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Full Length Research Paper

Phylogenetic relationships of *Chaetomium* isolates based on the internal transcribed spacer region of the rRNA gene cluster

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Molecular characterization of 18 *Chaetomium* isolates collected from India based on the internal transcribed spacer (ITS) region of the rRNA gene sequences was done. Phylogenetic analysis of full length ITS region showed that *Chaetomium globosum* isolates, Cg1, Cg2, Cg6, Cg11 and Cg15, *Chaetomium* spp. isolates, C16, C17 and a *Chaetomium perlucidum* isolate formed a group with American isolates of *Chaetomium* spp., SW287, SW271 and CL024, thereby supporting the close relationships among these isolates. Other *C. globosum* isolates, Cg3, Cg4, Cg5, Cg10, Cg12, Cg13 and Cg14 clustered with European isolates, UOA/HCPF 9215 and UOA/HCPF 9860 and an Australian isolate NC1. Isolates Cg7, Cg8, and Cg9 were closely related to the Australian isolates but distantly related to the isolates from New Zealand. However, all these isolates clustered in the same Australia group as evident in the evolutionary history analysis using parsimony method. European isolate MU-2009 and Australian isolates NA26 were separated from the rest of the isolates and did not cluster in any of the groups formed. Results indicate that, different isolates of the *Chaetomium* spp. may have different life strategies and specialized in surviving diverse climates.

Key words: *Chaetomium*, rRNA sequences, internal transcribed spacer (ITS), phylogeny.

INTRODUCTION

Most fungi which belong to Chaetomiaceae family are distributed widely and occur on a variety of substrata, including soil and dung, from the tropics to temperate regions, with an excellent characteristic of cellulose degradation. The potential of *Chaetomium* spp. has also been recognized as biological control agent by various workers (Die Pietro et al., 1992; Soytong et al., 2001; Aggarwal et al., 2004). Many taxonomic studies have been conducted to arrange this group of fungi into a suitable classification scheme (Ames, 1963; Seth, 1970; Dreyfuss, 1976; Arx et al., 1986). However, due to limited number of morphological and developmental characters, the genetic and evolutionary relationships among *Chaetomium* isolates are uncertain. As a result, the phylogenies among these isolates are hardly reflected.

Internal transcribed spacer (ITS) is between the rRNA of 18 and 28S, which is divided into two segments by 5.8S, namely ITS1 and ITS2. ITS is a highly repetitive sequence in nuclear genome, and moreover, there is conserved evolution of intra-site and inter-site among the repetition units by unequal crossing over and gene

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conversion. Thus, the sequences among different ITS copies tend to be similar or totally conserved. With high-speed evolution, ITS can provide more variable sites and informative sites, which has been confirmed as an important molecular marker in the study of systematics and evolution concerning fungal populations. Since mid-1980s, ITS region of the rRNA gene sequences has been widely used in fungal systematic evolution and genetic relationships, as it can better demonstrate the intra-family, inter-genus and inter-species genetic relationship (Berbee and Taylor, 1992a, b, c; Hausner et al., 1993; Saenz et al., 1994; Spatafora and Blackwell, 1993, 1994; Swann and Taylor, 1993; Wingfield et al., 1994; Bryan et al., 1995; Sherriff et al., 1995).

In this study, the direct sequencing of ITS-PCR products was adopted to find out the evolutionary relationships among *Chaetomium* species isolated from India and from the diverse geographic origins. The genetic relationships of isolates found in diverse climates were discussed at molecular level, in order to provide basis for study on systematic evolution of the genus *Chaetomium*.

MATERIALS AND METHODS

Establishment of *Chaetomium* spp. cultures

Eighteen (18) isolates of *Chaetomium* species sampled for this study were isolated from wheat leaves /Indian soils and named based on their possession of particular morphological characters (Table 1). The fungal isolation was done on potato dextrose agar (PDA) and single spore pure colonies were maintained on PDA slants. All isolates were sequenced by the authors whereas the sequences for another 18 isolates were derived from previous studies (Graham et al., 2009; Hall et al., 2003; Oh et al., 2005; Syed et al., 2009; Unterseher and Schnittler, 2009; Meyer et al., 2011).

Extraction of DNA from *Chaetomium* spp.

DNA was extracted from *Chaetomium* species isolates by following CTAB method (Murray and Thompson, 1980). The nucleic acid pellet obtained was washed with 70% ethanol and dried at room temperature. Finally, the nucleic acid was dissolved in Tris EDTA (TE) and stored at -20°C. Agarose gel of 1.0% concentration was used to examine the quality of DNA by electrophoresis detection, and concentration of DNA was measured by spectrophotometer. The DNA solution was diluted with aseptic water to get the final concentration of 100 ng.

