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A New Subspecies of a Ciliate *Euplotes musicola* Isolated from Industrial Effluents

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Abstract.- The copper resistant ciliates RE-1 and RE-2 isolated from industrial effluents and tentatively identified on microscopic observation as members of the genus *Euplotes* were subjected to SS rRNA gene analysis. The nucleotide sequence of the two SS rRNAs from these ciliates were deposited in GenBank under accession numbers DQ917684 and EU103618. Phylogenetic analysis revealed that RE-1 belonging to the *muscicola* group was most closely related to *Euplotes muscicola,* while RE-2 belonging to the *adiculatus* group was most closely related to *Euplotes adiculatus,* with which they showed the fewest differences in their SS rDNA sequences. The nucleotide sequences of closely related *Euplotes* spp. were aligned and all the sequences were compared to check the species variations. In the nucleotide sequence of RE-1, fewer variations were observed in the regions 323-516 and 906-1303 when compared with other species of the group. General mutations are more frequent among the species in both groups of *Euplotes* as more than 160 general variations were observed among the species of *muscicola* group, while around 100 general base pair differences were detected in *adiculatus* group. On the basis of the results of this study as well as microscopic observations new subspecies *Euplotes muscicola lahorensis* subsp. nov. is being reported.

Key words: Ribotyping of ciliates, copper resistant ciliate, SSrRNA gene.

INTRODUCTION

The ciliate identification is principally based upon morphological and ultrastructural characteristics (Foissner *et al.*, 1999; Finlay and Fenchel, 1999; APHA, 1989; Curds, 1982; Curds *et al.*, 1983). It is often very difficult and complicated to study the composition of the ciliate community of a given environment. Species definition or affiliation of the organisms to a single species may become problematic, especially in the cases of morphological similarity. So to find the middle ground, uncertain and undecided species are lumped together in species complexes *e.g. Vorticella convallaria*-complex, *Vorticella aquadulcis*-complex, etc. (Foissner *et al*., 1992).

The introduction of modern molecular methods based on DNA analysis and fingerprints, especially the methods targeted at ribosomal RNA operon endowed with exact insight into similarity studies of microorganisms. The examples of application of molecular methods for identification of bacteria are numerous, likewise, there are articles which deal with identification of protozoa (Lynn *et al*., 2000: Regensbogenova *et al.*, 2004). Molecular characters, specifically the sequences of the small (SSrRNA) and large (LSrRNA) subunits of rRNA genes and some protein-coding genes make a new database available, with which phylogenetic hypotheses that have been primarily based on morphological observations can be tested (Greenwood *et al.*, 1991b; Baroin-Tourancheau *et al.*, 1998; Hirt *et al.*, 1995; Hammerschmidt *et al.*, 1996; Clark, 1997; Bernhard and Schlegel, 1998; Stechmann *et al.*, 1998; Struder-Kypke *et al.*, 2000). Takeshi *et al.* (2002) used the sequence of SS rRNA gene as a marker for the identification of vorticellid ciliates. Jerome and Lynn (1996) used a riboprinting strategy for identification of morphologically difficult to differentiate species of the *Tetrahymena pyriformis* complex. Only in a few cases these techniques have been applied to distinguish species within a genus *e.g., Tetrahymena* (Nanney *et al.*, 1998; Struder-Kypke *et al.*, 2001) and *Paramecium* (Struder-Kypke *et al.*, 2000b). SSrRNA have been commonly used to identify ciliate species and to re-evaluate the phylogenetic relationships of many ciliate groups (Ragan *et al.*, 1996; Stoeck *et al.*, 1998)

The cosmopolitan, hypotrich genus *Euplotes* (Class Spirotrichea, Order Euplotida) is remarkable among ciliates for its species richness (Kusch *et al.*, 2000). Attributable to its abundance, its ubiquitous distribution, and the simplicity of culturing this

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ciliate, *Euplotes* species have been comprehensively and expansively studied. Numerous features have been used to find out species in the genus *Euplotes e.g.* Tuffrau (1960) used the number of dorsal kineties, the number of frontoventral cirri (cirrotype), the argyrome pattern on the dorsal surface (dargyrome), and the form of the macronucleus. Based primarily on differences in the dargyrome pattern, Curds (1975) divided *Euplotes* species into six groups, but in a reconsideration (Gates and Curds, 1979), these six were reduced to three groups and referred to as the dargyrome types *e.g.* single-, double-, or multiple-. Afterwards Borror and Hill (1995) recognized four groups, by investigating cortical structure, data from morphometric analysis, ecology, endosymbionts and morphogenetic patterns, each one being considered a separate genus. Lastly, random amplified polymorphic DNA fingerprinting has been used in an attempt to separate species in *Euplotes.* However, this appears to be of limited use for the construction of phylogenetic relationships in this species (Chen *et al.*, 2000). Petroni *et al.* (2002), by use of molecular analysis, discovered taxonomic and phylogenetic relationships within the genus *Euplotes*. They have determined and aligned the nearly complete SSrRNA sequences of eleven *Euplotes* species, representing diverse dargyrome types and habitats. The main objective achieved in this study is the identification of copper resistant *Euplotes* by amplification, cloning and sequencing of the SS rRNA gene and performing sequence alignments.

MATERIALS AND METHODS

Culture collection

Already established cultures of *Euplotes* were taken from Cell and Molecular Biology Laboratory (CMBL), University of the Punjab, Lahore. The ciliate *Euplotes* RE-1 was isolated from the tannery effluents of Kasur and *Euplotes* RE-2, from the industrial effluents of Sialkot. Initial identification of protozoa was done by observing their body shape, morphological features, movements and behavior (Edmondson, 1966; APHA, 1989; Curds, 1982; Curds *et al*., 1983) using Zeiss Axiostar plus microscope.

Culture maintenance and growth media

The ciliate cultures of RE-1 and RE-2 were maintained in Bold-basal salt medium [$NaNO_3$ (0.25 g/l), $CaCl_2.H_2O$ (0.025 g/l), $MgSO_4.7H_2O$ (0.075 g/l), K_2HPO_4 (0.075 g/l), KH_2PO_4 (0.175 g/l), NaCl (0.025 g/l), EDTA (0.05 g/l), KOH (0.031 g/l), $FeSO_4.7H_2O$ (0.04 g/l), H_2SO_4 (0.001 M), H_3BO_3 (0.01142 g/l), $ZnSO_4.7H_2O$ (0.00881 g/l), $MnCl_2.4H_2O$ (0.00144 g/l), MoO_3 (0.00071 g/l), $CuSO_4.5H_2O$ (0.00157 g/l) and $Co(NO_3)_2.6H_2O$ (0.00049 g/l)], diluted 1:1000 with distilled water, with 5-7 wheat grains (Shakoori *et al*., 2004). The pH of the medium was adjusted at 7.3 - 7.6 and kept at room temperature (27±2°C) in normal day light. The growth of culture was observed daily by counting number of protozoan cells in the medium under light microscope. Every time, readings in triplicate were taken and their means were calculated (Haq *et al*., 2000). Growth curves were prepared by plotting a graph between time (days) of incubation along the X-axis and number of cells per ml along the Y-axis.

Copper resistance

To study the effect of copper on growth of ciliates, two sets of cultures, control and treated, each of three sterilized 250 ml flasks containing 50 ml of Bold-basal salt medium (pH 7.5) supplemented with 5-7 wheat grains, were inoculated with the culture and incubated at 28±2°C. Copper stress was given by adding $CuSO_4.5H_2O$ stock solution (50 mg/ml) in the medium. In control set, no metal ions were added to the medium. Continuous observation under microscope showed the growth of RE-1 and RE-2.

Isolation and analysis of genomic DNA

High molecular weight genomic DNA was isolated from vegetatively growing cells of RE-1 and RE-2 after starving for 15 h in 10 mM Tris-HCl (pH 7.5) before harvesting at 6741 × g (Eppendorf MiniSpin Plus Personal Microcentrifuge). The cell pellet was re-suspended in 0.5 ml lysis buffer (42% urea, 0.30 M NaCl, 10 mM Tris-HCl (pH 7.5), 10 mM EDTA, 1% SDS), and gently shaken till homogeneity. Genomic DNA was extracted by phenol: chloroform extraction method (Sambrook *et al.*, 1989). The upper aqueous phase was separated

and DNA was precipitated with absolute ethanol. The DNA pellet was washed with 500 µl of 75% ethanol, air dried and dissolved in sterile de-ionized H_2O. For removal of RNA from genomic DNA 5 µl RNAase (10 mg/ml) was added in the isolated genomic DNA. It was run on 1% agarose gel in TAE buffer and DNA concentration was determined by taking its absorbance at 260 nm and A_{260}/A_{280} ratio was calculated to check its quality.

Molecular identification of metal resistant ciliates

For molecular identification of the copper resistant ciliates RE-1 and RE-2 either genomic DNA or whole organism was used in the PCR to amplify SS rRNA gene (18 S rDNA).

Primer designing

Different sets of primers were selected and designed for the amplification of SS rRNA gene (Table I).

Table I.- Primers used to clon e and sequence SS rRNA gene of RT-1, RE-1 and RE-2.

Primer Name	Sequence (5´ – 3´)	Length (nt)
EukFor	AATATGGTTGATCCTGCCAGT	21
EukRev	TGATCCTTCTGCAGGTTCACCTAC	24
M13 F	GTAAAACGACGGCCAGT	17
M13 R	CAGGAAACAGCTATGAC	17
SS rRNA-4F	YAGAGGTGAAATTCT	15
SS rRNA-5R	GGTGGTGCATGGCCG	15
IntEukFor	GCGAGGAACAATGGGAGGGC	20
IntEukRev	CCKCCTTCAAGATTCAYAATTTC	23

Y, C+T; K, G+T.

Primers based on conserved regions in eukaryotic 18S rRNA genes (EukFor and EukRev) were the same as described by Regensbogenova *et al*. (2004). Positively screened clones were sequenced with M13 forward, M13 reverse, one forward and one reverse internal universal 18S primers (Elwood *et al*., 1985). Subsequently, one forward internal primer (IntEukFor) and one reverse internal primer (IntEukRev) were designed using online web programs such as Primer3 etc. to get the complete sequence of the amplified product.

