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Phenotypic and Molecular Characterization of *Chaetomium globosum* (Gustav Kunze) from Different Microhabitats of Tamil Nadu, India

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The biocontrol agent *Chaetomium globosum* is widely prevalent in soil and other cellulose containing substrates. Fifteen isolates of *C. globosum* was isolated from different microhabitats of Tamil Nadu using baiting, direct isolation from decomposed materials and through serial dilution technique. All the fifteen isolates were phenotypically identified by their colony growth and perithecia. The colour of the colony varied from greenish white to yellowish white with radial and fluffy mycelial growth pattern. The pycnidia of different *Chaetomium* isolates varied in their shapes like ovoid, lemoniform, elliptical or slightly elliptical with an apical germ pore. The size of ascomata varied from 105.1 – 593.45 x 51.21 - 387.41 µm dia. Comparison of perithecial production between the *C.globosum* isolates indicated that the number of perithecia was more in TNAU-Cg 101 and TNAU-Cg 105 isolates. Ascospores were brown in colour and size varied from 8 - 14 µm dia. The sporulation was higher in TNAU-Cg101, TNAU-Cg 102 and TNAU-Cg105 isolates. Besides, all fifteen isolates were confirmed as *C. globosum*, by amplification through internal transcribed spacer rDNA sequences bearing the accession numbers MK587669, MK590290, MK592857, MK603862, MK592858, MK828135, MK821416, MK823129, MK828197, MK603941, MK828136, MK757844, MK820066, MK828133 and MK828134. Phylogenetic analysis of the fifteen *C. globosum* isolates, indicated that all the isolates were clustered into a single group.

Introduction

The genus *Chaetomium* was first established by Gustav Kunze in 1817. It is one of the largest saprophytic ascomycetes fungi having more than 300 genera (Rodriguez *et al.*, 2002), belongs to the class Sordariomycetes, order Sordariales and family Chaetomiaceae. It is present in different microhabitat which includes soil, decomposed cotton, paper, moistened wall, damaged buildings, plant rhizoplane as well as rhizosphere (Prokhorov and Linnik, 2009). *Chaetomium* produces an imperfect Acremonium – like stage on culture media and identified by the presence of flask shaped perithecia superficially surrounded by

long, dark and stiff ascomatal hairs. *Chaetomium* spp. were normally identified by morphological character, the type of ascomatal hairs were erect, flexuous or coiled globose (Udagawa, 1979), colony character (Petcharat and Soytong, 1991), ovate or obovate ascomata, evanescent asci, slightly fusiform or clavate; lemoniform or ovate ascospore with an apical germ pore (Wang *et al.,* 2016). *Chaetomium* species are strictly saprophytic organism and have been shown to be antagonist against several plant pathogens, e.g. *Thielaviopsis paradoxa* (Soytong *et al.,* 2005), *Phytophthora palmivora,* (Hung *et al.,* 2015), *P. infestans* (Shanthiyaa *et al.,* 2013), *Pythium ultimum* (Kean *et al.,* 2010), *Alternaria brassicicola* (Vannacci and Harman, 1987), *Pyricularia oryzae* (Song *et al.,* 2016, *Cochliobolus sativus* (Aggarwal *et al.,* 2004).

Materials and Methods

Collection of samples

Soil sample and decomposed material from different ecosystem were collected from Coimbatore, Coonnor, Ooty, Salem and Yercaud of Tamil Nadu. *Chaetomium* spp., was isolated from rhizoplane soil of *Delonix regia, Saccharum officinarum, Polianthes tuberosa, Camellia sinensis, Opuntia ficus-indica, Citrus aurantifolia* and decomposed waste material from cotton, paper, litter, paddy straw, areca leaf plates and soil from compost pit. Samples were collected in butter paper covers and brought to the laboratory and preserved in a refrigerator at 5^{o}C for further studies.

