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# Characterization of *Gloeotrichia ghosei* BTA 9020 an isolate of culture collection of Algae, University of Allahabad with emphasis on lipid profiling and fatty acid composition

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**ABSTRACT**

*The present investigation deals with the cultural studies, pigment analysis, lipid profiling, total sugar content, extracellular ammonium excretion and molecular characterization of cyanobacterium Gloeotrichia ghosei BTA 9020 (NCBI accession no. KM435243). Initially isolate was identified and grouped on the basis of morphological features followed by chemotaxonomical and molecular attributes. The study revealed that investigated organism produced good amount of secondary metabolites in culture conditions during decline phase of growth; unsaturated fatty acid, namely; palmitic acid C16:0 was found highest (25.1%) followed by linolelaidic acid C18:2n6t (21.9%). The 16S rRNA sequence was compared with the retrieved cultures from NCBI GenBank database. Phylogenetic analysis was largely consistent which was obtained from 16S rRNA gene sequence analysis. Phylogenetic trees were constructed by UPGMA, maximum-parsimony and neighbour joining and the findings indicate that the genus Gloeotrichia is distantly related to the genus Calothrix and Rivularia.*

**Keywords:** *Gloeotrichia*, Lipid profiling, Phylogenetic relationship, Secondary metabolites 16S rRNA

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## INTRODUCTION

Cyanobacteria are prokaryotic micro-organisms that resemble gram negative bacteria in structure but possess oxygen evolving photosynthetic system similar to that of eukaryotic algae and higher plants [1]. They are a large and diverse group of organisms that can be found in areas ranging from deserts or glaciers to soil or hot springs. *Gloeotrichia* is a cyanobacterium made up of filaments bound together to form a round, spiked colony between 1-3 mm in diameter [2]. *Gloeotrichia* species has strongly tapering filaments and form mucilaginous spheres akinetes [3, 4]. Most species of the genus *Gloeotrichia* grow attached to different substrata in the littoral zone of lakes [5] and the only truly planktonic species is *Gloeotrichia echinulata* [6]. Like many species of cyanobacteria, the organism contains gas vesicles which allow them to float into the water column and are capable of producing toxins [7, 8]. *Gloeotrichia*, is commonly found in well mixed mesotrophic and eutrophic lakes at temperate latitudes. In the benthos, it begins colonial formation in June and July and assimilates large amounts of phosphorus from nutrient rich sediments. The acquired phosphorus is significantly above its immediate needs and the colony stored the phosphorus for subsequent growth and divisions once in the water column. These reserves allow *Gloeotrichia* sp. to

thrive under phosphorus limitation in the epilimnion when other phytoplankton cannot [9]. Fatty acids in general are of commercial value and many pharmaceutical agents. In an observation and analysis, fatty acids GLA, palmitic acid, linoleic and oleic acid reported as the predominant [10]. Under the right conditions it has been observed that populations of *Gloeotrichia* sp. has progressed from little or none present in the plankton community in one year, to complete dominance in the following season [11]. Due to its dominant characteristics and complex life cycle, the presence of *Gloeotrichia* has become a cause of concern with regard to the greater ecosystems.

## MATERIALS AND METHODS

**Strain and growth condition:** The studied strain was obtained from Department of Botany, University of Allahabad, Allahabad, U.P, India which was originally isolated from the submerged rice fields of Allahabad during August to October. Unialgal biomass was inoculated in Erlenmeyer flask containing BG-11 (-N) broth medium [12]. The flasks were kept in culture room under light: dark cycles of 14:10h conditions maintained at 28±2ºC under illumination provided by cool white fluorescent tubes of 54-67µmol photons $m^{-2}s^{-1}$. The flasks were shaken manually on daily basis to prevent cell clumping.

**Estimation of chlorophyll-a:** Homogenized algal cells of 10ml were centrifuged at 6500 rpm for 10 minutes (refrigerated centrifuged Eppendorf model No. 5430 R ) thereafter supernatant discarded. Transferred the algal pellet to a test tube and added 10ml of 90% methanol. Shake the contents and placed the tubes in a water bath at 60˚C for 30 minutes. Optical density (O.D.) was recorded at 665nm by using UV-Vis spectrophotometer model 1800 Shimadzu applying the method described [13].