Amplification of SS rRNA gene

For amplification of SS rRNA gene of the two ciliates RE-1 and RE-2, a single or a few protozoa cells of each ciliate culture was picked from *in vitro* culture under the microscope, washed with sterile water and put into 50 µl of separate reaction mixtures containing 0.5 mM of each deoxynucleoside triphosphate (dNTPs), 100 pmol of each primer, 1X Taq buffer with $(NH_4)_2SO_4$ - $MgCl_2$ (Fermentas), 2 mM $MgCl_2$ and 0.5 U of Taq DNA polymerase (Fermentas # EP 0402). In another case 350-400 ng of genomic DNA of each ciliate was used in the separate PCR reactions instead of protozoan cell of ciliate culture. Primers based on conserved regions in eukaryotic18S rRNA genes and the reaction conditions described by Regensbogenova *et al*. (2004) were used with little modification. An initial denaturation step at 95ºC for 5 min was followed by 35 cycles each of 94ºC for 1 min, 52ºC for 1 min and 72ºC for 1 min, and a final extension at 72ºC for 10 min in a Gene Amp Thermal Cycler 2720 (Applied Biosystem, Singapore). Quality and quantity of amplified DNA (the nearly complete SS rDNA gene) were determined by electrophoresis in 1.5 % agarose gel (Sambrook and Russell, 2001).

DNA extraction (Gene clean)

PCR products were purified from agarose gel in 1X TAE buffer, using DNA Extraction Kit (Fermentas # K0513) using prescribed protocol.

Ligation into cloning vector

The nearly complete SS rRNA gene obtained (after gene clean) was ligated in pTZ57 R/T cloning vector using Fermentas InsTAclone™ PCR Cloning Kit (# K 1214) in 3: 1 insert: vector ratio according to the prescribed protocol. Vector pTZ57 R without insert and vector pTZ57 R/T with insert was also incubated with the experimental tubes as positive and negative controls, respectively.

Competent cell preparation and transformation

Competent cells of *E. coli* DH5α cells were prepared acoording to the method of Sambrook and Russell, 2001. Competent cells were transformed with the recombinant DNA (ligated in pTZ57 R/T cloning vector) and the transformants were screened using X-Gal/IPTG and ampicillin selection.

Confirmation of positive clones and sequence analysis

For the confirmation of positive clones (white colonies), plasmid DNA was isolated by miniprep method (Sambrook and Russell, 2001) and subjected to double digestion with *Eco*R1 (Fermentas #ER0272) and *Hind*III (Fermentas #ER0501) using Tango Y (2X) buffer. Restricted fragments were analyzed on 1.5 % agarose gel.

For nucleotide sequencing, plasmids of the positive clones were prepared using QIAprep® Spin Miniprep Kit. The clones were sequenced in Beckman Coulter CEQ 8000 Automated Genetic Capillary Analyzer. For sequencing initially M13 forward (Fermentas # S0100) and M13 reverse (Fermentas # S0101) sequencing primers were used. Complete sequence of the SS rRNA gene was obtained by sequencing with internal universal SS rRNA primers (Elwood *et al.*, 1985), one oligonucleotide complementary to evolutionary conserved region of the coding strand of eukaryotic SS rRNA genes (SS rRNA-4F; Eukaryotic location 892→906) and the other complementary to evolutionary conserved region of the noncoding strand of eukaryotic SS rRNA genes (SS rRNA-5R, eukaryotic location 1262→1277). Moreover, forward internal primer (IntEukFor) and reverse internal primer (IntEukRev), designed using online web programs Primer3 (v. 0.4.0, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), were also used to get the complete desired sequences of the SS rRNA gene of ciliates. Nucleotide sequences of the primers used are mentioned in Table I.

The sequences of SS rRNA genes were deposited in NCBI database GenBank. The alignment of the sequences was done using CLUSTAL W (Thompson *et al.*, 1994). Homologues were selected from result of BLASTn search of GenBank. Sequence analysis was done using web available programs at NCBI and DDBJ. The sequences were compared to check the variations.

Phylogenetic analysis

After aligning the sequences, phylogenetic relationship of SS rRNA genes from these identified species were determined. Nucleotide sequences of other related *Euplotes* species were available from GenBank under accession numbers given in Table II. Since several species of *Euplotes* show identical SS rDNA sequences, not all sequenced *Euplotes* species were included in the phylogenetic analysis.

Table II.- Previously reported SS rRNA gene sequences of various closely related species of *Euplotes* taken from GenBank.

Name of organism	DB source accession No.	Author
Euplotes aediculatus	AF164136	Prescott *et al.*, 1999*
Euplotes charon	AF492705	Li and Song, 2006
Euplotes crassus	AJ310492	Bernhard *et al*., 2001
Euplotes encysticus	EF535728	Li *et al*., 2007*
Euplotes euryhalinus	EF094968	Vallesi *et al*., 2008º
Euplotes eurystomus 1	AJ310491	Bernhard *et al*., 2001
Euplotes eurystomus 2	AF452707	Chen and Song, 2001b*
Euplotes focardii	EF094961	Vallesi *et al*., 2008º
Euplotes harpa	AJ811015	Vannini *et al*., 2005
Euplotes magnicirratus	AJ549210	Vannini *et al*., 2004
Euplotes minuta	AY361908	DiGiuseppe & Dini,2007*
Euplotes muscicola	AJ310491	Petroni *et al*., 2002
Euplotes muscorum	DQ661046	Hadded & Kloetzel, 2007
Euplotes octocarinatus	AJ310489	Bernhard *et al*., 2001
Euplotes parawoodruffi	AF452708	Chen and Song, 2001b*
Euplotes parkei	AJ305247	Petroni *et al.* , 2002
Euplotes patella	EF094964	Vallesi *et al*., 2008*
Euplotes rariseta	AJ305248	Petroni *et al.*, 2002
Euplotes vannus	EF094957	Vallesi *et al*., 2008º
Euplotes woodruffi	AF452710	Chen and Song, 2002*

* Unpublished data

º Submitted to GenBank in 2006 but published in 2008

To test the relationships within the genus *Euplotes*, the two ophryoglenid species *Ichthyophthirius multifiliis* U17354 (Wright and Lynn, 1995) and *Ophryoglena cantenula* U17355 (Wright and Lynn, 1995) were chosen as outgroup species.

The phylogenetic tree was constructed using MEGA 3.1 (Kumar *et al*., 2004). Genetic distances were calculated with the DNADIST program of the PHYLIP package, ver. 3.51c (Felsenstein, 1993) based on the Kimura 2-parameter model (Kimura, 1980). To construct sequence identity matrix CLUSTALX program was used and this matrix showed percentage sequence similarity among SS rRNA gene sequences of different *Euplotes* species. The alignment was pair-wise, calculated by using an open gap penalty of 100% and unit gap penalty of

0%. Similarity matrix was calculated with a gap penalty of 0% and after discarding unknown bases. The programs FITCH (Fitch-Margoliash least squares method (Fitch and Margoliash, 1967) and NEIGHBOR (neighbor-joining method (Saitou and Nei, 1987) of this package were used to construct distance trees. Bootstrap analysis was performed using the same software package to test the statistical reliability of the topology of the neighbor-joining tree with 1000 bootstrap resamples of the data.

RESULTS

Microscopic observations

On microscopic observation RE-1 and RE-2 were tentatively identified as member of the genus *Euplotes*. This genus is characterized by rows of fused larger cilia running along the ventral (bottom) surface. The organisms used these large cilia, that is tufted together to form cirri, to position themselves for feeding and movement. The size of RE-2 is slightly larger than that of RE-1 (Fig. 1). Basic cell shape of RE-1 was ovoid (ellipsoid), while that of RE-2 was slightly elongated and ovoid and both had funnel-shaped buccal cavity. Both cultures were very interesting ciliates with a transparent body. They had a band-like macronucleus (the big backward "C"). From the side, both RE-1 and RE-2, were quite thin and can be seen using their cirri and "walking" along objects *e.g.* walking on the edge of an air bubble.

Effect of copper on growth of ciliates

Primarily the cultures of both *Euplotes* RE-1 and RE-2 were maintained in Bold basal salt medium. Growth curves, plotted between time (days) of incubation and number of cells per ml revealed gradual increase in the number of cells in the medium. Both the ciliates, *Euplotes* RE-1 and RE-2, showed good growth in Bold-basal salt medium (Fig. 2). *Euplotes* RE-1 attained maximum growth on 5th day in Bold-basal salt medium in which the cell number increased from 0.4 x 10^3 cells ml^{-1} at the time of inoculation to 32 x 10^3 cells ml^{-1} (80 fold). In contrast, *Euplotes* RE-2 attained maximum growth on 4th day. In Bold-basal salt medium the increase in cell count was 89.47 fold

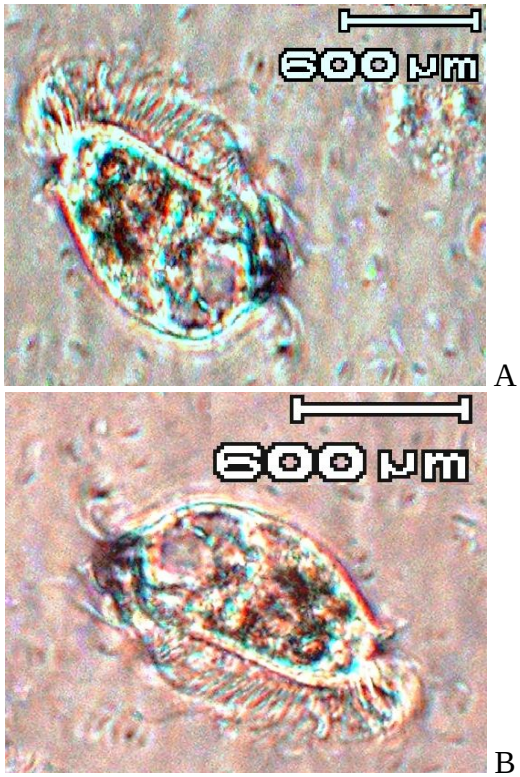

Fig. 1. Microscopic image of RE-1 (A), and RE-2 (B). On ribotyping these isolates were respectively identified as *Euplotes muscicola* and *Euplotes adiculatus*.