Isolation of *C. globosum*

Chaetomium were normally isolated by baiting and soil plate technique method (Wang *et al.,* 2016, Soytong *et al.,* 2001). *Chaetomium* was isolated through serial dilution technique by collecting the soil samples from various ecosystem and the collected soil were weighed each of 1 g and serially diluted, poured in sterile petriplate over which glucose-ammonium nitrate agar (GANA) medium (10 g glucose, 1 g NH_4NO_3, 1 g Difco bacto yeast extract, 0.5 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 20 g agar, 0.06 g rose bengal, 0.03 g streptomycin, 1,000 ml distilled water) was poured. Then the fungus was incubated for 7-9 days at 20^{o}C. It was observed under microscope for the presence of morphological identifications like ascomatal formation, mycelial appendages, presence of perithecia, ascus and acsospores, *Chaetomium* ascomatal formation occurs, and they were placed in Potato Dextrose Agar (PDA) medium supplemented with rifampicin 0.1 g. Finally pure culture was obtained by means of single spore isolation method (Pornsuriya *et al.,* 2008). Similarly *Chaetomium* was isolated by baiting technique. The cellulose enriched soil samples collected from different ecosystem of Tamil Nadu which includes compost pit soil, rhizoplane soil from *D. regia, S. officinarum, P. tuberosa, C. sinensis, O. ficus-indica* and *C. aurantifolia* were isolated through baiting technique. From that each soil sample (10 g) were transferred into sterilized petriplate and moistoined with distilled water. The moistened soil samples were baited with sterilized paddy straw bits, after 35 - 40 days of incubation at 20^{o}C, *Chaetomium* ascomatal growth was observed over the baited material that was transferred into water agar medium and incubated for 4 days. Then the single colony were transferred into Potato dextrose agar medium amended with antibiotic rifampicin 0.1 g dissolved in 1ml of ethanol / 100ml of PDA medium as described by (Pornsuriya *et al.,* 2008). Further it was isolated by direct isolation from decomposed material. The *Chaetomium* were isolated from ascomatal structures developed over decomposed cotton waste, paddy straw, paper

waste, litter waste and areca leaf plate, appearing as olive green to black colour perithecia were examined under the stereo microscope and were incubated in Potato Dextrose Agar (PDA) medium.

Morphological characterization of *Chaetomium* isolates

Isolated *Chaetomium* species were characterized morphologically using Phase contrast microscope based on characters like ascomatal hairs or lateral and terminal hairs may be straight, spiral, coiled, hooked etc., evanescent asci and based on the shape, colour and size of ascospores (Prokhorov and Linnik, 2011) (Fig. 1).

Scanning Electron Microscopy (SEM)

C. globosum - TNAU-Cg 101 were selected for further studies because they showed rapid mycelial growth, highest ascomatal size and produces more number of sporulation in petriplate were cultured for 9 days at 20°C± 1°C and perithecia were scrapped and directly mounted on standard copper SEM stubs using double-sided adhesive tape. The samples were sputter coated, when bombarded with electrons, biological specimens ineffectually dissipate the resulting charge, which can cause imaging artifacts. So, coating the specimens with a thin layer of a conductive metal with gold alloy using EMITECH ion sputter coater to a thickness of 300N, minimizes damage to specimens and improves topographical contrast for improved imaging by SEM. The photographs were taken at 15 KV using FAI QUANTA 250 Model SEM, images were captured and documented.

Molecular characterization

Genomic DNA was extracted from the pure culture of *Chaetomium* sp. using Cetyl Trimethyl Ammonium Bromide (CTAB) method (Murray and Thompson, 1980). The individual isolates were inoculated into Potato Dextrose Broth and incubated at 20±2^{o}C for 7 days. After 7 days, the mycelial mat formed over broth was harvested and the dried mat was powdered using liquid nitrogen. Using CTAB method the genomic DNA was extracted and subjected to PCR with primer pair ITS 1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.,* 1990). The Mastercycler gradient PCR (Eppendorf) was performed with 25µl reaction volume. The thermal cycling program was as follows: Initial denaturation at 94^{o}C for 2 minutes, denaturation at 94 ^{o}C for 1 minute, annealing at 55 ^{o}C for 1 minute, extension at 72 ^{o}C for 2 minutes, final extension at 72 ^{o}C for 10 minutes and 4 ^{o}C for infinity for final holding the samples. A negative control was used instead of DNA template. For each PCR reaction 20 µl were examined by agarose gel electrophoresis. The PCR product was analyzed on 1.2 % agarose gel, stained with ethidium bromide and viewed under UV-transilluminator. For identification, the amplified fragments were sequenced and confirmed using NCBI database.

