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Intraspecific rDNA-ITS Polymorphism within *Clavariadelphus subfastigiatus* (Basidiomycota: Gomphoid-phalloid Clade) from Pakistan

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Abstract

The genetic diversity of Clavariod fungus; *Clavariadelphus subfastigiatus* from Pakistan was examined at the Internal Transcribed Space of rDNA level. From seven (07) investigated strains of *C. subfastigiatus*, two haplotypes with 0.5714 haplotype diversity were observed. These strains showed intraspecific variations in ITS1-5.8S-ITS2 region at 10 sites with very little detectable genetic divergence. We propose these isolates as a *C. subfastigiatus*-Complex with some Indels and morpho-anatomic homogeneity. The topological distribution of this complex has also been discussed phylogenetically. © 2013 Friends Science Publishers

Keywords: Bootstrap; Hotspot; KPK; Percent identities; Moist temperate

Introduction

Clavariadelphus Donk. has widespread distribution in temperate regions of the world and has 19 recognize species (Kirk *et al.,* 2008). A few of them are symbiotic and remaining are saprobic. Its basidiomes are typically solitary, occasionally subcaespitose, filiform to clavate, ligulate or turbinate, in some species truncate and sterile at the expanded apex without marginal growth, light yellow or light orchraceous, brown, rufescent or ferruginous, flesh rather firm, not brittle, spongy in large fruitbodies (Methven, 1990). Three taxa of this genus have so far been reported from Dungagali, Murree hills, Pakistan, viz. *C. pistillaris* (L.) Donk, *C. truncatus* (Quel.) Donk and *C. truncatus* var. *atrobrunneus* Corner (Ahmad *et al*., 1997).

Identification of fungi is difficult and many authors have discussed about species identification and recognition (Avise and Wollenberg 1997; Mayden, 1997; Taylor *et al*., 2000; Wheeler and Meier, 2000; Mallet, 2001; Hey, 2001). One approach is to identify the species on morphological basis by recognizing its phenetic peculiarity. This is primarily based on classification of the organisms on the basis of overall visual similarity in traits. More than 100,000 species have been described so far by this method (Kirk *et al*., 2008). These types of studies mainly rely upon the use of macroscopic and microscopic characteristics of fruiting mushroom. The main drawback in this recognition is the plasticity in phenotypes of mushrooms, which is often confusing and results in species complexes, for example, the morphological species *Armillaria mellea* (Vahl) P. Kumm. (Anderson and Stasovski, 1992).

The Himalayan Moist Temperate Forests are hotspots for macrofungi (Ahmad *et al*., 1997). Previously, these forests were included in the DFO Indo-Burma regime (Myers *et al*., 2000) among 25 biodiversity hotspots. However, they were recently separated from the Indo-Burma hotspot and designated as a new hotspot included in the recognized 34 hotspots of biodiversity (Mittermeier *et al*., 2011). In the present work, we successfully sequenced 7 isolates of *C. subfastigiatus* collected from these forests of Pakistan. We found intraspecific variations in hyperdiverse ITS1/5.8s/ITS2 region and detected two haplotypes. Hanif *et al.* (2012) used same region for investigating ectomycorrhizae associated with *Cedrus deodara* (Roxb.) G.Don. from Pakistan.

Materials and Methods

Sampling Details

The sampling site was located in the moist temperate forests of Pakistan. These forests are dominated by coniferous trees and show high basidiomycete biodiversity (Khalid, 1998; Niazi, 2008). Basidiomata of *C. subfastigiatus* were collected from Himalayan Moist Temperate Forests of Pakistan. The temperature ranges here from -4 to 25°C. Soil is loamy with gravels and rock stones of variable sizes. The selection of the sampling site is based on the richness and abundance of *Aphyllophorales*. During the exploration of *Aphyllophorales*, a species of *Clavariadelphus,* different from the known species from Pakistan, was collected from Himalayan Moist Temperate Forests, associated with *Pinus wallichiana* A. B. Jacks. Field notes were prepared and this fungus was given a tentative number and vouchered. It was morphologically characterized following Reid (1984). Small portions from the hymenium (about 1 cm) were placed in

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2% CTAB buffer in 1.5 mL eppendorf vials and kept frozen at -20°C for molecular characterization. The collected specimen was dried with a fan heater overnight and kept in vouchered Ziploc bags. The measurements of spores, basidia, cystidia and sterigmata were taken and drawings were made with the aid of a camera lucida. Basidiospores were observed at 1600X.