(from 0.38 x 10^3 cells ml^{-1} at the time of inoculation to 34 x 10^3 cells ml^{-1} on 4th day). A gradual increase in the number of cells, both in the control and treated cultures is obvious. The lag phase was prolonged in both the ciliate cultures in the copper containing medium (Fig. 2). In *Euplotes* RE-1 the maximum growth was attained on 7th day and in RE-2 the maximum growth was achieved on 5th day in the copper treated cultures. The decrease in the cell counts of *Euplotes* RE-1 and RE-2 in copper containing medium was 2.4 and 2.15 fold, respectively. The reduction in cell population of *Euplotes* RE-1 was 83.91%, while that of RE-2 was 82.84% after eight days of incubation in copper containing media.

Ribotyping

PCR amplification of the small subunit ribosomal (18S) RNA gene from genomic DNA (Fig. 3B) yielded a fragment of approximately 1800

bp in length (Fig. 3A). These SS rDNA were cloned in pTZ57R/T cloning vector and *E. coli* DH5α cells were transformed with this recombinant DNA. Positively screened clones were confirmed by double restriction using *Eco*RI and *Hind*III restriction enzymes (Fig. 4).

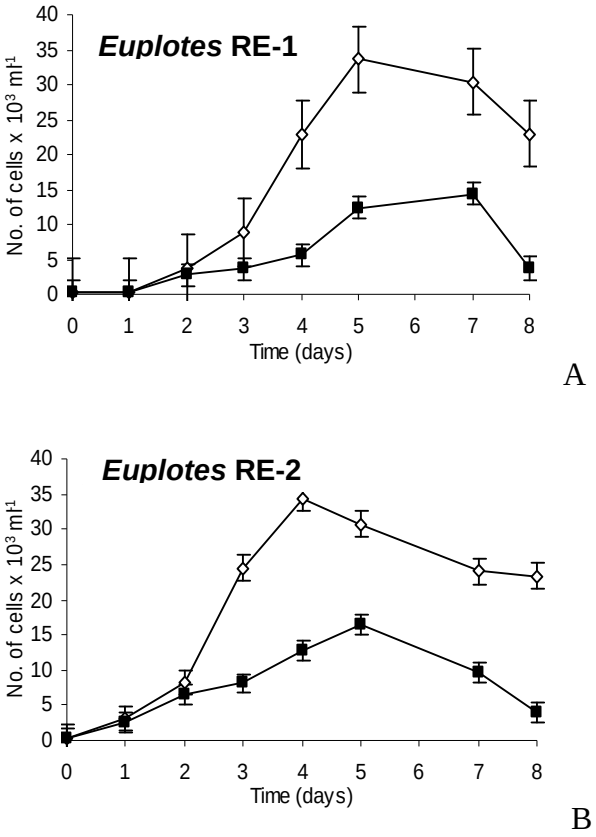

Fig. 2. Effect of copper on the growth of ciliates RE-1 (A), and RE-2 (B) in Bold basal salt medium at pH 7.5 and 28 ± 2°C (◊, Control; ■, Cu^{2+} treated).

SS rRNA genes of ciliates RE-1 and RE-2 were 1818 bp and 1875 bp long and were deposited in GenBank under Accession numbers DQ917684 and EU103618, respectively. On the basis of BLASTn search results of 1818 bp SS rDNA sequence of RE-1 and 1875 bp sequence of RE-2 proved them to be *Euplotes muscicola* and *Euplotes adiculatus,* respectively. These search results also confirmed the initial microscopic identification results.

Phylogenetic analysis

The alignment of nucleotide sequences of closely related *Euplotes* spp. selected from BLASTn search results was done using CLUSTALW. All the sequences were compared to check the species variations (Tables I and II of supplementary data).

Nucleotide differences between *Euplotes* spp., after the alignment, are shown in Tables V and VI. In the nucleotide sequence of *E. muscicola* SBSrc, the one belonging to the *muscicola* group* of *Euplotes* sp., fewer variations (general and specific mutations) were observed in the regions 323-516 and 906-1303 when compared with other species of the group (Table I of supplementary data). More base pair differences (general mutations) were observed in the other regions. Most of the points of differences between the SS rRNA sequences of the *E. muscicola* SBSrc and other members of the group are clustered within two regions: ~ 506-575 and ~ 1464-1457 (using numbers from *E. muscicola* SBSrc sequence). Seventeen specific mutations were observed, the common ones being T → G/A transversions and T → C transitions. Specific deletion of nucleotides A at position 549, T at position 1487, C at positions 559 and 566 and G at position 1386 was also observed in *E. muscicola* SBSrc. There was a deletion of T at position 124 in both *E. muscicola* and *E. muscicola* SBSrc. Moreover, A → G transition at position 876 and C → A transversion at position 1391 was also detected which are also the variations of both *E. muscicola* and *E. muscicola* SBSrc with other species of the *muscicola* group.

Table II (supplementary data) shows the base pair differences (specific and general mutations) in the nucleotide sequences of SS rDNA of *Euplotes adiculatus* and *E. adiculatus lahorensis* compared with other species of *adiculatus* group*. There

*Based on phylogenetic analysis, the various species of *Euplotes* have been divided into two groups *viz. muscicola* and *adiculatus*. The former group includes *E. muscicola, E. muscicola* SBSrc, *E. charon, E. magnicirratus, E. eurihalinus, E. muscorum, E. encysticus, E. rariseta, E. parkei, E. focardii, E. crassus, E. vannus* and *E. minuta*. The later group includes species *E. adiculatus, E. adiculatus lahorensis, E. parawoodruffi, E. woodruffi, E. eurystomus* 1, *E. eurystomus* 2, *E. harpa, E. patella* and *E. octocarinatus*.

*Supplementary data available on request by corresponding author.

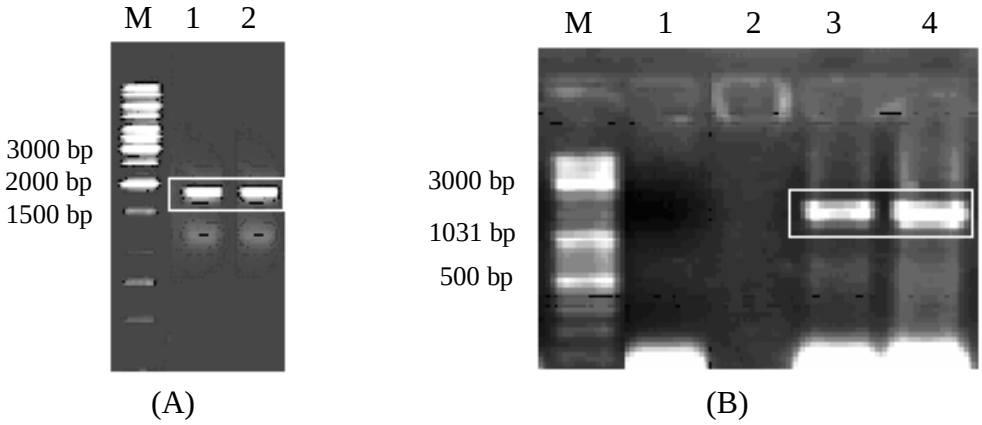

Fig. 3. PCR amplification of SS rRNA gene of copper resistant ciliates (A) using whole ciliate cell (lane 1, RE-1 and lane 2, RE-2) and (B) using genomic DNA as template (lane 1, negative control; lane 2, RE-1; and lane 3, RE-2). M represents marker lane. Ciliate small-subunit ribosomal RNAs encoded by the nucleus vary in length from 1762 nucleotides in RT-1 to 1818 and 1875 bp in RE-1 and RE-2, respectively.

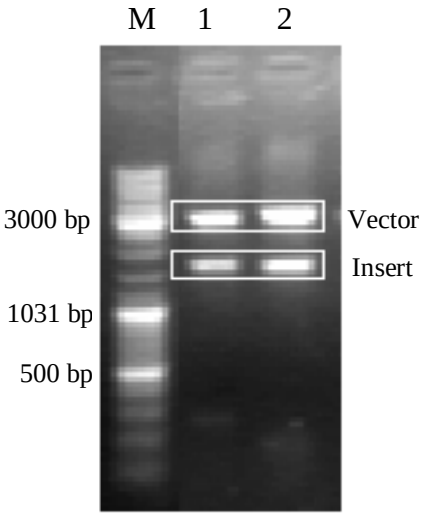

Fig. 4. Restriction of pTZ57R/T containing SS rRNA gene of copper resistant ciliates with *Eco*RI and *Hind*III. M represents marker lane. Other lanes are as follows: 1, RE-1 (1818 bp); and 2, RE-2 (1875 bp). The upper band represents the restricted vector (2.886 kb), whereas the lower band represents insert.

were observed four general variations in the region 1027-1428; A → G and G → A being the only transitions and A → T, the only transvertion observed. Ten specific mutations were observed when the nucleotide sequence of *E. adiculatus lahorensis* was compared with the nucleotide sequences of *E. adiculatus* and other species of the *adiculatus* group. Out of these specific variations three were G → A and C → T transitions while seven were the specific transversions *viz*., A → T, C → A, G → C, G → T, T → A and T → G. No specific deletions and insertions were observed. General mutations are more frequent among the species in both groups of *Euplotes* as more than 160 general variations were observed among the species of *muscicola* group while around 100 general base pair differences were detected in *adiculatus* group.

Comparisons of the two groups of *Euplotes* sp. (*Euplotes muscicola* group and *Euplotes adiculatus* group) SS rRNA sequences discovered that universal or ciliate-specific sequences (regions that are conserved among among all *Euplotes* sp.) were interspersed among semi-conserved sequences (regions of intermediate conservation) and non-conserved sequences (regions that display very high rates of genetic drift). The semi-conserved sequences are of use for the construction of quantitative molecular phylogenies concerning distantly related organisms, whereas the non conserved regions are important for resolving close phylogenetic relationships. Because of not having sequence variation, the highly conserved regions do not give information about sequence divergence; however, they are potentially helpful for rapidly sequencing SS rRNA genes.