Results and Discussion

C. globosum is the potential biocontrol antagonist against several plant pathogenic fungi and bacteria (Zhang *et al.,* 2013). *C.globosum* was isolated from different sources in various microhabitats of Tamil Nadu viz., Coimbatore, Coonnoor, Ooty, Yercaud and Salem. Fifteen *C. globosum* were isolated and cultured on PDA medium (Table 1). Isolation of *Chaetomium* through baiting technique took 35-40 days and through serial dilution took 10-12 days. Ascomata from decomposed material were identified using steromicroscope and

transferred into PDA medium, took 9 days for complete mycelial growth. Comparing to serial dilution and baiting technique, ascomatal structures formed over decomposed material were simple and quicker method for *Chaetomium* isolation.

Isolation of *Chaetomium* by different techniques

Seven isolates of *C. globosum* were obtained by baiting technique from rhizoplane of *D. regia. C. sinensis, S. officinarum, P. tuberosa, C. sinensis, O. ficus-indica* and soil from compost pit were designated as TNAU- Cg 101, TNAU- Cg 105, TNAU- Cg 106, TNAU- Cg 109, TNAU- Cg 110, TNAU- Cg 112, and TNAU- Cg 114. Isolate TNAU-Cg 113 was obtained through serial dilution technique. Yadav and Bagool (2015) reported *Chaetomium* spp., were isolated from samples of deteriorated papers by serial dilution method using Czepek Dox Agar (CZA) supplemented with a trace quantity of streptopenicillin. Similarly *C. globosum* were also isolated by carefully picking the ascomata from decaying material, with sterilized forceps and needles and by placing it over the PDA medium amended with rifampicin and incubating them at 20^{o}C for 7-9 days and finally by means of single spore isolation method, pure culture of *Chaetomium* were obtained. Seven isolates were obtained by direct picking of ascomata, developed over the decomposed material was placed directly on the PDA medium. They were designated as TNAU-Cg 102, TNAU-Cg 1032, TNAU-Cg 104, TNAU-Cg 107, TNAU-Cg 108, TNAU-Cg 111, TNAU-Cg 115.

Table.1 Isolates of *Chaetomium* spp. collected from different ecosystem of Tamil Nadu

Sl. No.	Isolate	Source	Origin	Latitude	Longitude
1	TNAU - Cg 101	Rhizoplane Soil of *Delonix regia*	Coimbatore	11° 0' 57.636" N	76°58' 13.116" E
2	TNAU - Cg 102	Decomposed cotton waste	Coimbatore	11° 0' 57.636" N	76°58' 13.116" E
3	TNAU - Cg 1032	Decomposed cotton waste	Coimbatore	11° 0' 57.636" N	76°58' 13.116" E
4	TNAU - Cg 104	Decomposed paper waste	Coonoor	11°20'29.4108" N	76°47'52.548" E
5	TNAU - Cg 105	Rhizoplane Soil –*Camellia sinensis*	Ooty	11°24'23.0904" N	76°41'35.682" E
6	TNAU - Cg 106	Rhizoplane soil –*Saccharum officinarum*	Coimbatore	11°0'24.804"N	76° 55' 5.448" E
7	TNAU - Cg 107	Decomposed paddy straw	Coimbatore	11°07'3.36"N	76°59'39.91" E
8	TNAU –Cg 108	Decomposed litter waste	Salem	11°39'51.5700" N	78°8'45.6396" E.
9	TNAU –Cg 109	Rhizoplane soil –*Polianthes tuberose*	Ooty	11°24'36.0000" N	76°41'59.9892"E
10	TNAU –Cg 110	Compost pit	Coimbatore	11°0'24.804"N	76° 55' 5.448" E
11	TNAU –Cg 111	Decomposed cotton waste	Coimbatore	11°0'24.804"N	76° 55' 5.448" E
12	TNAU –Cg 112	Rhizoplane Soil –*Camellia sinensis*	Ooty	11°24'36.0000" N	76°41'59.9892"E
13	TNAU –Cg 113	Rhizoplane soil- *Citrus aurantifolia*	Yercaud	11°46'30.9"N	78°12'33.33"E
14	TNAU –Cg 114	Rhizosphere soil – *Opuntia ficus-indica*	Coimbatore	11°0'24.804"N	76° 55' 5.448" E
15	TNAU –Cg 115	Areca leaf plates	Ooty	11°24'36.0000" N	76°41'59.9892"E.