**Estimation of phycobiliproteins (PBS):** Homogenized algal cells of 10ml were centrifuged at 6500 rpm for 10 min. Subject the pellet to repeated freezing and thawing in 5ml of 0.05M phosphate buffer containing 100ml each of 0.1M solution of $K_2HPO_4$ and $KH_2PO_4$ .Centrifuged the suspension to remove cell debris yielding a blue coloured supernatant and quantitated followed the method [14]. Optical density (O.D.) recorded at 615, 652 and 562 by using UV-Vis spectrophotometer model 1800 Shimadzu.

**Estimation of extracellular ammonium excretion:** Ammonia excretion was determined by the method described by [15]. 05 ml culture filtrate obtained by filtration of homogenized algal suspension filtered through Whatmann's filter paper was taken. Added 0.2 ml mixed phenol (2g of reagent grade phenol dissolved 100 ml of 95% ethyl alcohol) thereafter added 0.2 ml reagent-A (0.15 g of sodium nitroprusside dissolved in 30 ml of distilled water and stored in amber colour bottle). Finally 0.5 ml reagent-B (10 g of trisodium citrate and 0.5 g of NaOH in 50 ml of distilled water with 20 ml of 1.5 N sodium hypochlorite soln), mixed thoroughly with the aid of vortex shaker and kept for 1 h for development of blue colour in dark place. The absorbance was measured at 640 O.D.

**Estimation of total carotenoids:** Estimation of total carotenoids was determined by the method described by [16]. 10 ml homogenized algal suspension was taken and centrifuged at 6500 rpm for 10 min. Discarded the supernatant and added 3 ml 85% acetone and subjected to repeat freezing and thawing until the pellet becomes colourless. Measured the volume of the extract and make up the final volume upto 10 ml with 85% acetone and measured O.D. at 450 nm using 85% acetone as blank and calculated the total amount of carotenoid in $\mu g ml^{-1}$.

**Estimation of total sugar:** Total sugar was estimated following the method described by [17]. 0.2 ml of the homogenized algal cell suspension was taken and added 0.8 ml of distilled water and 4 ml anthrone reagent (dissolved 0.1 g of anthrone and 1.0 g of thiourea in freshly prepared 100 ml of 75% sulphuric acid) and shaken gently. Tubes were kept in a boiling water bath for 15 min by covering the mouth of tube with aluminium foil to prevent evaporation. After cooling the tubes the absorbance was measured at 620 nm and total sugar content was calculated from the standard graph.

**Lipid profiling:** Lipid profiling method followed as per described by [18]. 15 ml of 2% $H_2SO_4$ in methanol solution was added to total biomass (300 mg) in round bottom (RB) flask. Refluxed the RB flask containing the biomass in heating mantle at 10°C for 4 h. Transferred the FAME solution to a separating funnel then added ethyl acetate and distilled water to the FAME solution contained inside the separating funnel. Two aqueous phase layer was formed. Then separated out the lower phase and the upper phase was retained and washed with distil water till it gives a pH 7.0 (checking through pH strip). Separated the extract into a conical flask and put excess amount of sodium sulphate ($Na_2SO_4$) into it and kept for 20 min. Extract was transferred to 50 ml RB flask and rotaevaporated at 65°C. Rinsed

the RB flask containing the FAME by putting few drops of dichloro-methane and transferred the solution to a vial. Took 1µl sample from vial using micro syringe and injected in GC. From the standard (SUPELCO$^{TM}$ 37 components) and its retention time, the fatty acid content was identified.

**Isolation of genomic DNA:** Chemical lysis is ineffective therefore mechanical disruption was done in present investigation by following Xanthogenate method [19].

**PCR amplification of the 16S rRNA gene:** To amplify the 16S rRNA gene segments was carried out for 50 µl of reaction mixture using 2 µl (50 ng) of extracted DNA using PCR master mix made up of 5 µl of 1X *Taq* buffer with 1.5 mM $MgCl_2$, 5 µl of 200 µM each of dNTPs solution, 0.25 µl of1.25 U *Taq* polymerase along with 1.5 µl of 0.3 µM each of cyanobacterial universal primer for 16S rDNA like (536f-GTGCCAGCAGCCGCGGTRATA) and reverse (1488R-GGTTACCTTGTTACGACTTCACC) with 34.75 µl of sterile double distilled water. After DNA amplification, 3 µl of DNA sample was mixed with 1 µl of loading dye by pipetting in and out and loaded the sample in 2% agarose gel in gel electrophoresis unit and ran for 45 mins at 60V. 200 bp DNA ladder was used as marker. After gel electrophoresis, gel was illuminated in gel documentation system and observed DNA bands.