DNA Extraction, PCR, Sequencing and Phylogenetic Analysis

DNA was extracted by modified CTAB method following Gardes and Bruns (1993). The hymenial tissue was removed with sterile forceps and rinsed with sterile H_2O. The extraction was modified for silica emulsion binding and purification (Gene-Clean; Q-Biogene, Irvine, CA, USA). Polymerase chain reaction (PCR) was carried out following the protocols described by Gardes and Bruns (1993), using the fungus-specific primers ITS1F and ITS4, which amplify the nuclear rDNA-ITS region. The hot-start enzyme JumpStart (Sigma, St Louis, MO, USA) was used to catalyze the PCR with 2 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 53°C, 40 s + 5 s per cycle at 72°C, and finishing with 5 min at 72°C. The PCR products were purified with QI Aquick (Qiagen Inc., Valencia, CA, USA), and sequenced bi-directionally using Big Dye 3.1 on an ABI 3730 DNA sequencer (Applied Biosystems, Foster City, CA, USA). The contigs were eventually edited in sequencher 4.5 (Gene Codes, Ann Arbor, MI, USA) at Jodrell Laboratory, Royal Botanical Gardens, Kew, UK. DNA sequence was submitted to BLAST and used to query the nucleotide collection using default settings. Phylogenetic placement of *C. subfastigiatus* was confirmed by Maximum likelihood. Phylogram was made by using 35 rDNA sequences including 7 sequences obtained from *C. subfastigiatus* from Pakistan (Table 2). All the sequences were aligned and adjusted manually by using MacClade 4.08. Percentage identities and genetic divergence were calculated using the software DNAStar provided by DNAStar, Inc. 3801 Regent Street Madison, WI 53705, USA. DNAsp ver. 5.10.01 was used to calculate haplotype diversity. Matrix of aligned sequences was used to build the phylogenetic tree using software Mega5 and DNAStar. Maximum likelihood and neighbor joining criteria were used for phylogenetic analyses. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of nucleotides substitutions per site. To calculate intraspecific variation, we created species-specific alignment using Sequencher v.4.1, ClustalX (Thompson *et al*., 1997).

Results

All *C. subfastigiatus* basidiomata were characterized morpho-anatomically and homogeneity was found (Fig. 4; Table 2). But these were genetically different and classified into two haplotypes.

Habit and Habitat of the Material Examined

On the ground, ectomycorrhizal, solitary, sometimes in groups of 2–3, under *Pinus wallichiana* A.B. Jackson. The material examined was from Pakistan, Khayber Pukhtunkhaw (KPK), Aubia, Khanspur, Hospital Range, 34° 01′ 23.92″ N, 73° 24′ 14.11″E, elevation 2159 m, 22 Aug 2009, collected by M. Hanif, MH228904.129 (LAH # MH228904); Pakistan, KPK, Aubia, Khanspur, Helipad, 34° 01′ 30.89″ N, 73° 25′ 18.78″E, elevation 1974 m, 12 Sep 2009, collected by M. Hanif, MH129901.126 (LAH # MH129901); same location, 08 Aug 2009, collected by M. Hanif, MH089901.99 (LAH # MH089901); same location, 16 Aug 2009, collected by M. Hanif, MH168901.93 (LAH # MH168901); same location, 30 June 2007, collected by M. Hanif, MH3060701.297 (LAH # MH3060701); Pakistan, KPK, Nathia Gali, Near Governor's House, 34° 04′ 18.18″ N, 73° 23′ 34.44″E, elevation 2408m, 16 Aug 2009, collected by M. Hanif, MH168902.277 (LAH # MH168902); same location, 16 Aug 2009, collected by M. Hanif, MH168903.278 (LAH # MH168903).