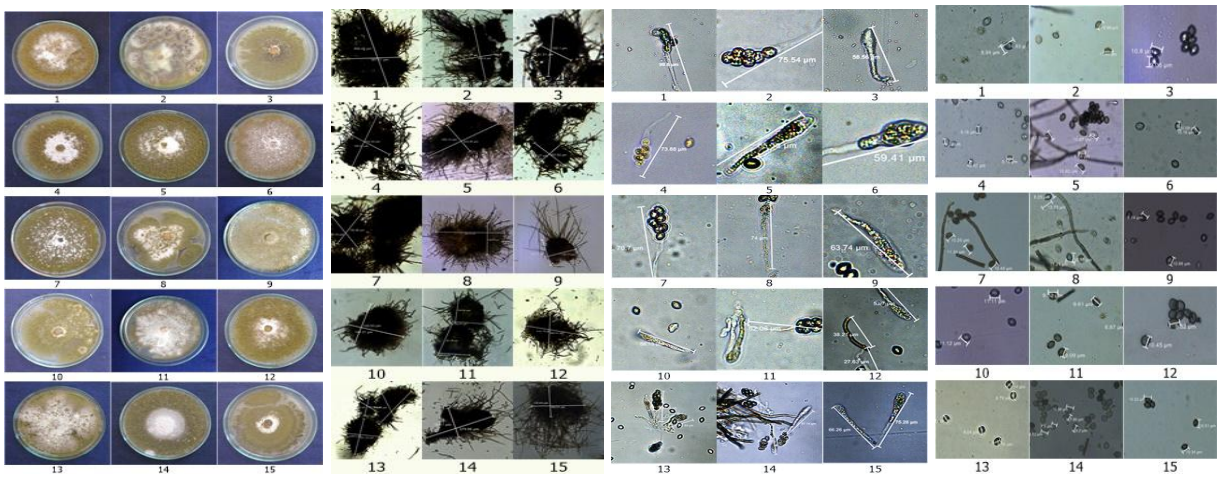
Table.2 Phenotypic characters of different *Chaetomium* spp. isolates on PDA medium