**Analysis of sequence data:** Nucleotide sequence obtained from DNA sequences was compared with the sequence available in the NCBI database using BLAST (http://www.ncbi.nml. nih.gov/ BLAST). Trees based on 16S rRNA were constructed using the available cyanobacterial gene sequences along with the sequence determined in this study using the neighbour-joining method [20, 21] by using Kimura 2-parameter model [22] and maximum parsimony [23] method contained in the MEGA 4.0 software [24]. Sequences were aligned using CLUSTALW to produce working alignment of 16S rDNA sequences for the target strains. The final alignments were obtained by manual refinement. Phylogenetic tree were constructed. The analysis of similarity matrix and phylogenetic tree was done. Statistical significance level of interior nodes was determined by bootstrap analysis (1,000 data re-samplings) [25] and values above 50% were reported.

## RESULTS AND DISCUSSION

Radiating colonies of heteropolar taperined filaments, each with a heterocyte and akinetes at the base and hair-like terminal cells at the opposite end (Fig. 1). Each filament has a sheath and the whole colony is enveloped in mucilage. Colonies form spherical yellow masses attached to surfaces. The whole colony enveloped by fine slime trichomes always oriented with heterocytes in to the centre of the colony. Trichomes rarely false branched; the branches separate soon from the mother trichome, but remain parallel and radially located within the colonial slime and form their own gelatinous sheaths. Colonies are joined to the substrate. Trichomes constricted at the cross walls and straight. Sheaths are always present, especially near apical parts of trichomes. Upon the basal heterocytes cylindrical akinetes develop in solitary at the end of the vegetation periods.

This unialgal *Gloeotrichia* strain yielded chlorophyll-a (1.13 and 1.57 µgml$^{-1}$) phycoerythin (7.17 and 26.7 µgml$^{-1}$), phycocyanin (7.28 and 19.6 µgml$^{-1}$) allophycocyanin (2.41 and 2.51 µgml$^{-1}$) total carotenoids (0.62 and 5.45 µgml$^{-1}$), total carbohydrates (8.33 and 16.8 µgml$^{-1}$) and total soluble proteins (29.0 and 59.3 µgml$^{-1}$) during 15$^{th}$ and 30$^{th}$ days growth cycle (Table 1).

Fatty acid composition and total lipids in *Gloeotrichia* species was examined and presented (Table 2). The species grown in BG-11 medium without nitrate was harvested at exponential phase. The GC analysis showed variation in n-saturated, unsaturated and long chain branched fatty acids (Fig. 2). Among the fatty acids detected most of them belonged to unsaturated fatty acids (more than 60%). In the present investigation, *Gloeotrichia* sp. showed high lipid content palmitic acid C16:0 followed by linolelaidic acid C18:2 and long chain fatty acids (C20:1 and C24:0) which are to be important for nutraceutical and pharmaceutical industry. Interesting observation noted that the investigated organism produced comparable and good quantity of secondary metabolites including natural colorant materials, soluble proteins in culture conditions during decline growth cycle of the organism.

*Gloeotrichia* sp. represents a morphologically distinct form-genus with heteropolar filaments which differ from other rivulariaceae by obligatory formation of akinetes, but they do not exist yet strains typical *Gloeotrichia* species in cultures. 16S rRNA sequences of cyanobacteria belong to non heterocystous filamentous and heterocystous (GQ859626.1, KM019945.1, GQ859627.1, AF334696.1, AB074504.1, DQ234827.1, DQ234828.1, AB039625.1, AB093486.1, AM230706.1, HF678491.1, AM230667.1, AM230674.1, AM230672.1, AM230665.1, AF067818.1,

AF092504.1, AM230668.1, KM019924.1, AF334694.1, AF062638.1, AJ344563.1, DQ072896.1, AM230677.1, KM435243.1, KM019918.1, AY768401.1, AB097922.1, JX827161.1 were obtained from GenBank and nucleotide sequence of the PCR amplified 16SrRNA gene of studied strain i.e. *Gloeotrichia* sp. To examine their phylogenetic relationship, both this 16S rRNA sequence of 29 other cyanobacteria of heterocystous group was taken. Inference of the relationship from nucleotide of known function that is structurally similar can be accomplished through the comparative analysis. The study presented phylogenetic or evolutionary positions of the one strain using 16SrRNA gene sequence (Fig. 3-5).