Phylogenetic Analysis

Sequencing of seven isolates of *C. subfastigiatus*-Complex yielding 625-675 nts fragments. The sequences were BLAST searched resulting in 98% similarity with *C. subfastigiatus* (EU669206.1). For phylogenetic analysis, rDNA ITS sequences were downloaded from GenBank and aligned with 7 isolates of *C. subfastigiatus* from Pakistan. The analysis involved 35 rDNA sequences. From the aligned datasheet, all ambiguous positions were removed for each sequence pair. In the aligned data sheet, total of 727 characters were subjected for phylogeny. Out of these, 276 characters were conserved, 410 were variable but parsimony uninformative, 333 were parsimony informative and 76 were singleton. All the gaps were treated as "missing" data.

Topological distribution of isolates of *C. subfastigiatus*-Complex and its phylogenetic recognition was inferred from maximum likelihood method. Evolutionary analysis was conducted in MEGA5 (Tamura *et al*., 2011). The Phylogram represented by three major clades formed by 35 species, and species of *Thelephora* Ehrh. ex Willd. were selected as outgroup (Table 3). In maximum likelihood consensus tree, all isolates of *C. subfastigiatus*-Complex clustered together in sub-clade-I of the clade-I (Fig. 1). Strong bootstrap frequency (90%) clustered *C. subfastigiatus*-Complex mh126, mh129 and mh99 within C1 and designed as Haplotype I, separated from other 4 isolates of this complex mh277, mh295 and mh278 within C2 and designed as Haplotype I. These 7 isolates clustered together with supportive bootstrap frequency (55%) in the same sub clade. These seven isolates shared morpho-anatomic characters. Sequences of *C. subfastigiatus*

Table 1: rDNA sequences downloaded from GenBank for molecular characterization and phylogenetic analyses

Fungal Species	Accession No.	Origin	Strains
Clavariadelphus ligula	EU526003.1	USA	OSC 1064245
C. mucronatus	EU526000.1	USA	OSC 1064138
C. occidentalis	EU834202.1	USA	OSC 114250
C. sachalinensis	EU834196.1	USA	OSC 96213
C. sachalinensis	EU624411.1	USA	p058i
Clavariadelphus sp.	DQ974709.1	USA	src121
C. subfastigiatus	HQ379937.1	Pakistan	MH129901.126
C. subfastigiatus	Unpublished	Pakistan	MH228904.99
C. subfastigiatus	Unpublished	Pakistan	MH089901.129
C. subfastigiatus	Unpublished	Pakistan	MH3060701.93
C. subfastigiatus	Unpublished	Pakistan	MH168901.277
C. subfastigiatus	Unpublished	Pakistan	MH168902.295
C. subfastigiatus	Unpublished	Pakistan	MH168903.278
C. subfastigiatus	EU669206.1	USA	p067i
C. subfastigiatus	EU669207.1	USA	p069i
C. truncatus	HQ650728.1	Canada	SMI278
C. truncatus	DQ097871.1	Canada	OUC99108
Gautieria crispa	DQ974732.1	USA	src627
G. monticola	AF377101.1	USA	SNF346CA
G. pterosperma	AF442137.1	USA	HDT45921
Gloeocantharellus purpurascens	AY872281.1	USA	104220
Gloeocantharellus sp.	EU118161.1	China	CYD-2007a
Gomphus clavatus	DQ365637.1	USA	Isolate 8.2
Kavinia alboviridis	GQ981506.1	England	KM141510
Lentaria dendroidea	EU118640.1	Sweden	SJ 98012 (GB)
L. surculus	HQ533048.1	New Zealand	PDD:95856
Phaeoclavulina flaccid	AF438554.2	Korea	K8
Ramaria cystidiophora	EU597077.1	Canada	UBCOGTR0419s
R. flava	AJ408367.1	SPAIN	MA-Fungi 48072
R. flavescens	AJ408366.1	SPAIN	MA-Fungi 48069
R. gracilis	EU258553.1	USA	-
Thelephora anthocephala	DQ974771.1	USA	src614
T. caryophyllea	GU234018.1	Netherlands	O75453
T. ganbajun	EU696946.1	China	Gb279
T. terrestris	AB634267.1	Japan	H227