Sl. No.	Isolates	Colony growth	Zonation	Margin	Topography	Matured colonies	Pigmentation
1	TNAU-Cg 101	Rapid	Without zonation	Smooth	Raised fluffy growth	Greenish white	Light yellow
2	TNAU-Cg 102	Rapid	Without zonation	Irregular	Raised fluffy growth	Olive greenish yellow	Dark yellow
3	TNAU-Cg 1032	Moderate	Concentric zonation	Light wavy	Flat mycelail growth	Dark green	Green
4	TNAU-Cg 104	Moderate	Concentric zonation	Smooth	Medium raised growth	Greenish white	Green
5	TNAU-Cg 105	Rapid	Concentric zonation	Smooth	Raised fluffy growth	Greenish white	Yellowish green
6	TNAU-Cg 106	Slow	Concentric zonation	Irregular	Flat mycelail growth	Light brown	Greenisg brown
7	TNAU-Cg 107	Slow	Concentric zonation	Smooth	Flat mycelail growth	Green	Green
8	TNAU-Cg 108	Moderate	Without zonation	Wavy	Medium raised growth	Whitis green	Grayish green
9	TNAU-Cg 109	Moderate	Concentric zonation	Smooth	Flat mycelail growth	Greenish grey	Green
10	TNAU-Cg 110	Moderate	Without zonation	Smooth	Flat mycelail growth	Green	Yellowish green
11	TNAU-Cg 111	Slow	Without zonation	Irregular	Raised fluffy growth	Whitish green	Yellowish white
12	TNAU-Cg 112	Moderate	Concentric zonation	Smooth	Medium raised growth	Green	Green
13	TNAU-Cg 113	Slow	Without zonation	Wavy	Raised mycelial growth	Whitish brown	Light orange
14	TNAU-Cg 114	Slow	Concentric zonation	Smooth	Medium raised growth	Green	Greenish white
15	TNAU-Cg 115	Moderate	Concentric zonation	Irregular	Flat mycelail growth	Green	Light brown

Table.3 Perithecia and ascospres characters of different *Chaetomium globosum* isolates on PDA and their accession numbers

Sl. No.	Isolates	Ascomata (Perithecia)	Ascomatal size (µm) (10x)	Ascomatal hairs	Ascospore size (µm) (40x)	Ascospore shape	Ascus size (µm) (40x)	Sporulation	Accession number
1	TNAU-Cg 101	Mycelial like appendage	593.45 x 387.41	Dark brown, ovate	14.45	Slightly elliptical	99.8	++++	**MK587669**
2	TNAU-Cg 102	Slightly coiled with loose hairs	484.57 x 248.90	Yellowish green	10.88	Lemon shaped	75.54	+++	**MK590290**
3	TNAU-Cg 1032	Undulated with irregular	105.1 x 51.21	Brown, ovate	10.8	Elliptical	58.56	++	**MK592857**
4	TNAU-Cg 104	Mycelial like appendage	242.94 x 159.21	Dark brown, ovate	8.16	Lemon shaped	73.88	++	**MK603862**
5	TNAU-Cg 105	Roughened straight hairs	522.05 x 254.13	Brown, ovate	12.10	Oval	85.12	++++	**MK592858**
6	TNAU-Cg 106	Slightly coiled	258.27 x 207.63	brown, globose	10.09	Slightly elliptical	59.41	+	**MK828135**
7	TNAU-Cg 107	Superficial, wavy	164.53 x 150.98	Light brown, globose	10.25	Lemon shaped	70.7	++	**MK821416**
8	TNAU-Cg 108	Straight with loose hairs	329.23 x 190.94	Light brown, ovate	8.25	Slightly elliptical	74	++	**MK823129**
9	TNAU-Cg 109	Straight with loose hairs	121.18 x 118.17	Light brown, ovate	9.14	lemoniform	63.74	+++	**MK828197**
10	TNAU-C.g 110	Slightly coiled, wavy	164.31 x 149.53	Brown, ovate	11.12	Slighltly elliptical	58.13	++	**MK603941**
11	TNAU-Cg 111	Straight with loose hairs	136.84 x 123.97	Black, oavate	9.32	Lemon shaped	52.08	+	**MK828136**
12	TNAU-Cg 112	Undulated, Coiled or straight	412.19 x 360.51	brown, ovate	9.52	Lemon shaped	53.7	++	**MK757844**
13	TNAU-Cg 113	Straight with less hairs	331.33 x 239.50	brown, globose	9.79	Slightly elliptical	47.82	++	**MK820066**
14	TNAU-Cg 114	Straight at lateral	379.88 x 194.10	brown, globose	10.36	Lemon shaped	57.14	++	**MK828133**
15	TNAU-Cg 115	Mycelail like appendage with tuff hairs	182.57 x 130.62	Light brown, ovate	10.22	Lemon shaped	75.28	++	**MK828134**