**Table 1: Biochemical characterization of *Gloeotrichia ghosei***

| Name and code of the strain & NCBI GenBank accession no. | Biochemical/ physiological characterization (Result in µg ml$^{-1}$) | | | |
|---|---|---|---|---|
| | | | 15$^{th}$ day | 30$^{th}$ day |
| *Gloeotrichia ghosei*. BTA9020<br>NCBI Accession No.: KM435243 | Total soluble protein | | 29.00±5.00 | 59.33±5.70 |
| | Total carbohydrates | | 8.33±1.15 | 16.00±0.00 |
| | Chlorophyll-a | | 1.13±0.31 | 1.57±0.13 |
| | Extracellular ammonium excretion | | 3.50±0.92 | 10.50±4.90 |
| | Phycobiliproteins | PE | 7.17±1.40 | 26.76±1.20 |
| | | PC | 7.28±0.90 | 19.60±1.20 |
| | | APC | 2.41±0.20 | 2.51±0.30 |
| | Carotenoids | | 0.62±0.30 | 5.45±2.90 |

**Table 2: Fatty acids and lipid profiling present in *Gloeotrichia ghosei***

| SN | Fatty acid composition | Fatty acid content (%) |
|---|---|---|
| 1 | Butyric Acid Methyl Ester (C4:0) | 4.48 |
| 2 | Caproic Acid Methyl Ester (C6:0) | 0.02 |
| 3 | Caprylic Acid Methyl Ester (C8:0) | 0.01 |
| 4 | Capric Acid Methyl Ester (C10:0) | 0.13 |
| 5 | Undecanoic Acid Methyl Ester (C11:0) | 0.13 |
| 6 | Lauric Acid Methyl Ester (C12:0) | 0.19 |
| 7 | Tridecanoic Acid Methyl Ester (C13:0) | 0.18 |
| 8 | Myristic Acid Methyl Ester (C14:0) | 0.10 |
| 9 | Myristoleic Acid Methyl Ester (C14:1) | 0.26 |
| 10 | Pentadecanoic Acid Methyl Ester (C15:0) | 0.56 |
| 11 | *cis*-10-Pentadecenoic Acid Methyl Ester (C15:1) | 7.40 |
| 12 | **Palmitic Acid Methyl Ester (C16:0)** | **25.10** |
| 13 | Palmitoleic Acid Methyl Ester (C16:1) | 4.54 |
| 14 | Heptadecanoic Acid Methyl Ester (C17:0) | 1.04 |
| 15 | *cis*-10-Heptadecenoic Acid Methyl Ester (C17:1) | 0.66 |
| 16 | Elaidic Acid Methyl Ester (C18:1n9t) | 3.56 |
| 17 | Oleic Acid Methyl Ester (C18:1n9c) | 7.39 |
| 18 | **Linolelaidic Acid Methyl Ester (C18:2n6t)** | **21.93** |
| 19 | Linoleic Acid Methyl Ester (C18:2n6c) | 3.33 |
| 20 | Arachidic Acid Methyl Ester (C20:0) | 5.13 |
| 21 | γ-Linolenic Acid Methyl Ester (C18:3n6) | 1.18 |
| 22 | *cis*-11-Eicosenoic Acid Methyl Ester (C20:1) | 5.78 |
| 23 | Linolenic Acid Methyl Ester (C18:3n3) | 0.13 |
| 24 | Heneicosanoic Acid Methyl Ester (C21:0) | 0.46 |
| 25 | *cis*-11,14-Eicosadienoic Acid Methyl Ester (C20:2) | 0.67 |
| 26 | Behenic Acid Methyl Ester (C22:0) | 0.32 |
| 27 | Erucic Acid Methyl Ester (C22:1n9) | 0.13 |
| 28 | Tricosanoic Acid Methyl Ester (C23:0) | 0.70 |
| 29 | *cis*-13,16-Docosadienoic Acid Methyl Ester (C22:2) | 0.10 |
| 30 | *cis*-5,8,11,14,17-Eicosapentaenoic Acid Methyl Ester (C20:5n3) | 3.86 |
| 31 | Nervonic Acid Methyl Ester (C24:1) | 0.52 |