Table 2: Morpho-anatomic Comparison of different isolates of *Clavariadelphus subfastigiatus*-Complex collected from KPK, Pakistan

Haplotypes of *Clavariadelphus subfastigiatus*-Complex	Basidioma size	Colour	Basidiospores	Basidia l×w (top)×w (bottom)	Cystidia	Habitat
C. subfastigiatus MH99	120 mm high, 7–14 mm diam., at base, enlarging towards the apex to 20 mm diam.	brownish orange, reddish golden fading to deep orange	7.50–9.0 µm × 4.0–5.50 µm	35–40 µm × 10–13 µm × 5–6 µm	32–49 µm × 7–8 µm × 3–5 µm	On ground with *Pinus wallichiana*
C. subfastigiatus MH126	115 mm high, 7–12 mm diam., at base, enlarging towards the apex to 19 mm diam.	brownish orange, reddish golden fading to deep orange	7.50–9.0 µm × 4.0–5.50 µm	34–38 µm × 10–14 µm × 6–7 µm	32–49 µm × 7–8 µm × 3–5 µm	On ground with *Pinus wallichiana*
C. subfastigiatus MH129	117 mm high, 9–16 mm diam., at base, enlarging towards the apex to 22 mm diam.	brownish orange, reddish golden fading to deep orange	7.50–9.0 µm × 4.0–5.50 µm	33–43 µm × 10–14 µm × 5.5–8 µm	32–49 µm × 7–8 µm × 3–5 µm	On ground with *Pinus wallichiana*
C. subfastigiatus MH93	120 mm high, 7–14 mm diam., at base, enlarging towards the apex to 20 mm diam.	brownish orange, reddish golden fading to deep orange	7.0–9.0 µm × 4.0–5. 0 µm	32–41 µm × 9–12 µm × 5–7 µm	32–49 µm × 7–8 µm × 3–5 µm	On ground with *Pinus wallichiana*
C. subfastigiatus MH277	120 mm high, 7–14 mm diam., at base, enlarging towards the apex to 20 mm diam.	brownish orange, reddish golden at tips	7.0–8.5 µm × 4.0–5. 0 µm	34–40 µm × 8–13 µm × 5–7 µm	32–49 µm × 7–8 µm × 3–5 µm	On ground with *Abies pindrow*
C. subfastigiatus MH278	120 mm high, 7–14 mm diam., at base, enlarging towards the apex to 20 mm diam.	brownish orange, reddish golden at tips	7.0–8.5 µm × 4.0–5. 0 µm	32–43 µm × 9–13 µm × 5–7.5 µm	32–49 µm × 7–8 µm × 3–5 µm	On ground with *Abies pindrow*
C. subfastigiatus MH295	120 mm high, 7–14 mm diam., at base, enlarging towards the apex to 20 mm diam.	brownish orange, reddish golden at tips	7.0–8.5 µm × 4.0–5. 0 µm	35–40 µm × 8–13 µm × 5–7 µm	32–49 µm × 7–8 µm × 3–5 µm	On ground with *Abies pindrow*

Table 3: Polymorphism at 10 positions within ITS1 + 2 rDNA sequences of 7 samples of *C. subfastigiatus*-Complex

