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Studies on Phylogeny of *Chaetomium* Species of India

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A B S T R A C T

A set of 44 *Chaetomium* isolates from Delhi-NCR region were collected and molecularly characterized and confirmed using ITS sequences from NCBI database as *C. atrobrunneum, C. brasiliense, C. elatum, C. funicola, C. globosum, C. megalocarpum, C. nigricolor* and *C. perlucidum.* Cluster analysis using maximum parsimony phylogenetic tree for 44 isolates of *Chaetomium* executed among the six gene regions *viz.*, actin, β-tubulin, calmodulin, ITS, *rpb2* and *tef-1*. The grouping of *Chaetomium* species using actin appeared either totally or partially heterogenous grouping. Even though with β-tubulin, the isolates of *Chaetomium* were not grouped in homogenous manner, interspecific diversity was higher in comparison to intraspecific diversity. Total heterogeneous grouping was observed for the *Chaetomium* species using the calmodulin sequences. Among all the regions studied in this study for grouping the most diversified grouping was observed with *rpb2* gene. Better homogeneity was observed even with *tef-1* region. But among all ITS was established as the best region for grouping of *Chaetomium* species.

Introduction

Chaetomium is a fungus can be exploited economically and commercially. This fungus is extensively used in degradation of cellulolytic material (Umikalsom *et al.*, 1997 & 1998). In the field of Agriculture this organism has been employed as a biocontrol for reducing the disease incidence against several plant pathogens (Soytong *et al.*, 2001, Aggarwal *et al.*, 2004, Dhingra, *et al.*, 2003). This fungus has wide distribution having more than 160 recognized species (Wang *et al.*, 2014). In India alone reports have been suggested that more than 60 species (http:// www.indiabiodiversity.org/species/) were occurring. The contemporary species concept for this fungus includes a broadly defined morphological diversity as well as a large number of synonymies with limited phylogenetic evidence (Wang *et al.*, 2016). Thus it is necessary to find an alternative method for accurate identification of the species and grouping of this genus. The advent of molecular tools for investigations in fungal identification has paved better way for easier and more accurate identification. Furthermore very limited knowledge is known for molecular identification for this fungus (Asgari and Zare, 2011, Sharma *et al.*, 2013). Thus an attempt has been made to identify species and accurate grouping for different species of *Chaetomium* based on molecular sequencing data of *Chaetomium*.

Materials and Methods

Collection, molecular identification and characterization of *Chaetomium* species

The investigation was started with collecting different samples from different parts of Delhi- NCR region. And the samples were isolated and identified based on basic generic character of *Chaetomium*. The details of the isolates collected was mentioned in the Table 1.Total forty four samples were confirmed as *Chaetomium* and were used for the present investigation. The main objective of the investigation was to construct the phylogenetic trees to differentiate *Chaetomium* species. For this purpose six gene regions were considered viz., ITS, *tef-1*, *rpb2* and β-tubulin, actin and calmodulin (Santamaria *et al.*, 2009). Initially molecular identification and characterization was carried out by amplification and sequencing of ITS region. Subsequently multigene phylogeny was undertaken to know the best region for grouping of species by using above said six regions.

Molecular characterization of collected isolates of *Chaetomium* species using ITS region

DNA extraction

Genomic DNA was extracted from all the forty four isolates of *Chaetomium* using monosporic cultures by CTAB (Cetyltrimethyl Ammonium Bromide) method (Culling, 1992). 0.2g of mycelium mat of seven days old was collected from potato dextrose broth and grounded in sterilized pestle and mortar using liquid nitrogen and transferred to 1.5 ml eppendorf micro tubes. 600 µl of preheated (60^{o}C) 2× CTAB extraction buffer (2 % (w/v) CTAB, 100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, pH 8.0) was added to the eppendorf micro tubes. The solution was incubated at 60^{o}C for one hour in water-bath with occasional gentle stirring. To this solution an equal volume of chloroform and isoamyl alcohol (24:1) was added and mixed thoroughly. The mixture was subsequently centrifuged at 10,000 rpm for 20 min at 24^{o}C. Aqueous phase was separated and transferred to a fresh tube. To this aqueous phase an equal volume of chloroform and isoamyl alcohol (24:1) was added and mixed thoroughly and centrifuged at 10,000 rpm for 20 min at 24^{o}C. These steps were repeated 2- 3 times till a clear aqueous phase was obtained. To this clear aqueous phase 0.6 volume of ice cold isopropanol and 0.1 volume of sodium acetate buffer (3 M) was added and incubated at −20^{o}C for 30 minutes. DNA was precipitated by centrifuging at 10,000 rpm for 10 min at 4^{o}C. The precipitate was treated with 75 % ethanol and centrifuged at 10,000 rpm for 10 min at 4^{o}C. Aqueous phase was discarded and DNA was dried under a regular air flow for 20 min, re-suspended in 70µl TE buffer and stored at −20^{o}C. The presence of DNA in the samples was further confirmed by separating them on 0.8 % agarose gel at 80 volts for 45 min using gel electrophoresis unit. The concentration of DNA was measured through spectrophotometrically using Nano drop 2000 spectrophotometer.

PCR amplification using internal transcribed spacer (ITS)

The molecular identification of the purified isolates was done by using with the sequencing of the internal transcribed spacer (ITS) sequences. The amplification of ITS region was carried out using universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGAT ATGC-3') (White *et al.*, 1990). The DNA fragment consisting of 3' end of the 18S rDNA, ITS1, the 5.8 rDNA, ITS2 and the 5' end of the 28S rDNA was amplified using ITS1 and ITS4 primers. PCR reactions were

carried out in 0.2ml thin walled PCR tubes with a total reaction volume of 25µl containing 12.5 µl of Dream *Taq* (2X) of ThermoScientific make (master mix consisting of buffer, dNTP's, $MgCl_2$, *Taq* DNA polymerase at appropriate concentrations and pre mix of loading dye), 1 µl (10 Pmol/µl) of each forward and reverse primers, 1 µl (100 ng/ µl) of DNA sample and nuclease free water. The PCR amplification conditions were initial denaturation at 94^{o}C for 5 min, 35 cycles of denaturation at 94^{o}C for 1 min, primer annealing at 55^{o}C for 1 min, primer extension at 72^{o}C for 2 min, followed by final primer extension at 72^{o}C for 5 min.