DISCUSSION

Heavy metal pollution corresponds to an important environmental problem due to the poisonous and noxious effect of metals; furthermore, their accumulation throughout the food chain leads to serious ecological and health problems. The heavy metals are bio-accumulated in the body of microorganisms and other aquatic plants and animals. The organisms utilize a range of strategies to reduce heavy metal toxicity depending on the nature of heavy metal and the organism under stress. An ample variety of microorganisms such as bacteria, fungi, algae, yeast and protozoa are originated in waters receiving industrial effluents. Their presence indicates their ability to resist the stressful conditions. Among protozoa, ciliates are fine candidates for use as whole cell biosensors to sense the presence and to find out the bio-available concentration of heavy metal ions in the natural samples (Martin-Gonzalez *et al.*, 2006).

Microscopic observation

Microscopic observation revealed the copper resistant ciliates to be the members of genera *Euplotes*. When these ciliates were exposed to sub-lethal concentration of copper, dense granules appeared in the cytoplasm. Martin-Gonzalez *et al.* (2006) also reported that when ciliated protozoa were exposed to sub-lethal concentrations of Cd or Zn, the most general and noticeable obvious change was the appearance of very electron-dense granules in the cytoplasm. They reported that initially, these granules or inclusions seemed to form inside electroluscent small vacuoles, but afterward the electron-dense material extended more or less uniformly/ consistently and the original vacuole disappeared. It is expected that these cytoplasmic granules correspond to complexes formed by both metallic cations (Cu^{2+}, Cd^{2+} and Zn^{2+}) and metallothioneins. At this stage, heavy metals are not bioavailable and thus they are not toxic to cells.

Growth media used

Information about the nutrition of ciliates is imperative to study them (Holz, 1964). In general Protozoa culture grows in salt medium supplemented with organic compounds (Weekers and Vogels, 1994). Both the ciliates *Euplotes* RE-1 and RE-2 showed good growth on Bold-basal salt medium which supports the fact that ciliates possess elaborate and complex mechanisms to manufacture their bio-molecules by using salts present in the environment or by utilizing scant organic molecules released in the culture medium by other organisms (Rehman *et al*., 2005).

Tolerance of ciliates to copper

Metal tolerance is defined as the maximum metal concentration at which organisms survived and multiplied. It is very intricate to establish comparisons of the toxic and deadly effects of heavy metals among the reported studies using ciliates, because of the diversity of the experimental conditions used (Nilsson, 1989; Martin-Gonzalez *et al*., 1999; Gutierrez *et al*., 2003). This study reports the growth and survival of two ciliates *Euplotes* RE-1 and RE-2 on Bold-basal salt medium in the presence of copper, which has hampered the growth of ciliates. The common trend was decreased cell growth with increasing copper concentration in the medium. Growth period was delayed when copper concentration was increased. In the literature it was reported previously (Sudo and Aiba, 1973; Brady *et al*., 1994) that a concentration of 25-27 ppm copper reduced the growth of *V. microstoma* and *Opercularia* sp. from activated sludge by 50%. Salvado *et al.* (2001) reported that the ciliate *Euplotes affinis* did not apparently suffer great losses at 24 hours, in the presence of 5-10 mg/l copper and in due course it increased its population, which is considered as a regular succession of species. Thus ciliates are proficient of surviving when metal concentrations higher than those normally found in water and environment are present, as a consequence of their adaptability and stability of their metabolic processes in the stress conditions. Brady *et al*. (1994) stated that repetitions growth in the medium containing same copper concentration resulted in improved growth, arguing for a mechanism of tolerance by adaptation. A strain of *T. pyriformis* tested by Nicolau *et al.* (2001) was more sensitive to Zn than to Cu, in contrast with earlier reports using the same species (Yamaguchi *et al*., 1973; Piccinni *et al*., 1987; Nilsson, 2003).

Table III.- Sequence Identity Matrix used to reconstruct phylogenetic relationships among different species of *Euplotes muscicola* group. Last three numerals of the GenBank accession numbers are given in the table to represent the organism's identity. The accession number and the homology scores of the organism identified in this study were written in bold.

Seq.	AF705	AJ210	EF968	DQ046	EF728	**DQ684**	AJ254	AJ248	AJ247	EF496	AJ492	EF957	AY908
AF705	1.000	0.977	0.950	0.939	0.901	**0.916**	0.929	0.928	0.909	0.911	0.909	0.897	0.911
AJ210	-	1.000	0.972	0.921	0.922	**0.937**	0.950	0.949	0.930	0.931	0.892	0.918	0.931
EF968	-	-	1.000	0.928	0.930	**0.943**	0.959	0.958	0.939	0.937	0.898	0.922	0.933
DQ046	-	-	-	1.000	0.925	**0.941**	0.937	0.924	0.910	0.909	0.906	0.894	0.905
EF728	-	-	-	-	1.000	**0.944**	0.938	0.926	0.907	0.906	0.865	0.890	0.903
DQ684	-	-	-	-	-	**1.000**	**0.963**	**0.942**	**0.926**	**0.923**	**0.882**	**0.906**	**0.921**
AJ254	-	-	-	-	-	-	1.000	0.949	0.939	0.937	0.892	0.916	0.929
AJ248	-	-	-	-	-	-	-	1.000	0.954	0.952	0.906	0.933	0.946
AJ247	-	-	-	-	-	-		-	1.000	0.893	0.921	0.947	0.958
EF496	-	-	-	-	-	-			-	1.000	0.915	0.944	0.953
AJ492	-	-	-	-	-	-			-	-	1.000	0.9569	0.937
EF957	-	-	-	-	-	-			-	-	-	1.000	0.967
AY908	-	-	-	-	-	-			-	-	-	-	1.000

Table IV.- Sequence Identity Matrix used to reconstruct phylogenetic relationships among different species of *Euplotes adiculatus* group. Last three numerals of the GenBank accession numbers are given in the table to represent the organism's identity. The accession number and the homology scores of the organism identified in this study were written in bold.

Sequences	AF708	AF710	AF136	AF707	**EU618**	AJ491	AJ015	EF964	AJ489
AF708	1.000	0.997	0.968	0.971	**0.967**	0.965	0.901	0.923	0.948
AF710	---	1.000	0.968	0.970	**0.966**	0.964	0.901	0.923	0.948
AF136	---	---	1.000	0.990	**0.987**	0.963	0.899	0.917	0.943
AF707	---	---	---	1.000	**0.990**	0.965	0.901	0.919	0.945
EU618	---	---	---	---	**1.000**	**0.962**	**0.898**	**0.917**	**0.942**
AJ491	---	---	---	---	---	1.000	0.905	0.928	0.954
AJ015	---	---	---	---	---	---	1.000	0.939	0.924
EF964	---	---	---	---	---	---	---	1.000	0.970
AJ489	---	---	---	---	---	---	---	---	1.000

Molecular identification of copper resistant ciliates

Kingdom Protista is subdivided into five Phyla, Phylum Ciliophora (ciliates) being one of them (Fig. 5). Ciliates are evolutionarily grouped with the entirely parasitic Apicomplexa and with the Dinoflagellates to form the Alveolates, indicating that studying them is likely to elucidate novel properties of these organisms with considerable medical and ecological impact. They are one of the better-characterized groups within the protozoa, yet they are remarkably diverse. Smith and Chao (2003) attempted to present monophyletic groups based on biochemistry, genetics and ultrastructure in newer classifications. The protists all together are paraphyletic that is why such systems often abandon or split up the kingdom and in its place treating the protists groups as separate lines of eukaryotes. Metal resistant ciliates, isolated from industrial waste water, were identified as *Euplotes muscicola* and *Euplotes adiculatus*, on the basis of microscopic observation as well as SS rDNA analysis. *Euplotes* belongs to the class Spirotrichiae; order Euplotida. The SSrDNA were compared and phylogenetic analysis was done with other species of the genus.

Phylogenetic analysis of Euplotes spp.

Euplotes, due to its abundance, ubiquitous distribution and the ease of culturing, have been extensively studied. In the past, Tuffrau (1960), Curds (1975), Gates and Curds (1979); and Borror and Hill (1995) considered a number of characteristics to determine species in the genus