Sample	Position in ITS1 + 2 alignment (679 nts)									
	39	52	83	133	172	220-221	228	441-442	502	566
C. subfastigiatus MH99	C	A	C	A	A	TG	G	--	A	T
C. subfastigiatus MH126	C	A	C	A	A	TG	G	--	A	T
C. subfastigiatus MH129	C	A	C	A	A	TG	G	--	A	T
C. subfastigiatus MH93	T	G	T	G	-	--	A	TG	C	C
C. subfastigiatus MH277	T	G	T	G	-	--	A	TG	C	C
C. subfastigiatus MH278	T	G	T	G	-	--	A	TG	C	C
C. subfastigiatus MH295	T	G	T	G	-	--	A	TG	C	C

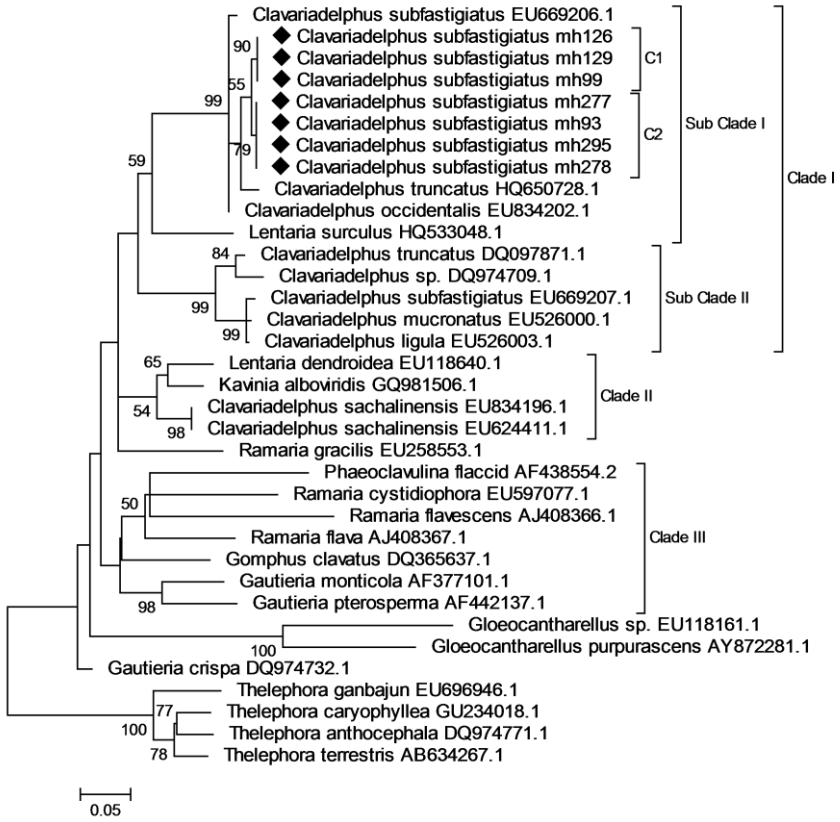

Fig. 1: Molecular Phylogenetic analysis by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein J. 1985). Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 35 nucleotide sequences. There were a total of 727 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2011)

(EU669206.1 and EU669207.1) from USA also clustered with *C. subfastigiatus* from Pakistan in major clad I. All these isolates shared 99-100% of their genetic characters with each other and had very little rDNA-ITS divergence (from identical to 2.1) compared with *Clavariadelphus* spp. included in the present analysis (Fig. 2). Species belonging to *Clavariadelphus* grouped into two adjacent sub clades of clade I intermixed with *Lentariaceae*. rDNA-ITS isolated from *C. subfastigiatus* from Pakistan showed 97.8% identity with *C. subfastigiatus* (EU669206.1), while the maximum genetic divergence (25.6) was observed with *C. sachalinensis* (S. Imai) Corner (EU834196.1 and EU624411.1). Intra-specific ITS variations could be inferred from different topological positions of *C. subfastigiatus* isolates. The *C. ligula* (Schaeff.) Donk 1933 and *C. mucronatus* Wells & Kempton were found to have same branch lengths with minor divergence (0.6) in rDNA-ITS and showed 97.8% identity (Fig. 2). These two morphologically divergent species seem to be convergent phylogenetically. BLAST analysis and phylogenetic