Sequencing of the amplified ITS fragments

The amplified products were separated on 1.2% agarose gel at 80 volts for 45 min using 1x TAE buffer (pH 8.0) containing ethidium bromide. The gels were photographed using gel documentation system. Amplicons of 500 to 650 bp were selected for sequencing the ITS region. For size selection a co-resolved 100 bp ladder was used. Sequencing of all the samples with distinct band was done through Eurofins Scientific, Bangalore.

Identification of *Chaetomium* species through ITS sequences

Molecular identification of *Chaetomium* spp. was done using nucleotide sequences of ITS region through NCBI (National Centre for Biotechnology Information) BLAST (Basic Local Alignment Search Tool) (webpage: http://blast.ncbi.nlm.nih.gov) and the sequences were submitted to NCBI Genbank.

Multigene (actin, β-tubulin, calmodulin, *rpb2* and *tef*-1) phylogeny of the isolates of *Chaetomium* spp.

The *Chaetomium* DNA isolated earlier for ITS amplification was used for the PCR amplification of the above genes.

PCR amplification of actin, β-tubulin, calmodulin, *rpb2* and *tef-1* genes

The genes (actin, β-tubulin, calmodulin, *rpb2* and *tef-1*) regions were amplified using the primers given in Table 2.

Sequencing of the amplified actin, β-tubulin, calmodulin, *rpb2*and *tef-1* fragments

The amplified products of actin, β-tubulin, calmodulin, *rpb2* and *tef-1*gene regions were separated on 1.2% agarose gel containing ethidium bromide at 80 volts for 45 min using 1x TAE buffer with pH 8.0. The amplified fragments of DNA were compared with ladder of 100 bp. The gels were photographed using gel documentation system. Sequencing of all the above regions of the samples with distinct band was done through Eurofins Scientific, Bangalore.

Phylogenetic analysis

Multiple sequence alignment of the above regions along with ITS region was performed using the Clustal W algorithm of MEGA 6.0 software. Phylogenetic tree was constructed using maximum parsimony (MP) analysis. Confidence values were assessed from 1000 bootstrap replicates of the original data.

Results and Discussion

Molecular identification and phylogenetic analysis of *Chaetomium* species

Molecular identification of *Chaetomium* isolates based on ITS region

PCR amplification of all the forty four isolates of *Chaetomium* was done using the primers ITS1 and ITS4. ITS is a conserved rDNA sequence that has been widely used both alone and in combination with other universal sequences, such as β-tubulin, actin, etc., to

identify, characterize, and to perform phylogenetic analysis of fungal isolates (Balazy *et al.,* 2008). The sequence length of ITS region was found to be 500-650bp (Approx.) (Fig. 1). Molecular identification of *Chaetomium* spp. using ITS region sequences was done through NCBI BLAST (webpage: http://blast.ncbi.nlm.nih.gov) for the species identification. The identification percentage was found to be 96-100%. The ITS sequences were submitted to NCBI and accession numbers were acquired (Table 3).

Multigene phylogenetic analysis

The identification of *Chaetomium* species confirmed through the molecular sequences of ITS region were used for further analysis. To perform multigene phylogenetic analysis, five regions were considered *viz.*, actin, β-tubulin. calmodulin, *rpb2* and *tef-1*along with ITS region.

PCR amplification of actin, β-tubulin. calmodulin, *rpb2* and *tef-1*was done using respective primers as shown in Table 2.The amplified products were separated and sequenced. An approximate length of 250 bp (Fig. 2), 500 bp (Fig. 4), 900 bp (Fig.6), 1050 bp (Fig.8) and 250 bp (Fig.10) of actin, β-tubulin, calmodulin, *rpb2* and *tef-1* respectively were obtained.

Only 23 isolates showed amplification of calmodulin gene. All the forward reaction sequenced data were used for the five regions of 44 samples along with ITS to construct dendrogram and evolutionary analyses.

The maximum parsimony tree was obtained using the subtree-pruning-regrafting (SPR) algorithm (Nei and Kumar, 2000). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches (Felsenstein, 1985).

Phylogenetic analysis of Actin region

It was evident from the dendrogram (Fig.3) that the different species of *Chaetomium* grouped into six major clusters. Two isolates of *C. perluicdum* (C-73 and C-81), six isolates of *C. atrobrunneum* (C-68, C-19, C-61, C-03, C-18 and C-20) and eight isolates of *C. megalocarpum* (C-48, C-22, C-21, C-23, C-70, C-65, C-77 and C-66) made into different Clusters (Clusters 3, 4 and 6). *C. brasiliense* isolates (C-07, C-45, C-50, C-46 and C-76) grouped in Cluster 1 along with one isolate of *C. nigricolor* (C-55). Three isolates of *C. globosum viz.*, C-42, C-62 and C-15 grouped with *C. funicola* isolates (C-12, C-16, C-80, and C-17) in Cluster 2. Most heterogeneous grouping was observed in Cluster 5 wherein 12 isolates of *C. globosum* (C-51, C-08, C-10, C-57, C-59, C-60, C-05, C-74, C-11, C-72, C-40 and C-58) were grouped with one isolate of *C.elatum* (C-02), one isolate of *C. atrobrunneum* (C-78) and one isolate of *C. funicola* (C-47).