Phylogenetic analysis of the 16S rRNA gene identified limited sequence diversity among the *Gloeotrichia* morphotype strains. Our results demonstrated that *Gloeotrichia*, *Calothrix* and *Tolypothrix* do not form a monophyletic group but instead display a high level of genetic diversity. The evolutionary distances suggested that they belong to at least five different genera. Our results also observed that the genus *Gloeotrichia* is distantly related to the genus *Calothrix*. We found correlations between genetic grouping and morphology in redundancy analysis. However, morphology alone was not sufficiently reliable to distinguish strains from different 16S rRNA gene clusters. The high level of diversity that we observed confirms the hypothesis that the Rivulariaceae groups are species rich. According to the sequences of planktic *Gloeotrichia* belong to type species rather to the vicinity of

nostocaceae that to Rivulariaceae [26]. *Gloeotrichia* additions also increased the richness and diversity of other phytoplankton taxa.

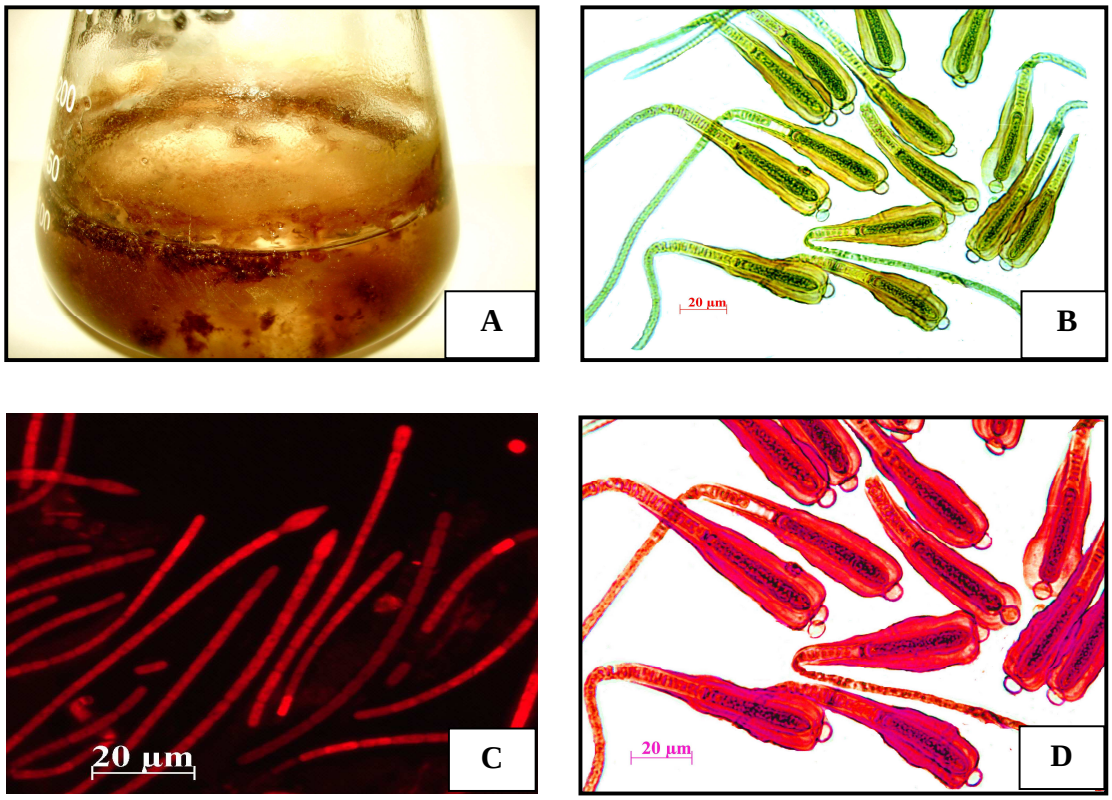
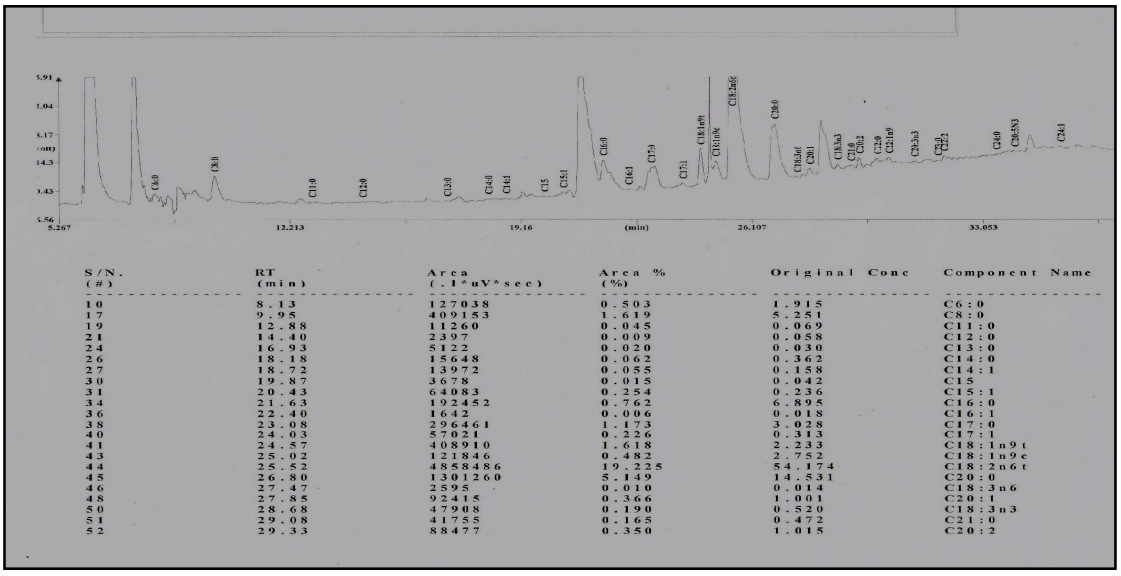