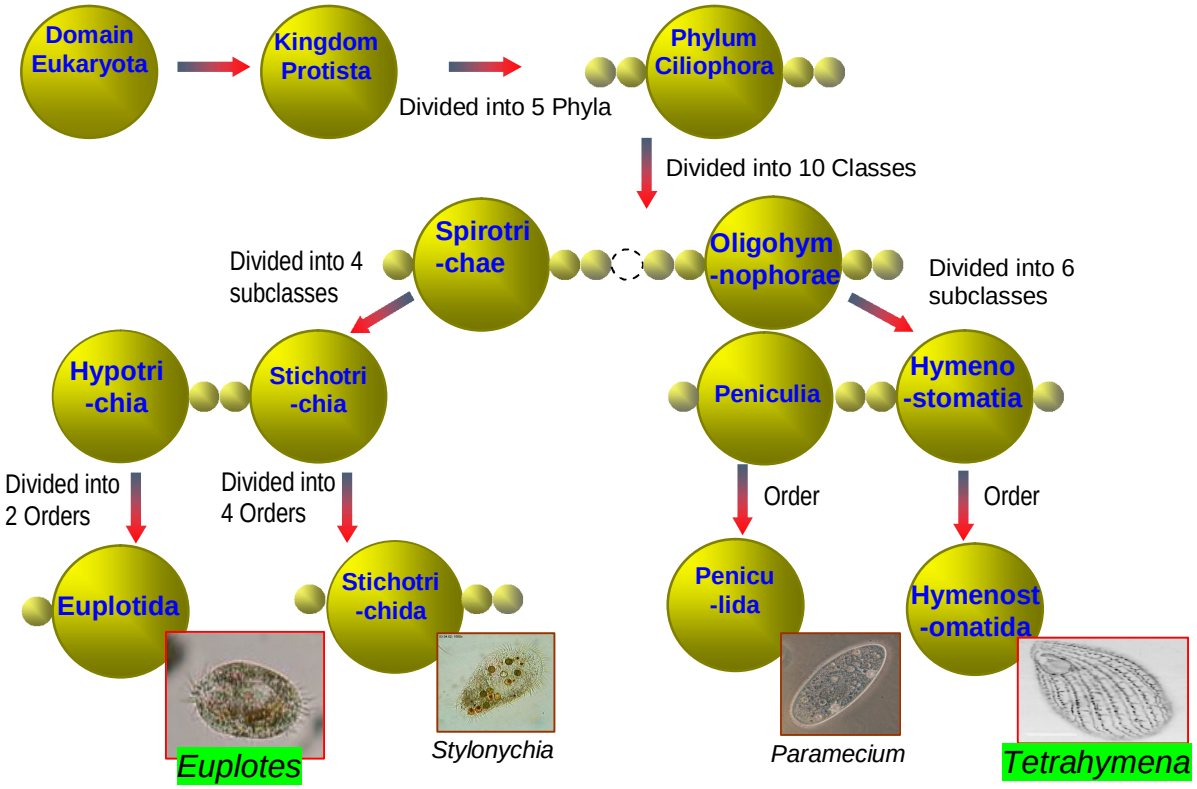

Fig. 5. Schematic diagram representing classification of ciliates up to Order level. The main ranks of classification of the representative organisms used in this study (highlighted ones) have been labeled. The number of yellow circles represent the number of Phyla / Classes / Subclasses / Orders. Classification of the organisms of closely resembling Orders was also shown.

Euplotes. Afterwards random amplified polymorphic DNA (RAPD) fingerprinting was used to separate species in *Euplotes* (Tan *et al.*, 1994; Chen *et al.*, 2000). Petroni *et al.* (2002) used molecular analysis to reveal taxonomic and phylogenetic relationships within 11 species (representing different dargyrome types and habitats) of this genus.

Based on phylogenetic analysis (Petroni *et al.*, 2003) the various species of *Euplotes* have been divided into two groups *viz.*, *muscicola* and *adiculatus*. The former group includes *E. muscicola, E. charon, E. magnicirratus, E. eurihalinus, E. muscorum, E. encysticus, E. rariseta, E. parkei, E. focardii, E. crassus, E. vannus* and *E. minuta*. The later group includes species *E. adiculatus, E. parawoodruffi, E. woodruffi, E. eurystomus, E. harpa, E. patella* and *E. octocarinatus*. Phylogenetic analysis indicated that one copper resistant locally isolated *Euplotes* belonged to the *muscicola* group, while the other belonged to the *adiculatus* group (Fig. 6). Moreover sequence identity matrices (Tables III and IV) revealed that the one belonging to the *muscicola* group was most closely related to *Euplotes muscicola,* while the other belonging to the *adiculatus* group was most closely related to *Euplotes adiculatus* with which they showed the fewest differences in their SS rDNA sequences.

On the basis of sequence homology (as indicated by phylogenetic tree) the local isolate *E. muscicola lahorensis* belonged to *Euplotes muscicola* with which its percentage homology was 99%. *E. muscicola lahorensis* showed 93% sequence similarity with both *E. muscorum* and *E. encysticus*, the clade of three being rooted at *E. muscicola*. On the basis of the results of this study *E. muscicola lahorensis* can be suggested to be a new subspecies of *E. muscicola* and named *Euplotes*

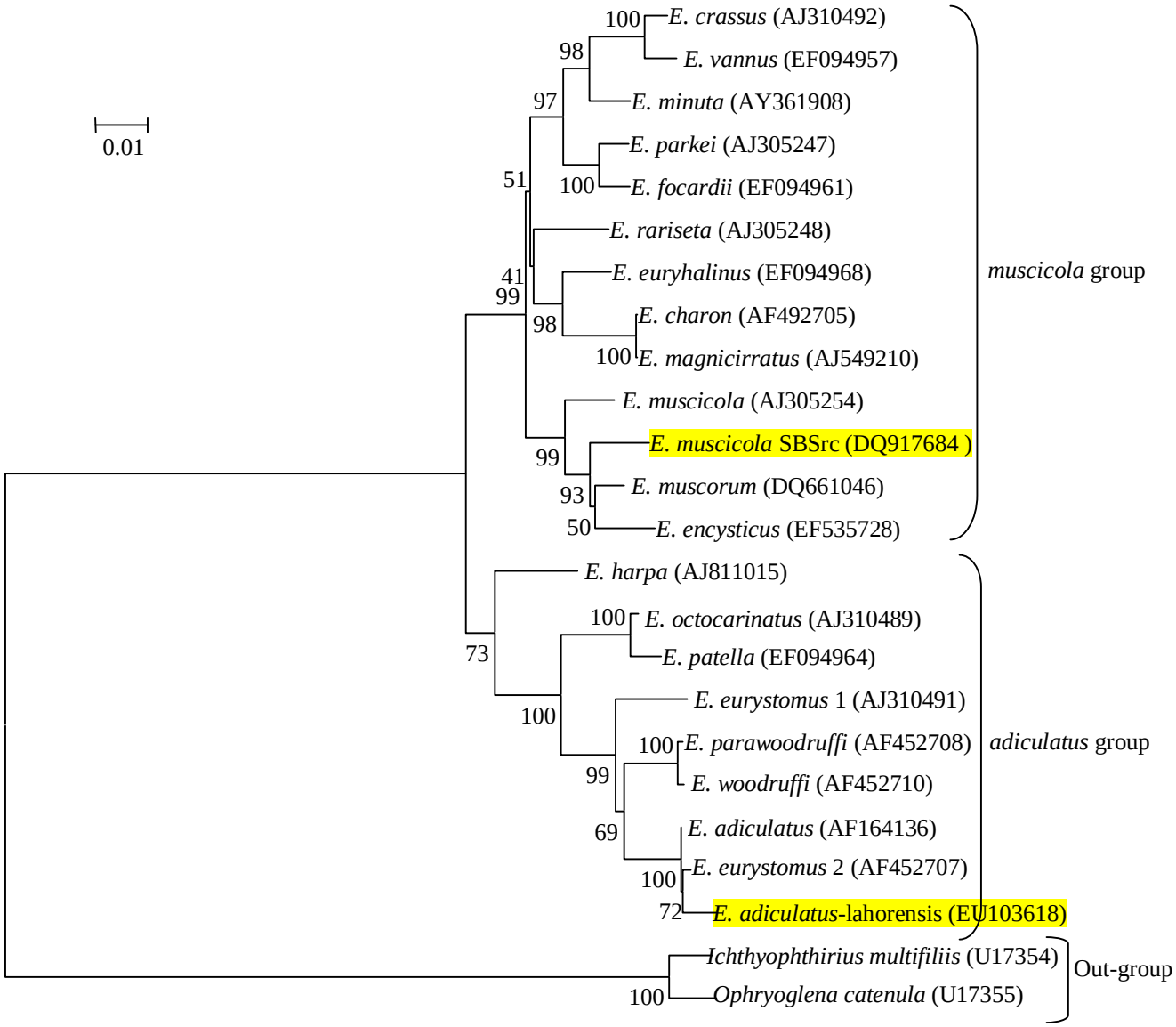

Fig. 6. A distance tree for *Euplotes* spp. inferred from SS rRNA gene sequences. The tree was derived from evolutionary distances produced by the Kimura-2- parameter correction model (Kimura, 1980). The numbers at the nodes represent the bootstrap percentages of 1,000 for the least squares method (Fitch and Margoliash, 1967) followed by the bootstrap values for the neighbor joining method of Saitou and Nei (1987). Evolutionary distance is represented by the branch length separating the species. The scale bar corresponds to 5 substitutions per 100 nucleotide positions.

muscicola lahorensis subsp. nov.

Likewise, the locally isolated *E. adiculatus lahorensis* shared high homology (≥ 99.9%) with *Euplotes adiculatus*. *E. eurystomus* 2 (AF452707; Chen and Song, 2001) also shared the same position in the tree but the SS rDNA sequence of this ciliate is based on unpublished data and is different from that of the type strain *E. eurystomus* 1 (AJ310491; Bernhard *et al.*, 2001). Phylogenetic analyses of RAPD patterns pointed to a separation of *E. adiculatus* strains into subgroups within one species, but all strains were genetically more analogous and alike to one another than to strains from two other *Euplotes* species. Crossings of the different *E. adiculatus* strains revealed them to belong to seven mating types of one gene pool

(Petroni *et al*., 2003). The frequent occurrences of conjugation in the studied populations explain the high genetic diversity observed in the genus *Euplotes*.

CONCLUSIONS

Three ciliates isolated from industrial effluent were identified (by amplifying SS rRNA gene) as *Euplotes muscicola* and *Euplotes adiculatus*. On the basis of the results of this study new subspecies *Euplotes muscicola lahorensis* subsp. nov. is being reported.

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Table I.- Supplementary Data: Variation (specific and general mutations) in the nucleotide sequence of SS rDNA of *Euplotes muscicola* and *Euplotes muscicola* SBSrc compared with other species of *muscicola* group.

Sequence Position 5′ → 3′

Species	*20*	*21*	*22*	*25*	*27*	*28*	*30*	*31*	*35*	*36*	*37*	*38*	*40*	*52*	*53*	*55*	*56*	*59*	*101*	*104*	*105*	*109*	*111*	*112*
E. muscicola	A	A	G	T	A	G	C	A	A	T	G	T	T	T	T	C	A	C	T	A	T	C	G	A
E. muscicola SBSrc	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	A	.
E. charon	.	.	.	.	.	.	.	.	.	.	.	.	.		_	.	.	.	.	.	.	.	.	.
E. magnicirratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	_	.	.	.	.	.	.	.	.	.
E. eurihalinus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E. muscorum	.	G	.	.	.	.	.	.	.	.	.	.	.	_	_	T	T	T	.	.	.	.	.	R
E. encysticus	T	C	T	G	_	_	G	G	C	A	C	C	G	.	.	.	.	.	.	.	.	.	.	.
E. rariseta	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E. parkei	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	_	A	.	.
E. focardii	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	_	A	.	.
E. crassus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	_	T	C	.
E. vannus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	_	A	.	.
E. minuta	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	_	A	.	.