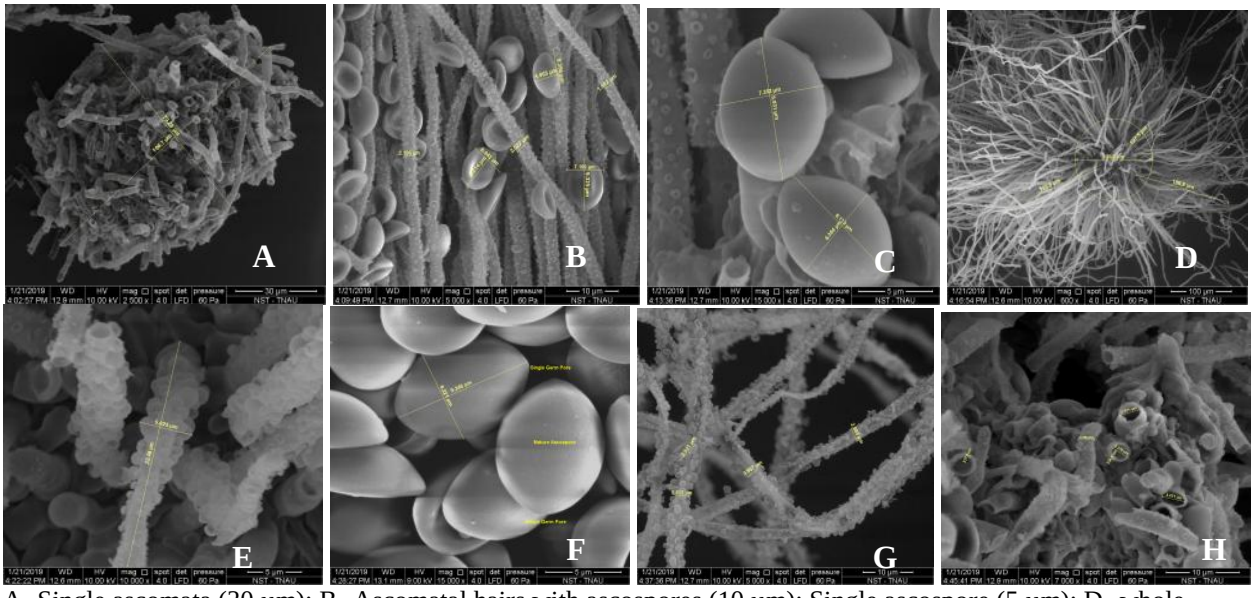
Fig.1 Morphological characters of different isolates of *Chaetomium globosum* on potato dextrose agar medium



A - Cultural charcter of 15 *C. globosum* isolates on PDA medium; B – Ascomata of 15 *C. globosum* isolates; C- Ascus of 15 *C. globosum* isolates; D- Ascospores of 15 *C. globosum* isolates