The grouping of *Chaetomium* species was good in Clusters 3, 4 and 6 using actin sequences but in the remaining clusters it appeared either totally or partially hetereogenous grouping.

Phylogenetic analysis of β-tubulin region

The dendrogram (Fig.5) showed that the different species of *Chaetomium* were grouped into six clusters. The isolates (C-70, C-77, C-65, C-66, C-21, C-22 and C-23) of *C. megalocarpum* were grouped in Cluster 1 yet one isolate (C-48) of this species grouped with other species of *Chaetomium* in cluster 4. In cluster 2 the isolates (C-51, C-08, C-10, C-05, C-42, C-72, C-59, C-11 and C-57) of *C. globosum* grouped with one isolate (C-02) of *C. elatum* and one isolate (C-07) of *C. brasiliense*. In cluster 3 all the isolates (C-12, C-80, C-16, C- 47 and C-17) of *C. funicola*

grouped with two isolates of *C. globosum viz.*, C-15 and C-40. Most heterogeneous grouping was observed in the cluster 4 in which four isolates (C-74, C-60, C-58 and C-62) of *C.globosum* were present with one isolate of each *C. megalocarpum* (C-48), *C. atrobrunneum* (C-68), *C. brasiliense* (C-45) and *C. nigricolor* (C-55).

In Cluster 5 six isolates of *C. atrobrunneum* (C-78, C-03, C-20, C-18, C-19 and C-61) two isolates of *C. perlucidum* (C-81 and C-73) and one isolate of *C. brasiliense* (C-76) grouped together. Cluster 6 was the smallest one with two isolates of *C. brasiliense* (C-46 and C-50). Even though, the isolates of *Chaetomium* were not grouped in homogenous manner, interspecific diversity was higher in comparison to intraspecific diversity.

Phylogenetic analysis of Calmodulin region

Heterogeneous grouping was observed for the *Chaetomium* species using the calmodulin sequences. All the species were grouped into three major clusters (Fig 7). In Cluster 1, *C. globosum* isolates (C-40, C-10, C-51 and C-05) grouped with two isolates (C-50 and C-07) of *C. brasiliense* and one isolate (C-02) of *C. elatum*. In Cluster 2, four isolates *viz.*, C-22, C-70, C-77 and C-23 of *C. megalocarpum* grouped with two isolates (C- 68 and C-78) of *C. atrobrunneum* and one isolate (C-55) of *C. nigricolor* and in Cluster 3, two isolates of each *C. globosum* (C-08 and C-72), *C. megalocarpum* (C-21 and C-48), *C. brasiliense* (C-76 and C-46) and *C. atrobrunneum* (C-19 and C-18) and one isolate of *C. funicola* (C-80) grouped together.

Table 1. List of *Chaetomium*isolates collected from different sources and places

Sl No	Isolate	Source	Place of Collection
1	C-02	Soil	New Delhi
2	C-03	Soil	New Delhi
3	C-05	Soil	New Delhi
4	C-07	Soil	New Delhi
5	C-08	Soil	New Delhi
6	C-10	Soil	New Delhi
7	C-11	Soil	New Delhi
8	C-12	Soil	New Delhi
9	C-15	Chilli seed	New Delhi
10	C-16	Mushroom Compost	New Delhi
11	C-17	Dried Cow Dung	New Delhi
12	C-18	Bottle brush tree leaf	New Delhi
13	C-19	Soybean seeds	New Delhi
14	C-20	Paper	New Delhi
15	C-21	Neem bark	New Delhi
16	C-22	Soil	Gurgoan
17	C-23	Soil	Gurgoan
18	C-40	Soil	Noida
19	C-42	Mushroom compost	Noida
20	C-45	Soil	Rohthak
21	C-46	Soil	Rohthak
22	C-47	Soil	Rohthak
23	C-48	Wheat grain	Rohthak
24	C-50	Soil	Hansi
25	C-51	Mushroom compost	Hansi
26	C-55	Mushroom compost	Sonipat
27	C-57	Soil	Hisar
28	C-58	Mushroom compost	Hisar
29	C-59	Sorghum seed	Hisar
30	C-60	Wheat grain	Hisar
31	C-61	Paper	Hisar
32	C-62	Neem Bark	Hisar
33	C-65	Mushroom compost	New Delhi
34	C-66	Fennel seed	New Delhi
35	C-68	Cardamom seed	New Delhi
36	C-70	Sorghum seed	New Delhi
37	C-72	Sorghum seed	New Delhi
38	C-73	Coriander seed	New Delhi
39	C-74	Cardamom seed	New Delhi
40	C-76	Coriander seed	New Delhi
41	C-77	Fennel seed	New Delhi
42	C-78	Paper	New Delhi
43	C-80	Fennel seed	New Delhi
44	C-81	Fenugreek seed	New Delhi

Table.2 Primers used for PCR amplification of different gene regions

Sl. No	Region for amplification	Primers	Primer sequences	Reference
1	Actin	ACT-512F	5'- ATGTGCAAGGCCGGTTTCGC-3'	Carbone and Kohn, 1999
		ACT-783R	5'- TACGAGTCCTTCTGGCCCAT-3'	
2	β-tubulin	Bt2aF	5'- GGTAACCAAATCGGTGCTGCTTTC-3'	Glass and Donaldson 1995
		Bt2bR	5'- AACATCAGTGTAGTGACCATTGGC-3'	
3	Calmodulin	CAL-228F	5'- GAGTTCAAGGAGGCCTTCTCCC-3'	Carbone and Kohn, 1999
		CAL-737R	5'- CATCTTTCTGGCCATCATGG-3'	
4	*rpb2*	fRPB2-5F	5'- GAYGAYMGWGATCAYTTYGG-3'	Liu *et al.*, 1999
		fRPB2-7cR	5'- CCCATRGCTTGYTTRCCCAT-3'	
5	*tef-1*	EF1-728F	5'- CATCGAGAAGTTCGAGAAGG-3'	Carbone and Kohn, 1999
		EF1-928R	5'- TACTTGGAAGGAACCCTTACC-3'	