**Fig. 1: Growth of *Gloeotrichia ghosei***
**A. Growth in liquid medium; B &D. Photomicrograph of *Gloeotrichia ghosei*;**
**C.Initial stage of *Gloeotrichia ghosei* stained with Nile red fluorescence dye (63x);**



| S/N. (#) | RT (min) | Area (.1*uV*sec) | Area % (%) | Original Conc | Component Name |
|---|---|---|---|---|---|
| 10 | 8.13 | 127038 | 0.503 | 1.915 | C6:0 |
| 17 | 9.95 | 409153 | 1.619 | 5.251 | C8:0 |
| 19 | 12.88 | 11260 | 0.045 | 0.069 | C11:0 |
| 21 | 14.40 | 2397 | 0.009 | 0.058 | C12:0 |
| 24 | 16.93 | 5122 | 0.020 | 0.030 | C13:0 |
| 26 | 18.18 | 15648 | 0.062 | 0.362 | C14:0 |
| 27 | 18.72 | 13972 | 0.055 | 0.158 | C14:1 |
| 30 | 19.87 | 3678 | 0.015 | 0.042 | C15 |
| 31 | 20.43 | 64083 | 0.254 | 0.236 | C15:1 |
| 34 | 21.63 | 192452 | 0.762 | 6.895 | C16:0 |
| 36 | 22.40 | 1642 | 0.006 | 0.018 | C16:1 |
| 38 | 23.08 | 296461 | 1.173 | 3.028 | C17:0 |
| 40 | 24.03 | 57021 | 0.226 | 0.313 | C17:1 |
| 41 | 24.57 | 408910 | 1.618 | 2.233 | C18:1n9t |
| 43 | 25.02 | 121846 | 0.482 | 2.752 | C18:1n9c |
| 44 | 25.52 | 4858486 | 19.225 | 54.174 | C18:2n6t |
| 45 | 26.80 | 1301260 | 5.149 | 14.531 | C20:0 |
| 46 | 27.47 | 2595 | 0.010 | 0.014 | C18:3n6 |
| 48 | 27.85 | 92415 | 0.366 | 1.001 | C20:1 |
| 50 | 28.68 | 47908 | 0.190 | 0.520 | C18:3n3 |
| 51 | 29.08 | 41755 | 0.165 | 0.472 | C21:0 |
| 52 | 29.33 | 88477 | 0.350 | 1.015 | C20:2 |