Sequence Position 5′ → 3′

Species	*113*	*114*	*114*	*115*	*116*	*117*	*118*	*119*	*120*	*121*	*122*	*124*	*125*	*129*	*155*	*174*	*177*	*183*	*187*	*191*
E. muscicola	C	T	_	A	T	A	T	T	C	G	T	_	A	T	A	A	G	A	A	T
E. muscicola SBSrc	.	.	T	.	.	.	.	.	.	A	.	_	.	.	.	.	.	.	.	.
E. charon	.	.	T	.	.	.	.	.	.	A	.	T	.	.	.	G	.	T	.	.
E. magnicirratus	.	.	T	.	.	.	.	.	.	A	.	T	.	.	.	G	.	T	.	.
E. eurihalinus	.	.	T	.	.	.	.	.	.	T	.	T	.	.	.	G	.	.	.	.
E. muscorum	.	.	T	.	.	_	_	.	.	W	.	T	.	_	.	.	A	.	R	Y
E. encysticus	.	.	T	.	.	.	.	.	.	A	.	T	.	.	.	.	.	.	.	.
E. rariseta	.	.	T	.	.	.	.	.	T	A	.	T	.	.	.	G	.	.	.	.
E. parkei	_	_	.	_	_	_	_	_	_	_	C	T	T	.	G	.	.	.	.	.
E. focardii	_	_	.	_	_	_	_	_	_	_	C	T	T	.	G	.	.	.	.	.
E. crassus	_	_	.	_	_	_	_	_	_	_	C	T	.	_	G	G	.	.	.	C
E. vannus	A	A	.	_	_	_	_	_	_	A	C	T	T	_	G	G	.	T	.	C
E. minuta	_	_	_	_	_	_	_	_	_	_	C	T	T	.	G	.	.	T	.	.

Continued…

Sequence Position 5′ → 3′

Species	*195*	*196*	*207*	*208*	*209*	*210*	*211*	*212*	*219*	*220*	*227*	*238*	*238*	*249*	*257*	*262*	*268*	*271*	*272*	*275*
E. muscicola	T	A	T	T	T	T	C	A	A	T	C	G		G	G	A	T	G	T	A
E. muscicola SBSrc	.	.	C	.	C	C	_	_	.	.	.	.	.	.	.	.	.	.	.	.
E. charon	.	.	.	A	.	.	_	_	G	C	.	.	.	.	.	.	C	.	.	G
E. magnicirratus	.	.	.	A	.	.	_	_	G	C	.	.	.	.	.	.	C	.	A	G
E. eurihalinus	.	.	.	.	A	.	_	_	G	C	.	.	.	.	.	.	.	.	.	.
E. muscorum	Y	R	.	.	C	C	_	_	.	.	.	.	.	.	.	.	.	.	.	.
E. encysticus	.	.	.	.	C	C	_	_	.	.	.	.	.	.	.	.	.	.	.	.
E. rariseta	.	.	.	.	C	A	_	_	.	.	.	.	.	.	.	.	.	.	.	.
E. parkei	.	.	.	.	C	_	_	_	.	.	.	.	.	.	T	G	.	_	.	T
E. focardii	.	.	.	.	C	_	_	_	.	.	.	.	.	.	T	G	.	.	.	.
E. crassus	.	.	.	_	C	_	_	_	.	.	_	T	G	_	.	.	.	_	.	.
E. vannus	.	.	.	_	C	_	_	_	.	.	.	.	.	.	.	.	.	_	.	.
E. minuta	.	.	.	.	C	_	_	_	.	.	.	.	.	.	.	.	C	_	.	.

Sequence Position 5′ → 3′

Species	*276*	*277*	*278*	*284*	*286*	*287*	*315*	*322*	*448*	*450*	*466*	*468*	*494*	*506*	*518*	*520*	*521*	*522*	*523*	*524*
E. muscicola	C	T	A	A	A	T	T	C	C	C	G	A	A	T	C	C	T	A	T	A
E. muscicola SBSrc	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	T	_	T	A	T
E. charon	T	C	G	.	.	.	C	A	T	T	.	.	.	.	.	.	A	.	.	T
E. magnicirratus	T	C	G	.	.	.	C	A	T	T	.	.	.	.	.	.	A	.	.	T
E. eurihalinus	.	C		.	.	.	.	A	.	.	.	.	.	.	.	.	A	.	.	T
E. muscorum	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T	G	T	A	T
E. encysticus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	_	T	A	T
E. rariseta	.	C	G	.	.	.	.	A	T	.	.	T	.	.	.	T	_	T	A	T
E. parkei	.	C	G	G	.	C	.	A	T	.	.	.	G	.	.	T	_	T	_	_
E. focardii	.	C	G	G	.	C	.	A	T	.	.	.	G	.	.	T	_	T	_	_
E. crassus	.	C	G	.	G	.	.	A	T	.	C	.	G	.	.	T	_	T	A	T
E. vannus	.	C	G	.	G	.	.	A	T	.	.	.	G	.	.	T	_	T	A	T
E. minuta	.	C	G	.	G	.	.	A	T	.	.	.	G	.	.	T	_	T	A	T

Continued…

Sequence Position 5′ → 3′

Species	*525*	*537*	*540*	*549*	*554*	*559*	*566*	*575*	*582*	*639*	*642*	*647*	*648*	*649*	*652*	*653*	*656*	*657*	*658*	*659*
E. muscicola	T	A	T	A	G	C	C	T	C	A	T	A	C	A	A	G	C	G	G	T
E. muscicola SBSrc	.	.	G	_	.	_	_	A	.	.	.	.	.	.	.	.	.	.	.	.
E. charon	.	G	.	.	N	.	.	.	T	.	.	.	.	.	_	.	T	.	.	C
E. magnicirratus	.	G	.	.	.	.	.	.	.	.	.	.	.	.	_	.	T	.	.	C
E. eurihalinus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.
E. muscorum	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.
E. encysticus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E. rariseta	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	T	.	.	.
E. parkei	C	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	A	.	C
E. focardii	C	.	.	.	.	.	.	.	.	.	T	.		.	.	.	.	A	.	C
E. crassus	.	.	.	.	.	.	.	.	.	.	T	.	T	C	.	.	T	A	.	C
E. vannus	.	.	.	.	.	.	.	.	.	.	T	.	T	C	.	.	T	A	.	C
E. minuta	.	.	.	.	.	.	.	.	.	G	T	.	T	T	.	A	T	.	.	C

Sequence Position 5′ → 3′

Species	*662*	*665*	*666*	*667*	*668*	*670*	*672*	*673*	*674*	*675*	*677*	*678*	*679*	*690*	*700*	*704*	*708*	*713*	*714*	*715*
E. muscicola	T	C	C	G	C	G	C	A	G	T	T	C	T	A	G	T	A	C	A	T
*E. muscicola*SBSrc	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.
E. charon	C	T	.	.	.	_	.	.	.	.	G	.	C	.	.	C	G	T	G	.
E. magnicirratus	C	T	.	.	.	_	.	.	.	.	G	.	C	.	.	C	G	T	G	.
E. eurihalinus	.	T	T	.	.	.	.	.	.	.	G	T	.	.	.	.	G	.	.	.
E. muscorum	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E. encysticus	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.
E. rariseta	.	T	.	A	.	.	.	.	.	C	.	T	.	.	C	C	G	.	.	.
E. parkei	.	T	T	.	T	.	.	.	.	.	.	.	.	G	.	.	G	.	.	C
E. focardii	.	T	T	.	T	.	.	.	.	.	.	.	.	G	.	.	G	T	.	.
E. crassus	.	T	T	A	.	.	T	G	A	A	.	.	.	.	.	.	G	.	G	.
E. vannus	.	T	T	A	.	.	T	G	A	A	.	.	.	.	.	.	G	.	G	.
E. minuta	.	T	.	A	.	.	.	G	A	A	.	.	.	.	.	.	G	T	G	.

Continued…

Sequence Position 5′ → 3′

Species	*726*	*727*	*731*	*736*	*737*	*738*	*739*	*740*	*741*	*742*	*743*	*744*	*745*	*746*	*747*	*748*	*749*	*750*	*751*	*752*
E. muscicola	C	G	T	T	A	C	T	T	T	A	C	C	C	T	T	T	T	T	T	C
E. muscicola SBSrc	.	.	C	.	.	T	.	.	.	.	.	.	.	.	.	.	C	.	.	.
E. charon	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	C	_	A	A
E. magnicirratus	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	C	_	.	A
E. eurihalinus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	_	.	A
E. muscorum	.	.	.	C	_	T	.	.	.	.	.	.	.	.	.	.	C	A	.	A
E. encysticus	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	C	_	.	A
E. rariseta	G	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	_	.	A
E. parkei	.	.	.	.	.	.	.	C	.	T	T	T	.	.	.	.	C	C	.	.
E. focardii	.	.	.	.	.	.	.	.	.	T	T	T	.	C	.	.	.	C	.	T
E. crassus	.	.	.	C	.	T	A	.	A	C	T	T	T	.	A	C	C	C	.	T
E. vannus	.	.	.	C	.	T	A	.	A	C	T	T	T	A	.	.	.	.	.	T
E. minuta	.	A	.	.	.	T	.	.	.	.	.	.	.	.	.	.	C	.	A	T

Sequence Position 5′ → 3′

Species	*753*	*754*	*755*	*756*	*758*	*759*	*760*	*762*	*766*	*767*	*768*	*768*	*769*	*770*	*771*	*772*	*772*	*773*	*774*	*775*
E. muscicola	T	A	T	A	A	T	A	T	T	T	A	_	G	A	T	C	_	T	T	T
*E. muscicola*SBSrc	.	.	.	.	T	C	.	.	.	A	.	.	C	.		A	.	.	.	.
E. charon	.	G	.	.	_	.	.	.	.	.	.	.	T	T	C	T	.	C	.	.
E. magnicirratus	.	G	.	.	_	.	.	.	.	.	.	.	T	T	C	T	.	C	.	.
E. eurihalinus	.	.	.	.	T	C	.	.	.	C	.	.	T	C	.	T	.	C	.	.
E. muscorum	.	.	.	.	T	C	.	.	.	A	.	.	C	T	.	T	.	.	.	.
E. encysticus	.	.	.	.	T	C	.	.	.	A	T	.	C	T	.	.	.	.	.	.
E. rariseta	.	.	.	.	T	C	.	.	.	.	.	T	C	T	C	T	.	C	.	.
E. parkei	.	.	.	.	T	C	.	.	A	.	.	T	C	.	.	T	.	.	.	.
E. focardii	.	.	.	.	T	C	.	.	.	.	.	T	C	T	.	T	A	.	.	.
E. crassus	_	_	_	_	T	.	C	A	.	.	.	T	T	G	.	_	.	_	_	_
E. vannus	A	C	C	C	T	.	C	A	.	.	.	T	T	T	.	_	.	_	_	_
E. minuta	A	T	A	_	T	C	.	.	.	.	.	T	C	T	.	C	.	_	.	.