Table 3. Identification of *Chaetomium* isolates based on NCBI Database using ITS region

Sl No	Isolate	ITS identification from NCBI Database	NCBI Accession number
1	C-02	*C. elatum*	KT357641
2	C-03	*C. atrobrunneum*	KT357642
3	C-05	*C. globosum*	KT357644
4	C-07	*C. brasiliense*	KT357646
5	C-08	*C. globosum*	KT357647
6	C-10	*C. globosum*	KT357648
7	C-11	*C. globosum*	KT357649
8	C-12	*C. funicola*	KT357650
9	C-15	*C. globosum*	KT357653
10	C-16	*C. funicola*	KT357654
11	C-17	*C.funicola*	KT357655
12	C-18	*C. atrobrunneum*	KT357656
13	C-19	*C. atrobrunneum*	KT357657
14	C-20	*C. atrobrunneum*	KT357658
15	C-21	*C. megalocarpum*	KT357659
16	C-22	*C. megalocarpum*	KT357660
17	C-23	*C. megalocarpum*	KT357661
18	C-40	*C. globosum*	KT357677
19	C-42	*C. globosum*	KT357679
20	C-45	*C. brasiliense*	KT357682
21	C-46	*C. brasiliense*	KT357683
22	C-47	*C. funicola*	KT357684
23	C-48	*C. megalocarpum*	KT357685
24	C-50	*C.brasiliense*	KT357687
25	C-51	*C. globosum*	KT357688
26	C-55	*C. globosum*	KT357692
27	C-57	*C. globosum*	KT357694
28	C-58	*C. globosum*	KT371324
29	C-59	*C. globosum*	KT371325
30	C-60	*C. globosum*	KT371326
31	C-61	*C. atrobrunneum*	KT371327
32	C-62	*C. globosum*	KT371328
33	C-65	*C. megalocarpum*	KT371330
34	C-66	*C. megalocarpum*	KT371331
35	C-68	*C. atrobrunneum*	KT371333
36	C-70	*C. megalocarpum*	KT371335
37	C-72	*C. globosum*	KT371336
38	C-73	*C. perlucidum*	KT371337
39	C-74	*C. globosum*	KT371338
40	C-76	*C. brasiliense*	KT371339
41	C-77	*C. megalocarpum*	KT371340
42	C-78	*C. atrobrunneum*	KT371341
43	C-80	*C. funicola*	KT371343
44	C-81	*C. perlucidum*	KT371344

