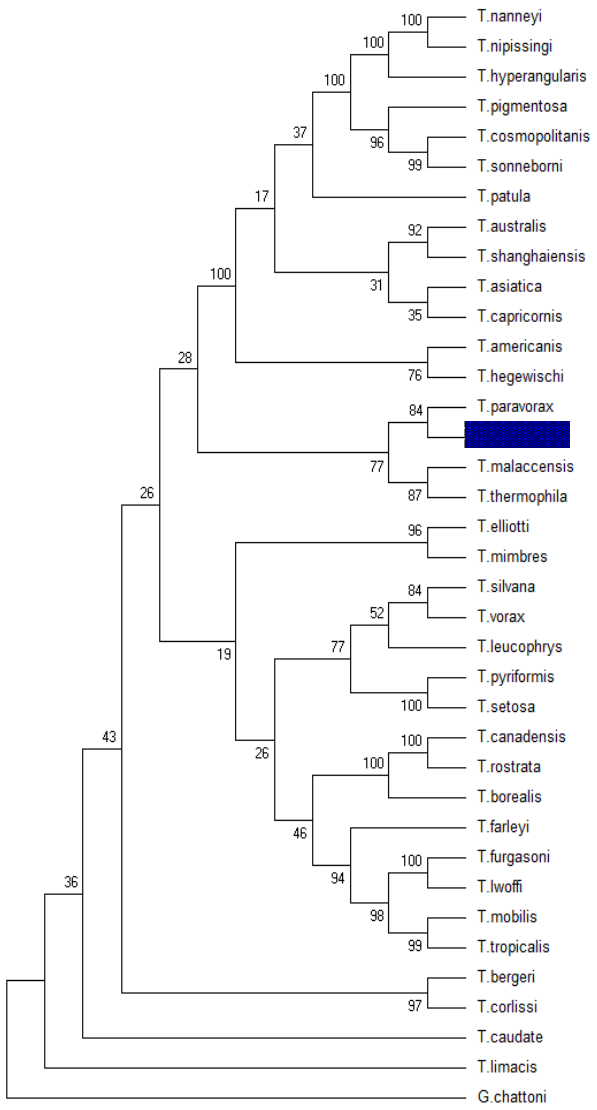


Fig.9. Phylogentic tree of *T. farahensis*, new species on the basis of the sequence of fragment of COX1 using neighbour joining method. *T. farahensis* falls in the same clade along with *T. thermophila* and *T. malaccensis* as observed in the tree based on SS RNA gene sequence.

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