Amplification, purification and sequencing of ITS region

PCR amplification was done on a thermal cycler (Bio-Rad Laboratories India Pvt. Ltd., India), with reaction volume of 25 µL. The reaction mix included double-stranded DNA template (about 100 ng), dNTPs Mix (200 µmol/L), ITS forward (ITS 1) and reverse (ITS 4) primers (0.1 µmol/L each) (White et al., 1990), Taq DNA polymerase (2.5 U), and 1X buffer. The process of amplification reaction was as follows: 2 min pre-denaturation at the temperature of 94°C, 1 min denaturation under the same condition, 1 min annealing at 55°C, and 2 min extension at 72°C. The above process was recycled for 30 times, and then extension was done for 10 min at 72°C. The amplified products of full length ITS regions were resolved by electrophoresis in 1.2% agarose gel in Tris-EDTA-acetate (TAE) buffer (1X), stained with ethidium bromide at 0.5 µg/ml, run at 80 V for 3 h, visualized under UV light, and photographed with gel documentation unit (Syngene Inc, Cambridge, UK).

The amplified segments included partial sequences of 18S and 28S ITS and full length ITS 1, 5.8S and ITS 4 region. Amplified products were purified using QIAquick® PCR Purification Kit (QIAGEN India Pvt. Ltd., India) and sequencing of the amplified products was done at the Department of Biochemistry of Delhi University, South Campus, New Delhi. All the sequences were submitted to NCBI database. The chromatograms from the automated sequencer were imported into the computer program MEGA5 (Tamura et al., 2011) for sequence assembly. Sequences generated in this study were aligned with other published sequences with the ClustalW of MEGA5 program. Minor adjustments of the alignment were done manually and maintained with the same program. Only unambiguously aligned sequences were used in analysis.

Phylogenetic analysis of *Chaetomium* species

The evolutionary history was inferred using the maximum parsimony method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). The MP tree was obtained using the tree-bisection-regrafting (TBR) algorithm (Nei and Kumar, 2000) with search level 3 in which the initial trees were obtained by the random addition of sequences (21 replicates). The analysis involved 38 nucleotide sequences including one closely related species, *Achaetomium strumarium* (Gene Bank accession No. AF048788) and a closest relative, *Neurospora crassa* (Gene Bank accession No. JN198494); a member of the Sordariomycetes within the Pezizomycotina clade. All ambiguous positions were removed for each sequence pair. There were a total of 764 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.

RESULTS

The direct sequencing of PCR products was used to obtain the ITS sequences of about 600 bp amplicon from the 18 *Chaetomium* isolates in India. Complete ITS sequence of all the isolates were separated into ITS1, ITS2 and 5.8S region and G+C content for all the three regions were calculated (Table 2). ITS1 region contains partial 18S ribosomal DNA site, while ITS2 region contains partial 28S ribosomal DNA site. The gross length of ITS sequences concerning 36 *Chaetomium* isolates ranged from 319 to 625 bp, with the maximum difference of 306 bp and the average of 522 bp. ITS 1 length varied from 71 to 227 bp, while ITS 2 length varied from 124 to 252 bp. 5.8S region was highly conserved having a length of 158 bp in all the isolates. The G+C content of ITS 1 region varied from 48.1 to 66.2%, while the G+C content of ITS 2 region ranged from 54.0 to

Table 1. *Chaetomium* isolates used in this study.