**Fig. 2: GC-FID analysis of Fatty Acid Methyl Esters derived from neutral lipids isolated from *Gloeotrichia ghosei***

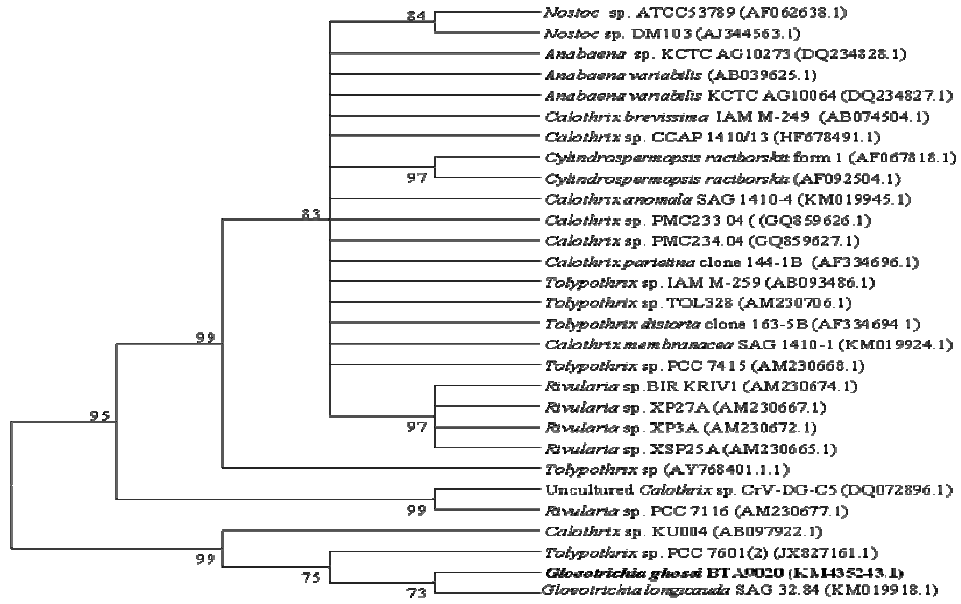



**Fig. 3: Maximum parsimony tree showing the relationship among 16S rRNA gene sequence of the strain including strains from GenBank. Value above node represent bootstrap support**

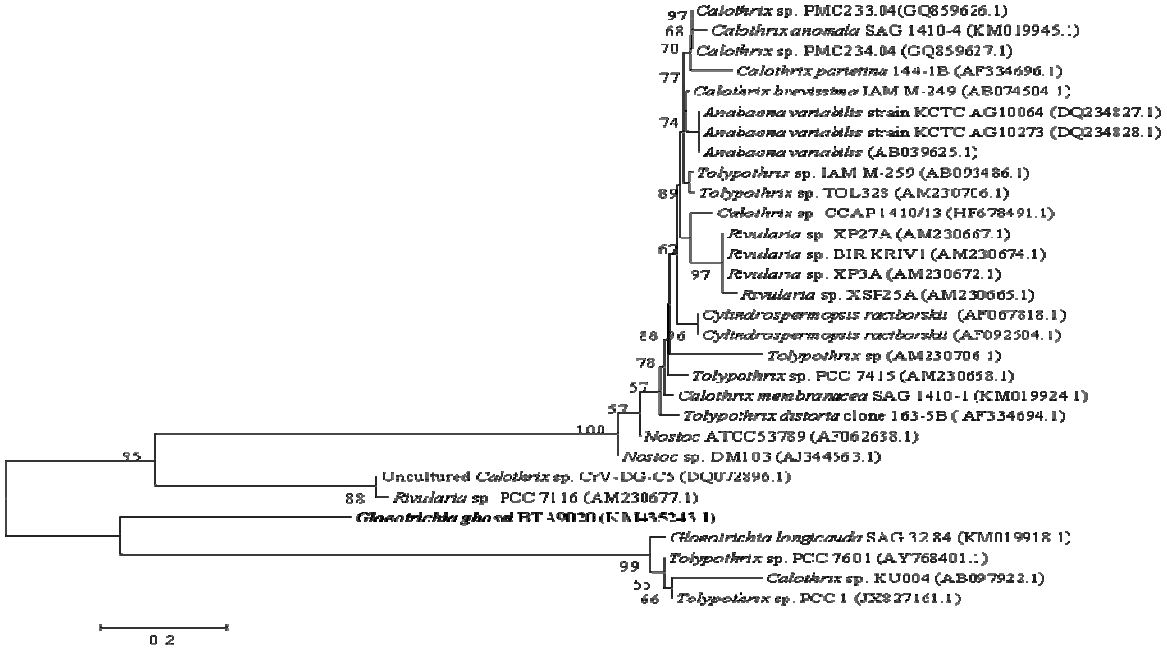



**Fig. 4: Neighbour-joining tree based on analysis of 16SrRNA genes showing the position of sequence obtained in this study (in bold). Numbers at nodes indicate bootstrap values greater than or equal to 55% for the NJ analysis. Bar 0.2 substitutions per nucleotide position**