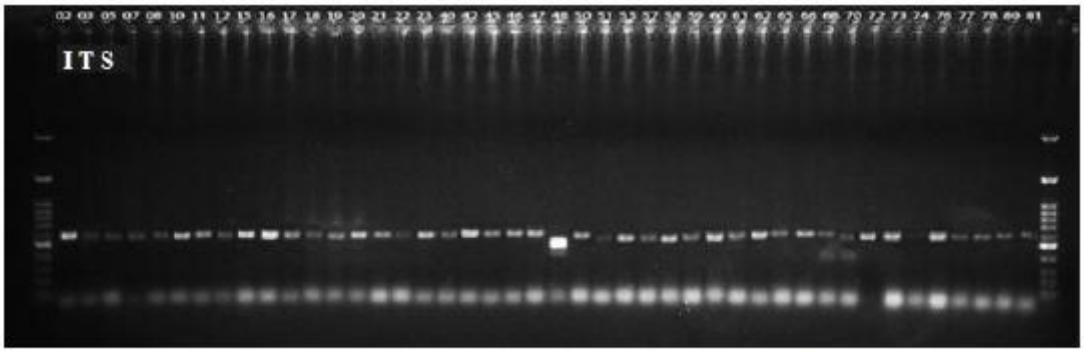

Fig 1: partial amplification of ITS region for 44 isolates of *Chaetomium*

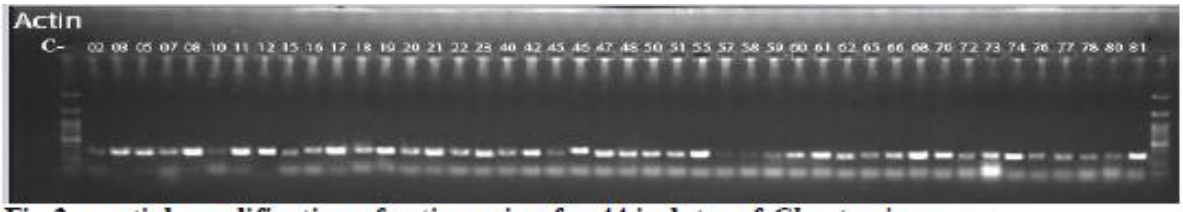

Fig 2: partial amplification of actin region for 44 isolates of *Chaetomium*

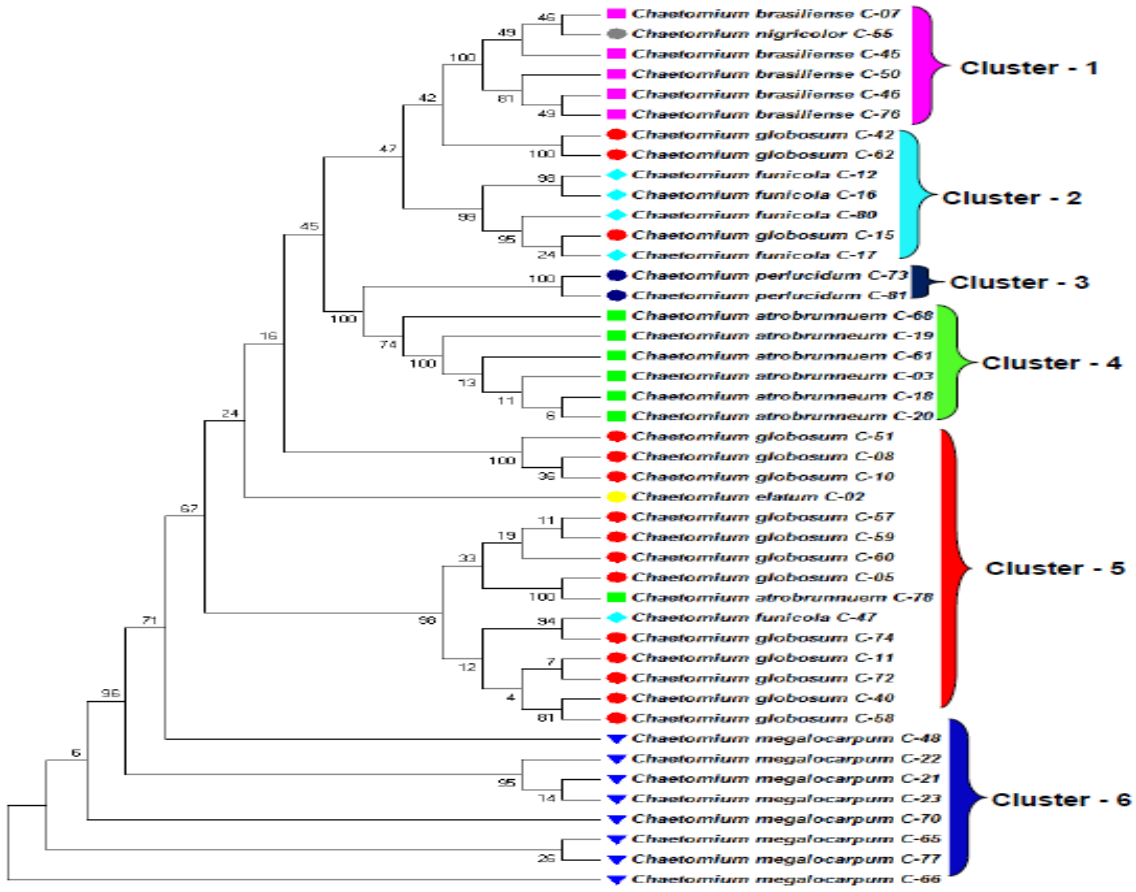

Fig 3: Phylogenetic relationship of 44 isolates of *Chaetomium* inferred by Actin sequences by using maximum parsimony analysis