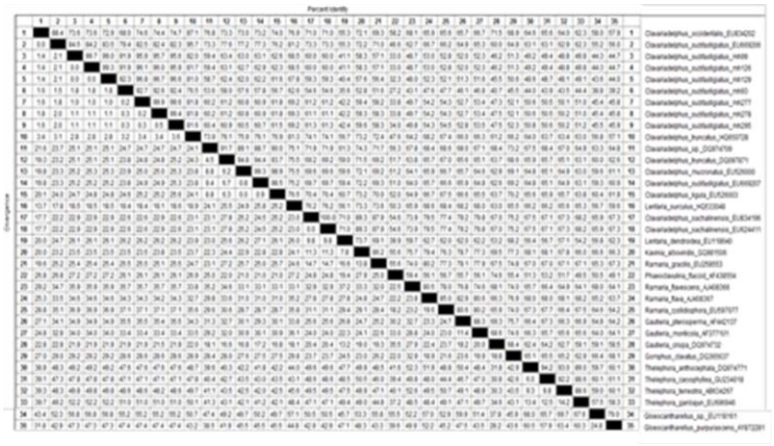

Fig. 2: Percent divergence is calculated by comparing sequence pairs in relation to the phylogeny reconstructed by MegAlign (DNASTAR). Percent identities compare sequences directly without accounting for phylogenetic relationships

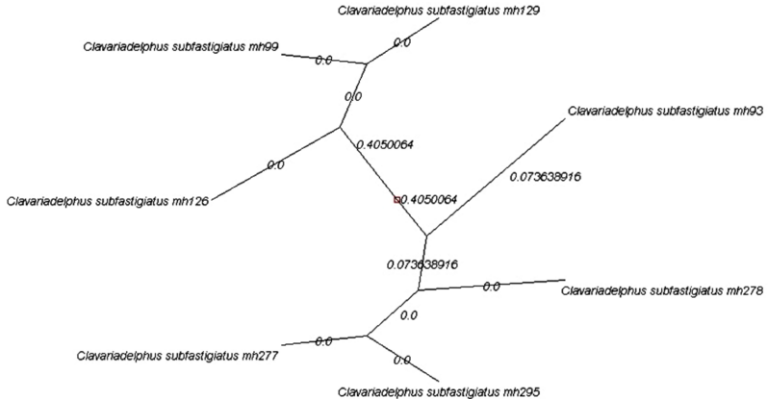

Fig. 3: Radial neighbour-joining (NJ) tree of 7 collections of *C. subfastigiatus*

Fig. 4: Haplotypes of *Clavariadelphus subfastigiatus* Wells and Kempton
Basidiomata (A-E): (A). MH228904.129 (B). MH129901.126 (C). MH089901.99 (D). MH168901.93 (D). MH3060701.297 (E). MH168902.277

placement of *C. subfastigiatus* with its replicate in the tree confirms its identification supported by morpho-anatomic characterization.

rDNA-ITS Sequence Analysis for Intraspecific Variations and Haplotype Analysis

We examined intra-specific variations in 7 sequences *C. subfastigiatus* and encountered two haplotypes forming species-complex. For the purpose of analysis, ITS sequences were considered to belong to different species if they differed in 3% or more base pairs across the ITS1/5.8s/ITS2 region. This 97% similarity criterion is similar to that of several previous studies (Izzo *et al*., 2005; Tedersoo *et al*., 2007). ITS variation within *C.*

subfastigiatus-complex ranged from identical to 2.1 (Fig. 2). The radial distance tree (Fig. 3) resulting from the NJ analysis shows separation between individuals of *C. subfastigiatus* (Fig. 3). These 2 haplotypes (h) separated from each other with 0.571 Haplotype/gene diversity (Hd).

We detected intraspecific variations within *C. subfastigiatus*-complex at 10 positions in the matrix of 679 bp. These were at 39, 52, 83, 133, 172, 220-221, 228, 441-442, 502 and 566 positions (Table 3).