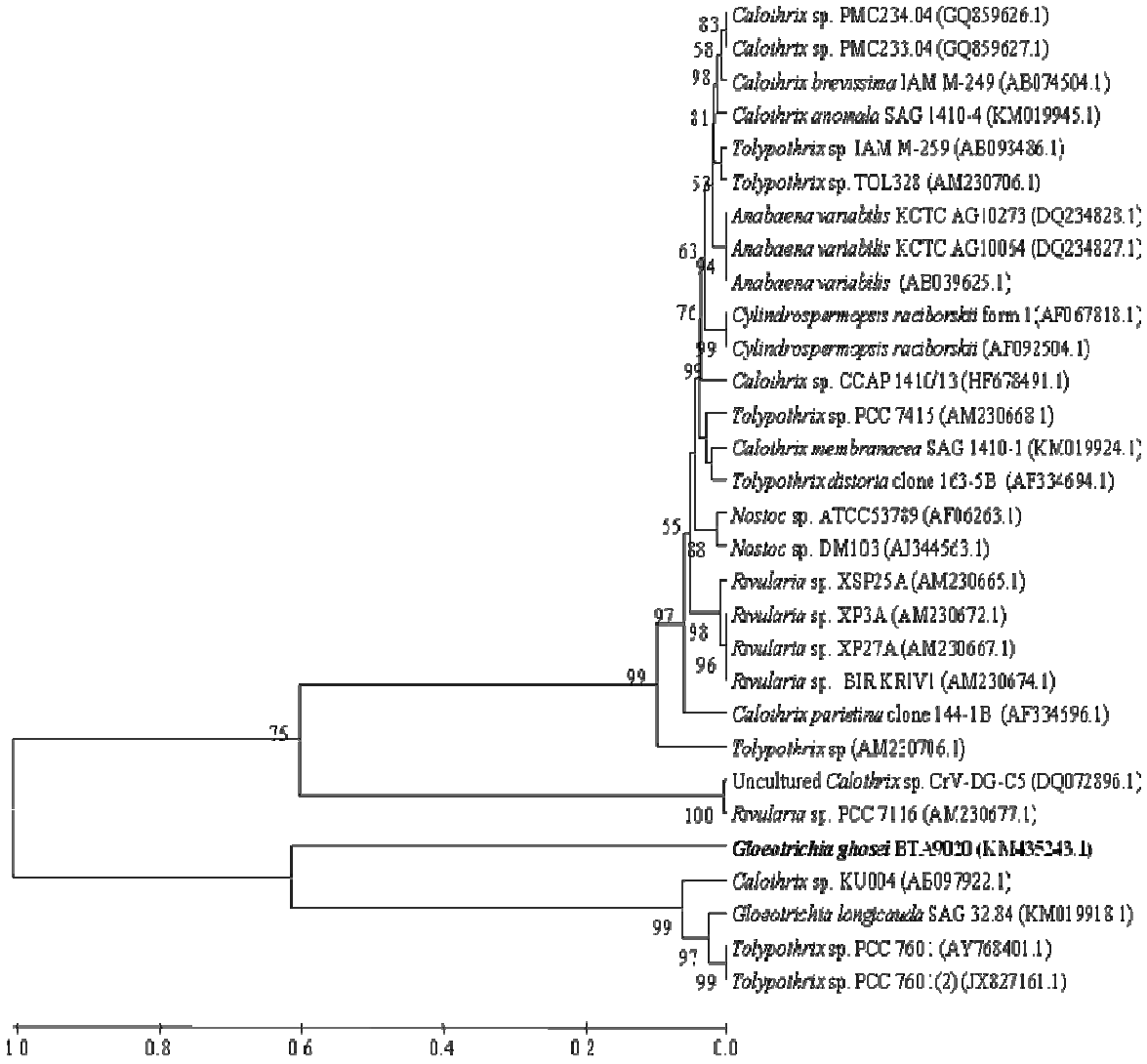



**Fig. 5: UPGMA clustering analysis of *Gloeotrichia ghosei***

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