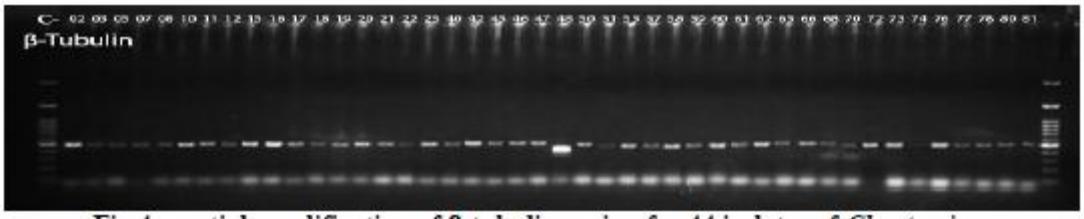

Fig 4: partial amplification of β-tubulin region for 44 isolates of *Chaetomium*

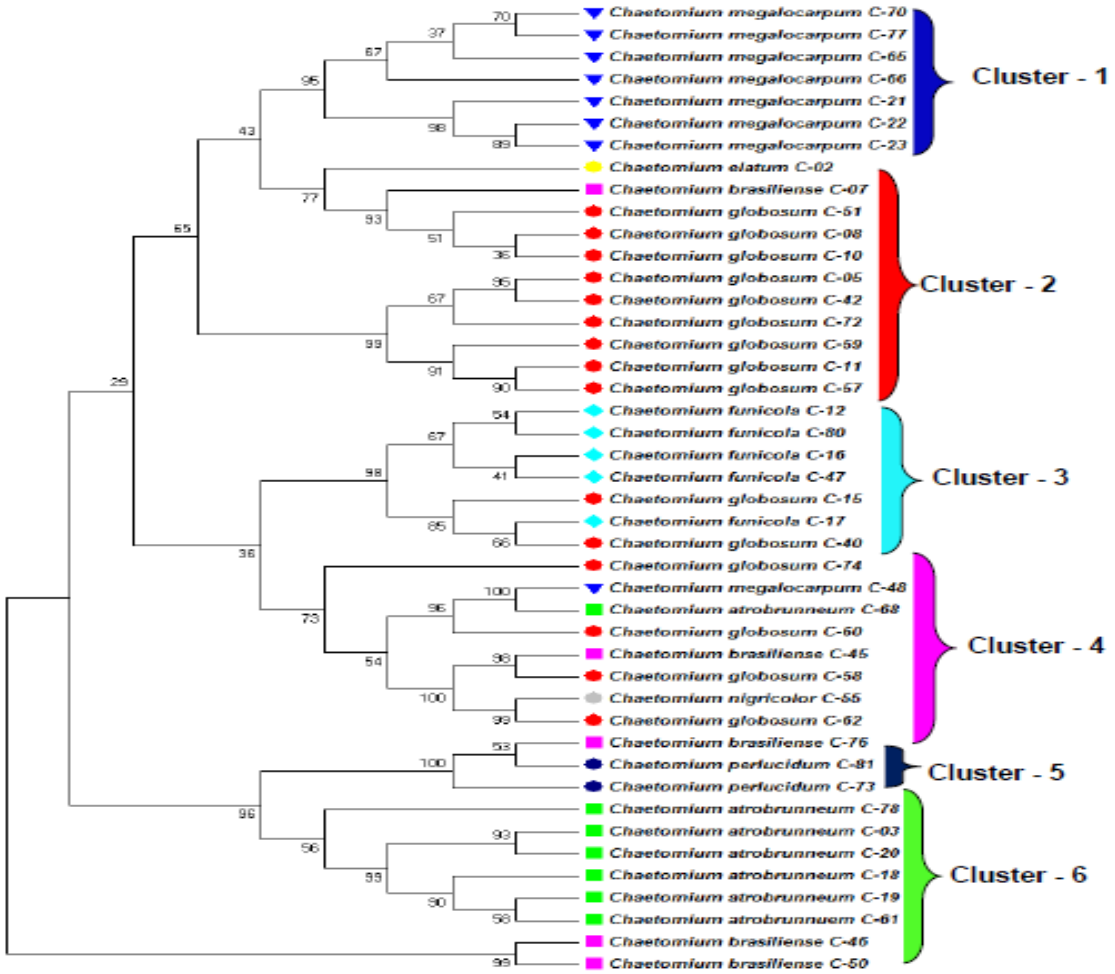

Fig 5: Phylogenetic relationship of 44 isolates of *Chaetomium* inferred by β-tubulin sequences by using maximum parsimony analysis

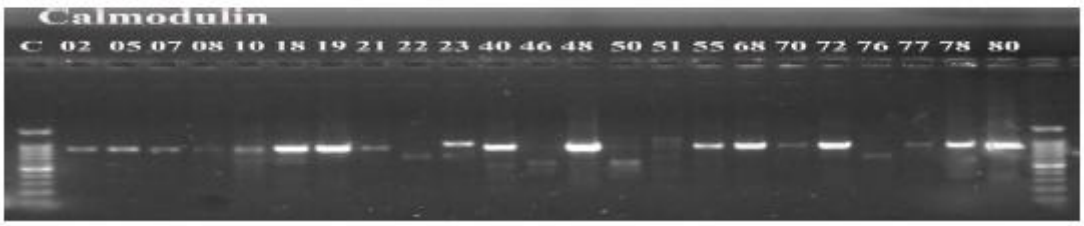

Fig 6: partial amplification of Calmodulin region for 23 isolates of *Chaetomium*

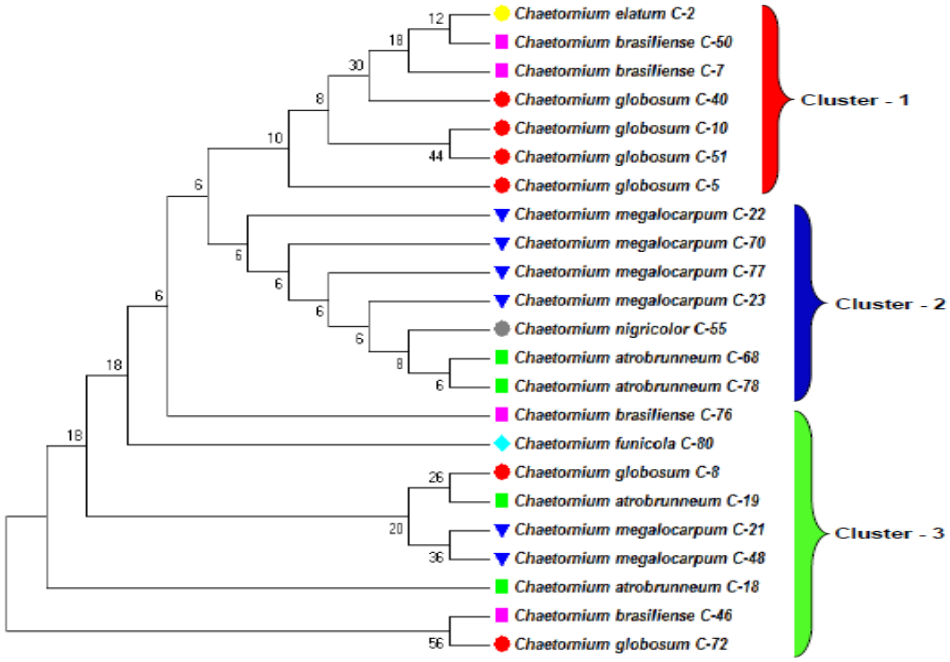

Fig 7: Phylogenetic relationship of 23 isolates of *Chaetomium* inferred by Calmodulin sequences by using maximum parsimony analysis