Species	Isolate	Geographic origin	Gene Bank accession number	Reference
C. globosum	Cg1	India	AY429048	This study
	Cg2	India	AY429049	This study
	Cg3	India	AY429050	This study
	Cg4	India	AY429051	This study
	Cg5	India	AY429052	This study
	Cg6	India	AY429053	This study
	Cg7	India	AY429054	This study
	Cg8	India	AY429055	This study
	Cg9	India	AY429056	This study
	Cg10	India	HQ224671	This study
	Cg11	India	HQ224665	This study
	Cg12	India	HQ224666	This study
	Cg13	India	HQ224667	This study
	Cg14	India	HQ224668	This study
	Cg15	India	HQ224669	This study
	Chaetomium globosum	America	AF423116	Oh et al. (2001)
	UOA/HCPF 9215	Greece	GQ376099	Meyer et al. (2011)
Chaetomium spp.	C16	India	HQ224670	This study
	C17	India	HQ316556	This study
	NSW3	Australia	EU035807	Syed et al. (2009)
	NSW2	Australia	EU035806	Syed et al. (2009)
	NC1	Australia	EU035805	Syed et al. (2009)
	NAD3	Australia	EU035804	Syed et al. (2009)
	NAD2	Australia	EU035803	Syed et al. (2009)
	NA26	Australia	EU035802	Syed et al. (2009)
	NA25	Australia	EU035801	Syed et al. (2009)
	NA16	Australia	EU035800	Syed et al. (2009)
	NA12	Australia	EU035799	Syed et al. (2009)
	ICMP 17488	New Zealand	EU770238	Graham et al. (2009)
	ICMP 17487	New Zealand	EU770237	Graham et al. (2009)
	SW287	America	AY234918	Hall et al. (2003)
	SW271	America	AY234917	Hall et al. (2003)
	CL024	America	AY234916	Hall et al. (2003)
C. perlucidum	*C. perlucidum*	India	HQ316557	This study
C. murorum	UOA/HCPF 9860	Greece	GQ376100	Meyer et al. (2011)
C. funicola	MU-2009	Germany	FN548164	Unterseher and Schnittler (2010)
A. strumarium		America	AF048788	Lee and Hanlin (1999)

66%. As the length of 5.8S region was highly conserved in all the isolates, the G+C content was also conserved that is 46.8% except for Cg7, Cg8 and *Chaetomium reflexum*.

As the default, gap was used to analyze 764 sites. There were 128 constant characters, 472 informative sites taking up 63.61%, and 528 variable sites representing 71.15%. The length of the simplest tree was 1193 steps, with consistency index (CI) of 0.809109 and retention index (RI) of 0.904600. The composite index was 0.755224 (0.731920) for all sites and parsimony-informative sites (in parentheses). Branches

Table 2. Base composition (bp) and %G + C content of ITS sequences of 18 isolates of *Chaetomium.*

Isolate	ITS1	%GC	5.8S	%GC	ITS2	%GC	Total length	%GC
Chaetomium globosum (Cg1)	150	49.7	158	46.8	251	60.7	559	53.7
C. globosum (Cg2)	160	48.1	158	46.8	251	61.4	569	53.6
C. globosum (Cg3)	175	58.3	158	46.8	234	59.3	567	55.7
C. globosum (Cg4)	167	59.3	158	46.8	234	60.7	559	56.4
C. globosum (Cg5)	146	61.6	158	46.8	214	59.3	518	56.2
C. globosum (Cg6)	151	49.0	158	46.8	252	60.7	561	53.7
C. globosum (Cg7)	226	58.4	158	46.2	175	61.7	559	56.0
C. globosum (Cg8)	175	58.3	158	46.2	233	59.2	566	55.3
C. globosum (Cg9)	227	56.8	158	46.8	223	57.4	598	54.4
C. globosum (Cg10)	177	55.9	158	46.8	174	60.9	509	54.8
C. globosum (Cg11)	71	66.2	158	46.8	124	57.3	356	55.9
C. globosum (Cg12)	145	62.1	158	46.8	158	63.3	461	57.3
C. globosum (Cg13)	157	59.9	158	46.8	160	63.8	465	56.8
C. globosum (Cg14)	149	61.1	158	46.8	161	62.7	468	56.8
C. globosum (Cg15)	117	62.4	158	46.8	141	66.0	416	56.4
C.perlucidum (Cp)	190	57.9	158	46.8	202	54.0	550	54.0
Chaetomium spp. (C16)	117	51.3	158	46.2	223	54.0	498	53.6
Chaetomium spp. (C17)	151	58.3	158	46.8	245	55.5	554	55.4

corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The consensus tree inferred from 261 most parsimonious trees is shown in Figure 1.

The isolates Cg7, Cg8 and Cg9 were closely related to the Australian isolates but distantly related to the isolates from New Zealand. However, all these isolates clustered in the same Australia group with 76% bootstrap value. Isolates Cg3, Cg4, Cg5, Cg10, Cg12, Cg13, and Cg14, clustered with European isolates UOA/HCPF 9215, UOA/HCPF 9860, and an Australian isolate NC1 to form a group named India with 84% bootstrap value. Both Australia and India groups were connected to each other by a small branch with 76% bootstrap value. Two large branches originating from this small branch separated a European isolate MU-2009 and an Australian isolate NA26 from the rest of the isolates with 58 and 73% bootstrap values respectively. Both these isolates did not cluster in any of the groups formed. The third group named as Indo/America was composed of eight Indian isolates viz., Cg1, Cg2, Cg6, Cg11, Cg15, C16, C17, *Chaetomium perlucidum*, three American isolates viz., SW271, CL024, SW287 and a closely related species, *Achaetomium strumarium* with the bootstrap value of 82%.