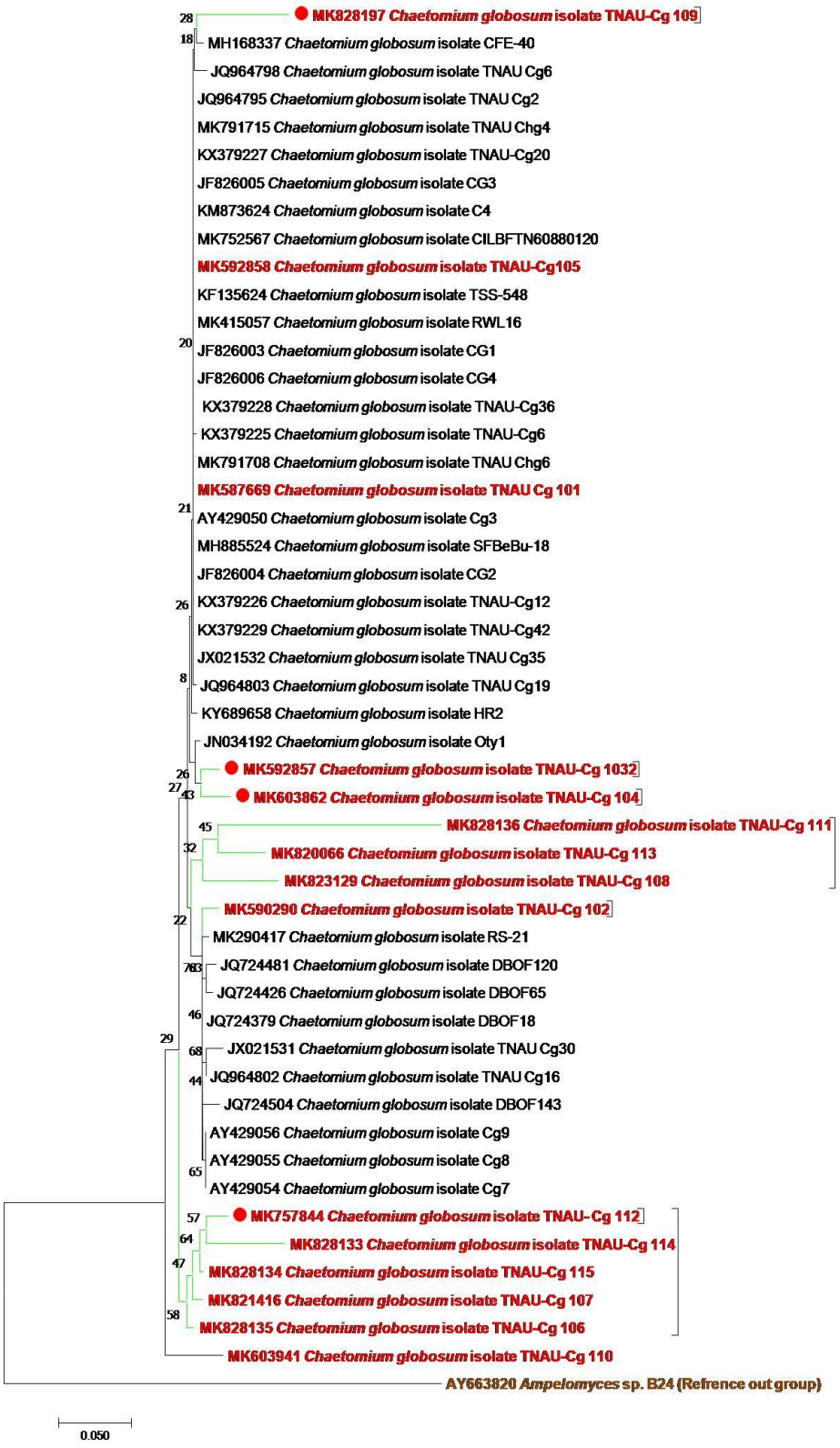
C. globosum isolate -TNAU-Cg 101; 2- *C. globosum isolate* -TNAU-Cg 102; 3- *C. globosum isolate* -TNAU-Cg 1032; 4- *C. globosum isolate* -TNAU-Cg 104; 5- *C. globosum isolate* -TNAU-Cg 105; 6- *C. globosum isolate* -TNAU-Cg 106; 7- *C. globosum isolate* -TNAU-Cg 107; 8- *C. globosum isolate* -TNAU-Cg 108; 9- *C. globosum isolate* -TNAU-Cg 109; 10- *C. globosum isolate* -TNAU-Cg 110; 11- *C. globosum isolate* -TNAU-Cg 111; 12- *C. globosum isolate* -TNAU-Cg 112; 13- *C. globosum isolate* -TNAU-Cg 113; 14- *C. globosum isolate* -TNAU-Cg 114; 15- *C. globosum isolate* -TNAU-Cg 115