Continued…

Sequence Position 5′ → 3′

Species	*780*	*818*	*838*	*839*	*844*	*848*	*849*	*850*	*851*	*858*	*866*	*867*	*867*	*869*	*871*	*876*	*881*	*896*	*897*	*901*
E. muscicola	C	A	C	A	T	T	A	T	T	T	A	T	_	A	T	A	G	A	A	T
E. muscicola SBSrc	.	.	.	.	.	A	_	_	.	.	.	.	G	.	G	.	.	.	.	.
E. charon	.	C	.	.	C	.	.	.	C	.	T	_	G	G	G	G	.	.	.	.
E. magnicirratus	.	C	.	.	C	.	.	.	C	.	T	_	G	G	G	G	.	.	.	.
E. eurihalinus	.	C	.	.	C	A	.	.	C	.	T	_	G	G	G	G	.	.	.	.
E. muscorum	.	.	.	.	C	A	_	_	.	.	T	C	G	.	G	G	.	.	.	.
E. encysticus	.	.	.	.	C	.	_	_	.	.	T	C	G	.	G	G	.	.	.	.
E. rariseta	.	C	.	T	C	.	.	.	.	.	C	.	.	.	G	G	.	.	.	.
E. parkei	.	A	T	T	C	.	.	.	.	.	C	.	.	.	G	G	.	.	.	.
E. focardii	.	A	T	T	C	.	.	.	.	.	C	.	.	.	G	G	A	.	.	.
E. crassus	T	T	T	T	C	.	.	.	.	_	_	C	.	.	G	G	.	G	T	.
E. vannus	T	T	T	T	C	.	.	.	.	_	T	C	.	.	G	G	.	G	T	.
E. minuta	T	T	T	T	C	.	.	.	.	.	T	C	.	.	G	G	.	G	T	-

Sequence Position 5′ → 3′

Species	*904*	*905*	*965*	*983*	*1075*	*1104*	*1105*	*1132*	*1136*	*1162*	*1203*	*1304*	*1306*	*1364*	*1386*	*1391*	*1406*
E. muscicola	C	A	T	_	G	C	C	C	T	A	G	A	T	T	G	C	_
E. muscicola SBSrc	_	_	.	.	A	.	.	A	_	.	.	.	.	.	_	.	T
E. charon	_	_	.	.	A	.	.	.	.	.	A	.	.	_	.	A	.
E. magnicirratus	_	_	.	.	A	.	.	.	.	.	A	.	.	_	.	A	.
E. eurihalinus	_	_	.	.	A	.	.	.	.	.	A	.	.	_	.	A	.
E. muscorum	_	_	.	.	A	.	.	A	.	R	.	_	A	_	.	.	.
E. encysticus	_	_	.	.	A	.	.	A	_	.	.	.	.	_	.	.	.
E. rariseta	_	_	C	.	A	.	.	.	.	.	A	.	.	_	.	A	.
E. parkei	_	_	.	.	A	.	.	.	.	.	A	.	.	_	.	A	.
E. focardii	_	_	.	.	A	.	.	.	.	.	A	.	.	_	.	A	.
E. crassus	_	_	.	A	A	_	_	.	.	.	A	.	.	_	.	A	.
E. vannus	_	_	.	T	A	.	.	.	.	.	A	.	.	_	.	A	.
E. minuta	-	-	.	.	A	.	.	.	.	.	A	.	.	_	.	A	.

Continued…

Sequence Position 5′ → 3′

Species	*1407*	*1408*	*1409*	*1410*	*1411*	*1413*	*1414*	*1415*	*1421*	*1424*	*1457*	*1459*	*1461*	*1463*	*1464*	*1465*
E. muscicola	C	C	A	T	T	A	C	C	T	C	A	G	A	G	T	_
E. muscicola SBSrc	.	.	.	.	.	.	.	_	.	.	C	A	G	A	G	T
E. charon	_	_	_	.	C	T	T	_	.	.	.	.	.	.	.	.
E. magnicirratus	_	_	_	.	C	T	T	_	.	.	.	.	.	.	.	.
E. eurihalinus	_	_	_	C	.	T	T	.	.	.	.	.	.	.	.	.
E. muscorum	_	.	.	.	.	.	.	_	.	.	.	.	.	.	.	T
E. encysticus	_	.	.	.	.	.	.	_	.	.	.	.	.	.	.	.
E. rariseta	_	.	T	.	.	.	.	_	G	.	.	.	.	.	.	.
E. parkei	T	.	T	.	.	_	_	_	.	A	.	.	.	.	.	.
E. focardii	T	.	T	.	A	_	_	_	.	A	.	.	.	.	.	.
E. crassus	T	.	.	.	.	T	T	A	.	A	.	.	.	.	.	.
E. vannus	T	_	.	.	A	_	_	_	.	A	.	.	.	.	.	.
E. minuta	.	-	-	.	.	-	-	-	.	A	.	.	.	.	.	.

Sequence Position 5′ → 3′

Species	*1487*	*1499*	*1502*	*1514*	*1536*	*1546*	*1550*	*1551*	*1552*	*1553*	*1558*	*1559*	*1560*	*1571*	*1579*	*1591*
E. muscicola	T	T	T	G	A	C	T	A	T	G	A	A	T	A	C	C
E. muscicola SBSrc	_	.	.	T	.	.	C	G	.	.		.	.	.	.	.
E. charon	.	.	.	.	G	.	.	T	C	.	G	.	.	.	.	.
E. magnicirratus	.	.	.	.	G	.	.	T	C	.	G	.	C	.	.	.
E. eurihalinus	.	.	.	.	G	.	.	.	.	.	G	.	C	.	.	.
E. muscorum	.	W	W	.	.	.	C	G	.	.		.	C	.	.	T
E. encysticus	.	.	.	.	.	.	C	G	.	.		.	.	.	.	.
E. rariseta	.	.	.	.	.	T	G	G	.	A		.	.	.	A	.
E. parkei	.	.	.	.	.	.	.	.	.	.		T	.	.	.	.
E. focardii	.	.	.	.	G	.	.	.	.	.	T	T	.	.	.	.
E. crassus	.	.	.	.	.	.	.	.	.	.	G	.	C	G	.	.
E. vannus	.	.	.	.	.	.	.	.	.	.	G	.	.	G	.	.
E. minuta	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.

Continued…

Sequence Position 5′ → 3′

Species	*1592*	*1633*	*1644*	*1650*	*1663*	*1664*	*1670*	*1672*	*1674*	*1675*	*1679*	*1739*	*1766*
E. muscicola	T	A	C	C	A	G	T	A	C	T	C	C	A
E. muscicola SBSrc	.	.	.	.	.	A	C	.	T	.	.	.	.
E. charon	.	.	T	T	.	.	C	.	.	.	T	.	.
E. magnicirratus	.	.	T	T	.	.	C	.	.	.	T	.	.
E. eurihalinus	.	.	T	T	G	.	.	.	.	.	T	T	.
E. muscorum	A	.	.	.	.	A	C	.	.	C	.	.	W
E. encysticus	.	.	.	.	.	A	C	.	T	.	.	.	.
E. rariseta	.	.	T	T	G	A	.	.	T	C	T	.	.
E. parkei	.	G	T	T	G	.	C	.	.	C	T	.	.
E. focardii	.	G	T	T		.	C	.	.	.	T	.	.
E. crassus	.	G	T	T	G	.	C	G	.	C	T	.	.
E. vannus	.	G	T	T	G	.	C	G	.	C	T	.	.
E. minuta	.	G	T	T	G	.	C	.	.	C	T	.	.

Sequence Position 5′ → 3′

Species	*1784*	*1788*	*1792*	*1788*	*1792*	*1794*	*1796*	*1799*	*1800*	*1804*	*1806*	*1807*
E. muscicola	A	G	A	G	A	_	A	G	G	G	G	A
E. muscicola SBSrc	.	.	.	.	.	.	.	.	.	.	.	.
E. charon	.	.	.	.	.	.	.	.	.	.	.	.
E. magnicirratus	.	.	.	.	.	.	.	.	.	.	.	.
E. eurihalinus	.	A	.	A	.	.	.	.	.	.	A	.
E. muscorum	.	.	.	.	.	.	.	.	.	.	.	.
E. encysticus	_	.	C	.	C	A	G	A	T	C	A	_
E. rariseta	.	.	.	.	.	.	.	.	.	.	.	.
E. parkei	.	.	.	.	.	.	.	.	.	.	.	.
E. focardii	.	.	.	.	.	.	.	.	.	.	.	.
E. crassus	.	.	.	.	.	.	.	.	.	.	.	.
E. vannus	.	.	.	.	.	.	.	.	.	.	.	.
E. minuta	.	.	.	.	.	.	.	.	.	.	.	.