DISCUSSION

Isolates of *Chaetomium* are cosmopolitan in distribution and occur on a variety of substrata, including soil and dung. They distribute widely in the diverse climates from the tropics to temperate regions. In spite of huge differences among geographical and ecological factors in the distribution area, the taxonomic position of *Chaetomium* is uncertain due to limited number of morphological and developmental characters. Like most perithecial ascomycetes, *Chaetomium* was originally placed in the all-encompassing order Sphaeriales (Ainsworth and Bisby, 1950, 1954; Martin, 1950; Alexopoulos, 1952; Ainsworth, 1961, 1971). Subsequently, Alexopoulos (1962) recognized the order Chaetomiales for *Chaetomium* and related genera. This was based on the presence of perithecial hairs, the lack of paraphyses, and the evanescent asci. Whiteside (1961), however, reported that paraphyses are present in *C. globosum*. Alexopoulos and Mims (1979) placed *Chaetomium* in the order Xylariales and then Eriksson (1982) included the genus in the Sordariales, a placement followed by Hawksworth et al. (1983) and most subsequent authors. Throughout these changes, the genus has remained in the family Chaetomiaceae. However, very few studies have been conducted to find out the genetic and evolutionary relationships among isolates within the Chaetomiaceae family. As a result, the phylogenies among *Chaetomium* isolates are hardly reflected.

In this study, although the lengths of individual ITS sequences of *Chaetomium* isolates studied were not consistent, the average length of 522 bp was more or less similar to that of the ITS sequences of other