Fig.1 SEM image of *C.globosum* TNAU-Cg 101



A- Single ascomata (30 µm); B- Ascomatal hairs with ascospores (10 µm); Single ascospore (5 µm); D- whole ascomata with ascomatal hairs (100 µm); E- Ascus (5 µm); F- Mature ascospore with germ pore (5 µm); G- ascomatal hairs (10 µm); H- opening of ascus wall with pore (µm)

Fig.3 Phylogenetic tree of different *Chaetomium globosum* isolates



Cultural and morphological characters of *C. globosum*

Usually the *Chaetomium* species were identified through morphological characters (Pornsuriya *et al.,* 2008, Von Arx *et al.,* 1986; Soytong and Quimio, 1989; Rodriguez *et al.*, 2002). The morphological characters of different isolates of *C.globosum* viz., mycelium, topography, colour, margin of colonies, zonation, colony growth, ascus and ascospores shape and size, were assessed (Table 2). The topography of all the fifteen isolates varied from raised fluffy growth to flat mycelial growth Zonation were observed in isolates like TNAU-Cg 1032, TNAU-Cg 104, TNAU-Cg 105, TNAU-Cg 106, TNAU-Cg 107, TNAU-Cg 109, TNAU-Cg 112, TNAU-Cg 114 and TNAU-Cg 115. Matured colonies were greenish white to olive greenish yellow. Pigmentation varied from greenish white to dark yellow. TNAU-Cg 102 and TNAU-Cg 105 were fast growing isolates which covered the plate within 9 days and TNAU-Cg 109 and TNAU-Cg 115 were slow growing isolates which required 15-17 days to cover the entire plate. The morphological observation through stereomicroscope revealed the presence of sub – globose ascomata or elongated perithecia. The size varied from 105.1 – 593.45 x 51.21 - 387.41 µm dia with straight or hooked or curled ascomatal hairs.

Then, the microscopic observation conformed that *Chaetomium* produced brown or olive brown color ascospores which was slightly elliptical or lemon shaped or oval around 8-14 µm dia with an single germpore and with an ascus wall of about 47- 99 µm dia. Perithecia and ascospres characters of different *Chaetomium* isolates on PDA medium are listed in Table 3. This was in accordance with Von Arx *et al.,* (1986) who reported that *C. globosum* was characterised with globose, ovate or obovate ostiolate ascomata; flexuous or coiled ascomatal hairs; evanescent asci, clavate or slightly fusiform; ascospores lemoniform, bilaterally flattened, 9–12 × 8–10 × 6–8 µm in size, with an apical germ pore. SEM image of *C.globosum* confirmed the presence of whole perithecia with osteolar pores. The hairs under SEM of entangled ascospores, for effective dispersal. The ascospores were smooth, oval in shape with 8-9 µm length and 6-7 µm width with the presence of single apical germ pore. The single whole ascomata showed tuft of slightly curved ascomatal hairs of different lengths varying from 150-170 µm (Fig. 2) Ahammed *et al.*,(2005) also reported that SEM image of ascospore were lemon shaped and with apical papillae at both ends indicating the presence of germpores.

Molecular characterization of *C.globosum*

The *Chaetomium* isolates were subjected to PCR using universal primer corresponding to 18S rDNA gene intervening sequencing. The results revealed that all the fifteen isolates were amplified with the amplicon size of 560bp. The genomic products were sequenced and all the 15 isolates were identified as *C. globosum.* The NCBI – BLAST search revealed that all the isolates had 92-99% homology with the deposited strains at NCBI. These isolates were submitted in NCBI database and assigned with accession numbers (Table 3). The Internal Transcribed Spacer (ITS) of rRNA gene were used for the molecular confirmation of *C. globosum* using the universal primer pair ITS1 and ITS4 which had an amplicon of 560 bp (Fig. 3). Sekhar *et al.,* (2018) also reported that combination with universal primer sequences ITS 1 and ITS 4 region was found to be 500-650bp. The phylogenetic analyses include 15 isolates, using MEGA 7 software with *Ampelomyces* sp. (AY663820) as reference out group with an boostrap value of 100% (Fig. 3).

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