Specific mutations of *Euplotes muscicola* SBSrc with the other species of the *muscicola* group are highlighted.

Table II.- Supplementary data: Variation (specific and general mutations) in the nucleotide sequences of SS rDNA of *Euplotes adiculatus* and *E. adiculatus lahorensis* compared with other species of *adiculatus* group.

Sequence Position 5′ → 3′

Species	*71*	*72*	*73*	*74*	*77*	*82*	*89*	*116*	*130*	*141*	*142*	*157*	*187*	*193*	*195*	*196*	*197*	*206*	*210*	*214*	*215*
E. adiculatus	_	A	T	T	T	A	A	T	G	T	A	A	G	G	G	A	A	G	C	C	G
E. adiculatus lahorensis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E. parawoodruffi	.	_	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E. woodruffi	.	_	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E. eurystomus 1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	_	.	T	.
E. eurystomus 2	.	.	.	.	.	G	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.
E. harpa	T	.	C	A	C	.	G	.	A	A	G	.	.	.	.	.	.	A	.	T	A
E. patella	.	.	.	.	.	.	.	A	A	.	.	.	A	A	A	G	.	T	T	T	A
E. octocarinatus	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	G	G	A	T	T	A

Sequence Position 5′ → 3′

Species	*215*	*227*	*242*	*280*	*282*	*286*	*287*	*292*	*296*	*297*	*296*	*305*	*306*	*308*	*310*	*375*	*380*	*410*
E. adiculatus	G	T	C	T	C	A	A	T	A	T	A	G	T	G	T	G	A	A
E. adiculatus lahorensis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T
E. parawoodruffi	.	.	.	_	.	.	.	C	_	_	T	.	.	.	.	.	.	.
E. woodruffi	.	.	.	_	T	.	.	C	_	_	T	.	.	.	.	.	.	.
E. eurystomus 1	.	.	A	.	.	.	.	.	.	.	.	C	C	T	G	_	.	.
E. eurystomus 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E. harpa	A	C	.	.	.	G	T	.	.	.	.	T	C	T	.	.	.	.
E. patella	A	.	.	.	.	G	.	.	.	.	.	T	C	T	.	.	C	.
E. octocarinatus	A	.	.	.	.	.	.	.	.	.	.	T	C	T	.	.	.	.

Continued…

Sequence Position 5′ → 3′

Species	*462*	*470*	*502*	*511*	*512*	*518*	*521*	*522*	*524*	*534*	*537*	*599*	*606*	*609*	*648*	*649*	*650*	*657*
E. adiculatus	T	G	C	A	C	G	T	G	T	C	A	A	A	C	A	G	G	C
E. adiculatus lahorensis	.	A	T	C	A	C	G	A	A	.	.	.	.	.	.	.	.	.
E. parawoodruffi	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E. woodruffi	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E. eurystomus 1	C	.	.	.	.	.	.	.	.	.	T	.	.	.	G	A	.	.
E. eurystomus 2	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E. harpa	C	.	.	.	.	.	.	.	.	.	.	T	T	T	.	.	_	T
E. patella	C	.	.	.	.	.	.	.	.	T	.	.		T	.	A	.	.
E. octocarinatus	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	A	.	.

Sequence Position 5′ → 3′

Species	*658*	*681*	*682*	*683*	*684*	*690*	*692*	*706*	*712*	*713*	*715*	*722*	*724*	*732*	*740*	*743*	*744*	*746*
E. adiculatus	G	C	C	G	A	T	C	T	G	T	C	A	T	C	G	_	T	T
E. adiculatus lahorensis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E. parawoodruffi	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	G	.
E. woodruffi	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	G	.
E. eurystomus 1	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	G	.	.
E. eurystomus 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E. harpa	.	T	.	.	T	C	T	A	T	C	.	C	C	T	T	G	.	C
E. patella	A	.	T	A	T	C	T	A	T	C	T	.	.	T	T	G	.	.
E. octocarinatus	.	.	T	A	T	C	T	.	.	.		.	.	T	T	G	.	.

Continued…

Sequence Position 5′ → 3′

Species	*747*	*750*	*752*	*757*	*758*	*759*	*760*	*761*	*763*	*764*	*766*	*768*	*774*	*781*	*784*	*786*	*835*	*842*
E. adiculatus	C	A	C	C	A	A	T	A	T	C	T	G	C	A	T	G	A	T
E. adiculatus lahorensis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
E. parawoodruffi	.	.	.	.	.	T	A	T	G	.	.	.	.	.	.	.	G	.
E. woodruffi	.	.	.	.	.	T	A	T	G	.	.	.	.	.	.	.	G	.
E. eurystomus 1	.	.	T	.	.	.	.	.	.	T	.	A	.	.	.	.	.	.
E. eurystomus 2	.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.
E. harpa	T	.	.	T	T	.	.	.	.	T	.	.	T	.	.	A	.	.
E. patella	.	C	.	T	T	.	.	.	.	T	C	A	.	C	C	A	.	.
E. octocarinatus	.	C	.	T	T	.	.	.	.	T	C	A	.	.	C	A	.	.

Sequence Position 5′ → 3′

Species	*843*	*850*	*866*	*868*	*872*	*874*	*875*	*876*	*878*	*879*	*882*	*890*	*898*	*899*	*911*	*926*	*929*	*930*
E. adiculatus	G	A	G	C	A	C	T	G	G	C	C	_	_	G	G	C	C	G
E. adiculatus lahorensis	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E. parawoodruffi	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	A
E. woodruffi	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	A
E. eurystomus 1	.	.	.	.	.	.	A	.	.	.	T	T	.	.	.	.	A	A
E. eurystomus 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
E. harpa	.	T	A	.	T	T	.	T	.	A	.	T	.	A	A	T	T	A
E. patella	.	.	A	T	T	T	.	T	A	A	T	T	G	A	.	.	A	A
E. octocarinatus	.	.	A	T	T	T	.	T	A	A	T	T	.	.	.	.	A	A

Continued…

Sequence Position 5′ → 3′

Species	*935*	*937*	*940*	*941*	*942*	*950*	*1001*	*1014*	*1015*	*1016*	*1026*	*1133*	*1198*	*1213*	*1266*
E. adiculatus	T	T	C	A	A	T	T	C	G	T	G	A	G	A	A
E. adiculatus lahorensis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E. parawoodruffi	.	_	G	G	G	.	A	T	C	.	.	T	.	.	.
E. woodruffi	.	_	G	G	G	.	A	T	C	.	.	T	.	.	G
E. eurystomus 1	.	.	G	G	G	C	.	T	T	A	.	T	.	.	.
E. eurystomus 2	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.
E. harpa	A	C	_	G	G	.	.	T	T	A	A	T	A	.	.
E. patella	.	.	A	G	G	.	.	T	T	A	.	T	.	.	.
E. octocarinatus	.	.	G	G	G	.	.	T	T	A	.	T	.	.	.

Sequence Position 5′ → 3′

Species	*1429*	*1431*	*1432*	*1434*	*1435*	*1436*	*1437*	*1438*	*1439*	*1440*	*1442*	*1443*	*1444*	*1445*	*1446*
E. adiculatus	G	T	C	C	C	T	T	T	T	C	A	T	A	A	G
E. adiculatus lahorensis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E. parawoodruffi	.	.	.	T	.	G	A	A	.	A	.	C	G	T	.
E. woodruffi	.	.	.	T	.	G	A	A	.	A	.	C	G	T	.
E. eurystomus 1	.	.	A	T	_	.	.	.	A	.	T	.	.	.	A
E. eurystomus 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E. harpa	A	C	T	T	.	.	C	.	C	T	.	C	G	.	.
E. patella	.	.	.	T	.	.	C	.	C	T	.	C	G	.	.
E. octocarinatus	.	.	.	T		.	C	.	C	T	.	C	G	.	.

Continued…

Sequence Position 5′ → 3′

Species	*1449*	*1450*	*1453*	*1579*	*1587*	*1607*	*1607*	*1619*	*1620*	*1623*	*1680*	*1687*	*1690*	*1691*	*1695*
E. adiculatus	T	T	C	T	A	G	_	A	A	_	T	C	A	C	C
E. adiculatus lahorensis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E. parawoodruffi	.	.	.	G	G	.	.	.	.	.	.	.	G	G	.
E. woodruffi	.	.	.	G	G	.	.		.	.	.	.	G	G	.
E. eurystomus 1	.	C	.	.	.	.	.	T	.	.	A	.	.	G	.
E. eurystomus 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E. harpa	.	.	T	.		A	C	C	T	A	.	.		G	T
E. patella	C	.	.	.	G	A	C	.	T	.	.	T	G	G	.
E. octocarinatus	C	.	.	.	G	A	C	.	T	.	.	T	G	G	.

Sequence Position 5′ → 3′

Species	*1701*	*1702*	*1706*	*1710*	*1755*	*1763*	*1764*	*1768*	*1772*	*1774*	*1791*	*1812*	*1816*	*1817*	*1818*	*1857*
E. adiculatus	G	T	T	T	T	A	T	G	_	C	C	C	A	C	T	G
E. adiculatus lahorensis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E. parawoodruffi	C	C	.	.	.	.	.	.	.	A	.	.	.	T	C	.
E. woodruffi	C	C	.	.	.	.	.	.	.	A	.	.	.	T	C	_
E. eurystomus 1	_	C	.	.	.	.	C	.	.	.	.	.	G	.	C	.
E. eurystomus 2	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
E. harpa	C	.	.	.	.	G	.	A	.	.	.	.	.	T	.	.
E. patella	C	C	C	.	A	G	.	.	.	.	T	T	.	T	C	.
E. octocarinatus	C	C	C	.	.	.	.	.	G	.	T	.	.	T	C	.

Specific mutations of *Euplotes adiculatus lahorensis* with the other species of the *adiculatus* group are highlighted.

Prescott, M.L., Harley, J., Donald, P. and Klein A. (1999). In 'Antimicrobial chemotherapy.' Microbiology 2nd edition . published by C. Brown Publishers, U.S.A. Pp 325