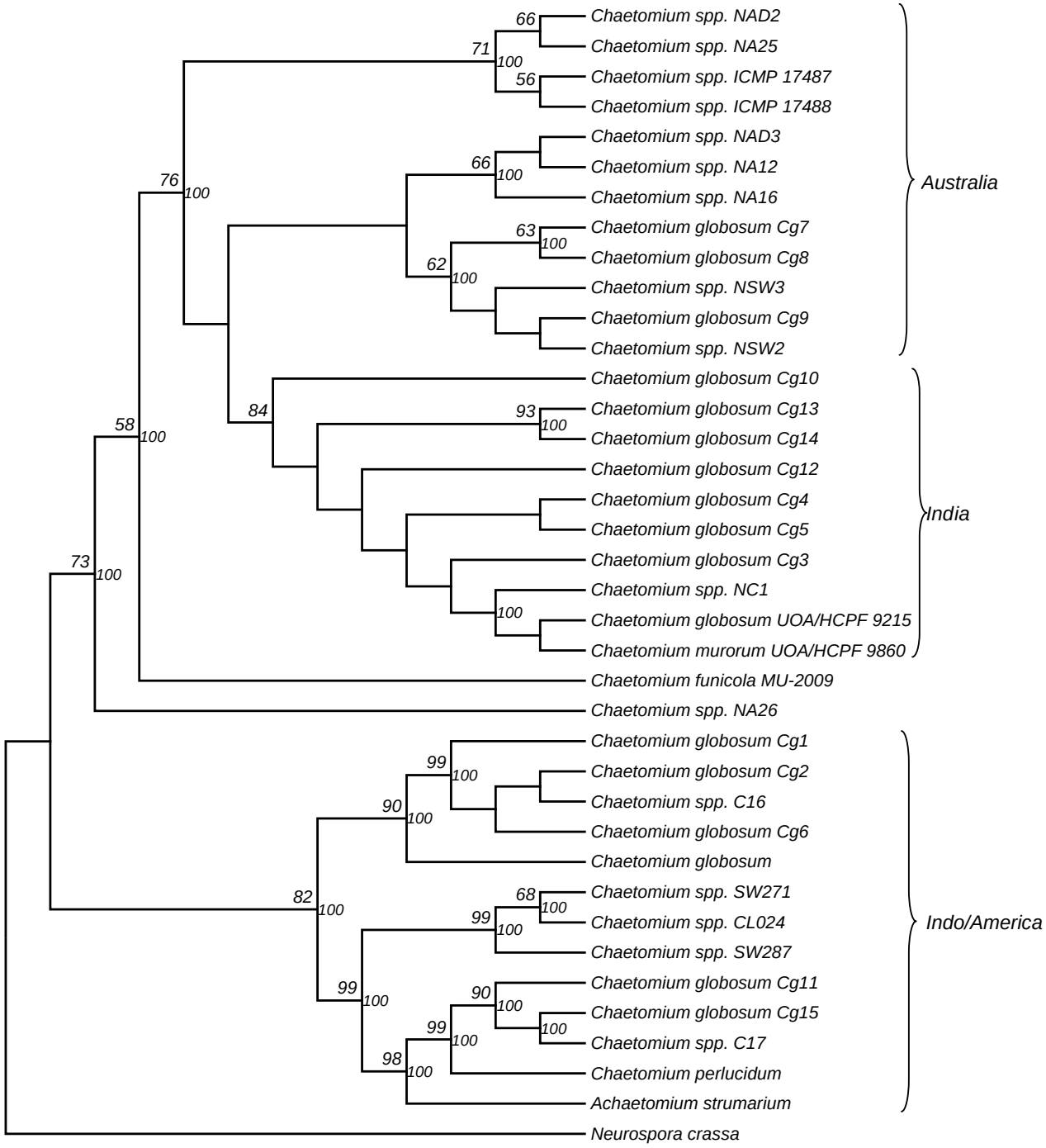

Figure 1. The bootstrap consensus tree of 261 most parsimonious trees inferred from 1000 replicates. The percentage of replicate trees and parsimonious trees, in which the associated taxa clustered together, are shown above the branches and inside the nodes respectively.

Chaetomium species such as *Chaetomium aureum*, *Chaetomium funicola* and *Chaetomium nepalense* (Lee and Hanlin, 1999). CI close to 0.9 obtained in this study, demonstrates that the phylogenetic relationship tree has considerable credibility. Most of the *Chaetomium* isolates in India showed a close relationship with the *Chaetomium*

isolates in America. On the other hand, very few isolates from India shared similarity with isolates from Australia. Nearly 39% of the Indian isolates could form a separate group though they clustered with two European and an Australian isolates. Genetic similarity among isolates within the groups suggests that some isolates of *Chaetomium* potentially inhabit many environments, from the tropics to temperate regions. However, the separation of a European isolate MU-2009 and an Australian isolate NA26 from the rest of the isolates suggests that the middle type of evolution, developing from a higher grade to a lower grade, possibly have occurred.

Syed et al. (2009) took the phylogenetic analysis to discuss the genetic relationship of plant endophytic and free-living *Chaetomium* species distributed in South East Asia, Europe and Australia. They pointed out that the *Chaetomium* species isolated from various substrates in South East Asia and Europe shared similar identity with isolates from leaf, seeds and soil collected from central and south eastern NSW (Australia). There was close genetic similarity among species of *Chaetomium* which potentially inhabit many environments.

A closely related species, *Achaetomium strumarium* (Synonym: *Chaetomium strumarium*) (Rai et al., 1964) was included in the phylogenetic analysis as out-group taxa. *A. strumarium* was distinguished among members of the genus *Chaetomium* in forming perithecia without the hairy ornamentations and ascocarps covered with pale, thin-walled, flexuous hairs (Rai et al., 1964; Abbott et al., 1995). Based on the morphological differences, the separate Achaetomiaceae family was proposed by Rai et al. (1970) for this species. However, based on the phylogenetic similarity with 98% bootstrap value between *A. strumarium* and *Chaetomium* isolates in India, this species has been recommended as a member of the *Chaetomiaceae* family in this study. Abbott et al. (1995) also recommended the placement of *A. strumarium* in the genus *Chaetomium*, on the basis of similarities of asci and ascospores, which was further supported by Lee and Hanlin (1999) based on their phylogenetic relationships.

Earlier, some studies were done on molecular characterization of Indian *Chaetomium* isolates based on randomly amplified polymorphic DNA (RAPD) (Ahammed et al., 2005) and universal rice prime (URP markers) (Aggarwal et al., 2008) but this is the first report on ITS sequence based phylogenetic analysis. The present study indicates that Indian *Chaetomium* isolates are genetically more similar to American, than to Australian, European and Oceanian (New Zealand) isolates. Taken together, the data indicate several interpretations. One possibility could be that an individual fungus may specialize to cycle in a particular habitat from the tropics to temperate regions. However, the possibility was ruled out as some of the isolates in India evolved in other continents and *vice versa*. Alternatively, different isolates of the same *Chaetomium* species may have different life strategies with some isolates specializing in surviving diverse climates. If the later hypothesis is supported by further work, then some *Chaetomium* species may be better attributed to the particular geographic origins.

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