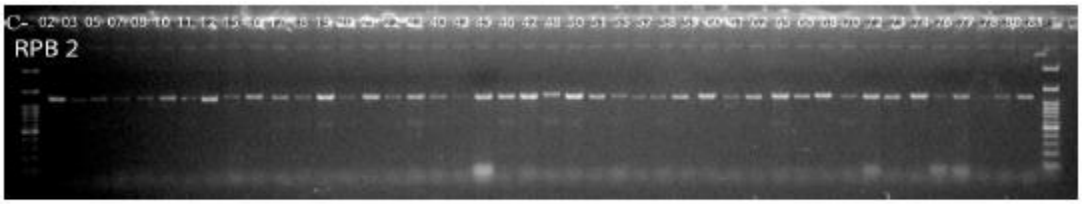

Fig.8:Amplification of *rpb2* gene in 44 isolates of *Chaetomium*

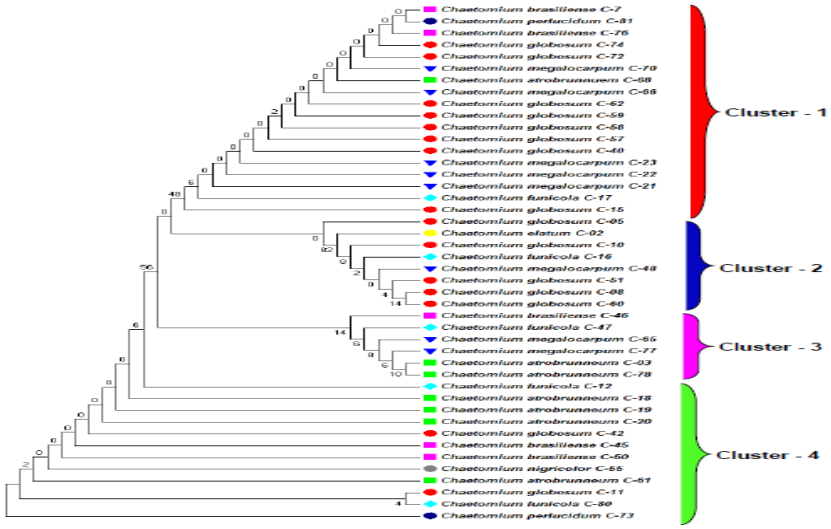

Fig 9: Phylogenetic relationship of 44 isolates of *Chaetomium* inferred by *rpb2*sequences by using maximum parsimony analysis

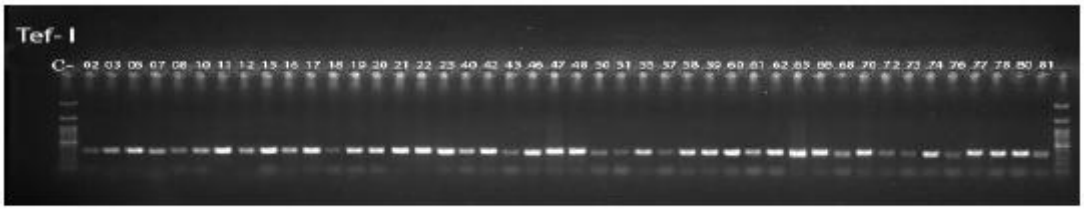

Fig 10: partial amplification of *tef-1* region for 44 isolates of *Chaetomium*

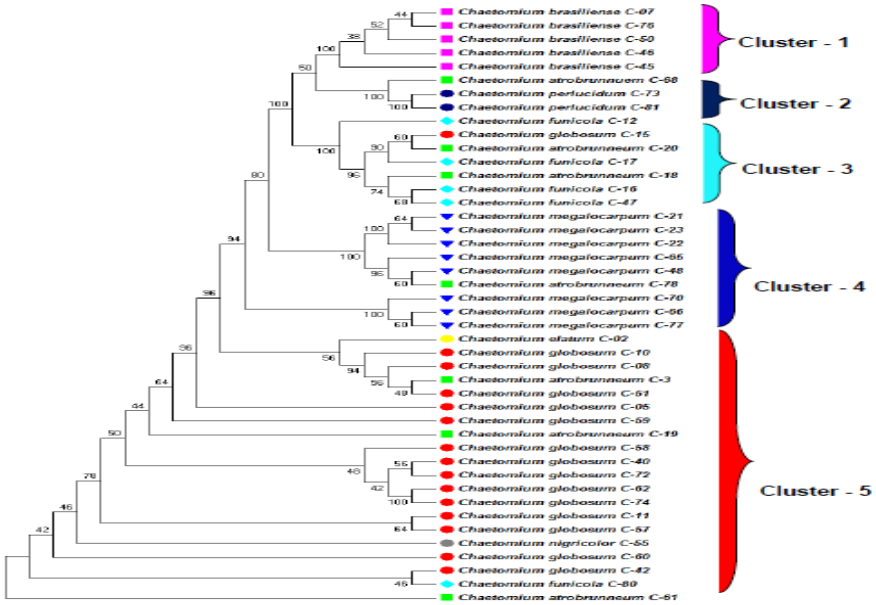

Fig 11: Phylogenetic relationship of 44 isolates of *Chaetomium* inferred by *tef-1* sequences by using maximum parsimony analysis

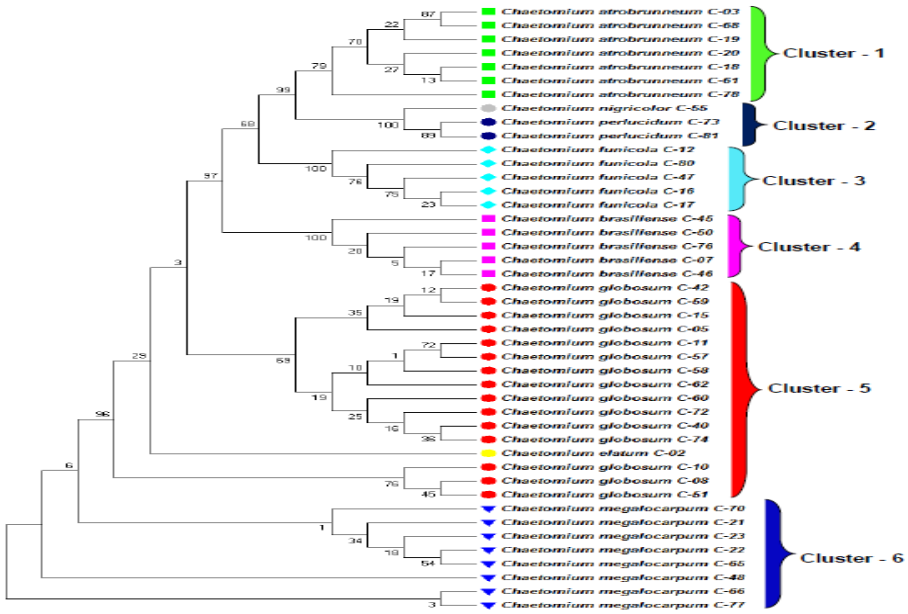

Fig 12: Phylogenetic relationship of 44 isolates of *Chaetomium* inferred by ITSsequences by using maximum parsimony analysis