Discussion

Originally described by Wells and Kempton (1968), *C. subfastigiatus* is characterized by its simple, subcylindric, clavate or broadly clavate basidiomata, smooth, becoming longitudinally rugose to rugulose, initially gray-red, dull red fading to salmon, gray-orange, apex obtuse or broadly rounded, often irregularly so, smooth, slowly pale brown to brown (Methven, 1990). This species is a new addition to the mycoflora of Pakistan. Previously 3 species of this genus were reported (Ahmad *et al*., 1997). Basidiomata of *C. subfastigiatus* collected from different localities of HMTF of Pakistan are almost homologous morphologically. However, when their rDNA sequences were compared, they showed intraspecific variations. All rDNA sequences belonging to *C. subfastigiatus* were grouped into 2 haplotypes; haplotype I comprised 3 isolates viz., mh126, mh129 and mh99 within C1 clade and haplotype II formed by 4 isolates viz., mh277, mh295 and mh278 within C2 clade (Fig. 2).

Although most fungal systematists can readily distinguish *Clavariadelphus* from other clavarioid, cantharelloid, craterelloid or gomphoid members of *Aphyllophorales* (Donk, 1933; Corner, 1950, 1970; Wells and Kempton 1968; Petersen, 1972; Petersen *et al.*, 1974), delimitation of infrageneric taxa has been proven difficult in many cases. A considerable part of this problem is due to subtle variations in basidiomata color, shape, growth habit and micro-morphological structures, especially in taxa with wide distributional ranges (Methven, 1990). For the present study, both morphological and molecular data confirm this specie as *C. subfastigiatus* when compared morphologically with its allies.

The BLAST analysis of all these isolates matched with an American isolate *C. subfastigiatus* (EU669206.1) with 98% identity. Two haplotypes differed in shared genetic characters in alignment matrix. Haplotype I shared 98% (621/634) genetic characters, while haplotype II also shared 98% (613/627) of genetic characters compared to American isolate.

The rDNA sequences of *C. subfastigiatus* samples from Pakistan displayed intra-specific variations within the species. These species occupied different topological positions despite having maximum similarity with each other in maximum likelihood tree. The phylogram showed the phylogenetic affinities of *C. subfastigiatus* with *C. truncatus* and *C. occidentalis* Methven on the basis of genetic characters studied so far (Fig. 1). All the basidiomes of *C. subfastigiatus* collected during 2008-2010 were amplified and sequenced. It is quite evident from phylogenetic placement of these sequences at three different positions. The similar intra-specific variations were reported by Grebenc *et al.* (2009). They studied such variations in *H. repandum* L. and *H. rufescens* Schaeff., while Olariaga *et al*. (2009) also reported intra-specific variations in *Clavulina J.* Schröt. species in their rDNA-ITS.

Intraspecific variations were observed within ITS1/5.8s/ITS2 regions with some Indels. We recorded polymorphism at 10 positions in the entire alignment of *C. subfastigiatus* isolates from Pakistan (Table 1). Both haplotypes of *C. subfastigiatus*-complex morpho-anatomically similar but different on molecular basis, as inferred from haplotype analysis. Haplotype I differed from haplotype II at seven positions. Haplotype I had C nucleotide, while haplotype II had T at position 39. This type of base substitutions were recorded at 6 more sites (52, 83, 133, 502 and 566) while deletions were also recorded at 3 positions. All these variations occurred in ITS1 and ITS2 regions of both haploytpes. Similar pattern of intraspecific variations have been reported by Hosoya *et al.* (2010).

In conclusion this study suggests the presence of intra-specific variation within rDNA-ITS of *C. subfastigiatus* isolates forming *C. subfastigiatus*-complex. Genetic differences delimited the 2 haplotypes collected locally for present investigation. The minor morphological differences of color could not be noticed at the time of collection. These differences may be due to environmental factors.

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