Phylogenetic analysis of *rpb2* region

It was inferred from the dendrogram (Fig. 9) that the different *Chaetomium* species grouped into four major clusters. Among all the regions studied for grouping the most diversified grouping was observed with this gene. In Cluster 1, the isolates of *C. globsum* (C-74, C-72, C-62, C-59, C-58, C-57, C-40 and C-15) grouped with *C.brasiliense* (C-07 and C-76), *C. perlucidum* (C-81), *C. megalocarpum* (C-70, C-66, C-23, C-22 and C-21), *C. atrobrunneum* (C-68) and *C.funicola* (C-17) made a grouping. In Cluster 2, the isolates (C-05, C-10, C-51, C-08 and C-60) of *C. globosum* were grouped with one isolate of each *C. elatum* (C-02), *C. funicola* (C-16) and *C. megalocarpum* (C-48). In the six member Cluster no.3, two isolates of *C. megalocarpum* (C-65 and C-77), two isolates of *C. atrobrunneum* (C-03 and C-78), one isolate of *C. brasiliense* (C-46) and one isolate of *C. funicola* (C-47) were found together. One isolate of *C. funicola* (C-12), three isolates of *C. atrobrunneum* (C-18, C-19, C-20 and C-61), two isolates of C. *globosum* (C-42 and C-11), two isolates of *C. brasiliense* (C-45 and C-50), one isolate of *C. nigricolor* (C-55) and one isolate of *C. perlucidum* (C-73) were present in Cluster 4.

Phylogenetic analysis of *tef-1* region

The phylogenetic grouping using *tef-1* sequences is depicted in Figure 11. Different *Chaetomium* species were grouped into five major clusters. All the isolates of *C. brasiliense* (C-07, C-76, C-50, C-46 and C-45) were grouped in Cluster 1. In the Cluster 4 all the isolates of *C. megalocarpum* (C-21, C-23, C-22, C-65, C-48, C-70, C-66 and C-77) were grouped along with one isolate of *C. atrobrunneum* (C-78). The isolates of *C. perlucidum viz.*, C-73 and C-81 with one isolate of *C. atrobrunneum* (C-68) grouped in Cluster 2. In Cluster 3 along with isolates of *C. funicola viz.*, C-12, C-17, C-16, and C-47 two isolates (C-20 and C-18) of *C. atrobrunneum* and one isolate (C-15) of *C. globosum* grouped together. In Cluster 5 all the isolates of *C. globosum* (C-10,C-08,C-51,C-05,C-59,C-58,C-40,C-72,C-62,C-74,C-11,C-57,C-60 and C-42) were found together along with isolates of other species *viz.*, *C. elatum* (C-02), *C. atrobrunneum* (C-3,C-19 and C-61), *C. nigricolor* (C-55) and *C. funicola* (C-80).

Phylogenetic analysis of ITS region

Most accurate grouping was obtained through ITS region sequences wherein *Chaetomium* species isolates were grouped into six major clusters as depicted in Fig. 12.Seven isolates of *C. atrobrunneum*(C-03, C-68, C-19, C-20, C-18, C-61 and C-78), five isolates of *C. funicola* (C-12, C-80, C-47, C-16 and C-17), five isolates of *C. brasiliense* (C-45, C-50, C-76,C-07 and C-46) and eight isolates of *C. megalocarpum* (C-70, C-21, C-23, C-22, C-65, C-48, C-66 and C-77) made into separate Clusters, Cluster 1, 3, 4 and 6 respectively., *C. nigricolor* (C-55) and *C. perlucidum* (C-73 and C-81) were found together in Cluster 2. Fifteen isolates of *C. globosum* (C-42, C-59, C-15, C-05, C-11, C-57, C-58, C-62, C-60, C-72, C-40, C-74, C-10, C-08 and C-51) in Cluster 5 made into a single group with one isolate of *C. elatum* (C-02).

All the species of *Chaetomium* (*C. globosum, C. atrobrunneum, C. brasiliense, C. elatum, C. cochliodes, C. funicola, C. nigicolor, C. megalocarpum* and *C. perlucidum*) which were authentically identified using ITS region were further analyzed for Phylogenetic grouping. Forty four isolates of *Chaetomium* were subjected to PCR amplification of genes *viz.*, actin, β-tubulin, calmodulin, *rpb2* and *tef-1*. Sequences of these regions were subjected to maximum parsimony phylogenetic analysis. The clustering obtained

was compared with ITS based clustering. In the present study ITS region gave best grouping for the *Chaetomium* species through phylogeny. The clustering of different species through ITS sequences matched with the earlier findings of Aggarwal *et al.,* (2013) in which ITS sequence data could clearly differentiate 18 different isolates of the *Chaetomium* spp. collected from different specialized life strategies surviving in diverse climates. Wang *et al.,* (2014) reported the multigene phylogenetic analyses with ribosomal ITS, partial ribosomal large subunits (28S rDNA), β-tubulin, the translation elongation factor 1α (TEF1-α), and the largest subunit of RNA polymerase II (*rpb1*) and recognized eight well-supported lineages within the monophyletic *C. indicum* group using ITS sequences. All these data sufficiently supplements the grouping of species of *Chaetomium* by using ITS sequences for phylogenetic analysis.

ITS gene region was the best region for *Chaetomium* species identification using NCBI database. ITS also found to be best region for the grouping of the *Chaetomium* species through phylogenetic tree.

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