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Research Article

Identification and Characterization of Ectomycorrhizal *Cortinarius* Species (Agaricales, Basidiomycetes) from Temperate Kashmir Himalaya, India, by ITS Barcoding

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The coniferous forests of Kashmir Himalayas provide a sustainable habitat for wide varieties of ectomycorrhizal fungi. The identification and characterization of many of these fungi however largely involves morphological descriptions of sporocarps alone, thus sometimes raising questions about the authenticity of these studies. The present study was carried out to identify and characterize ectomycorrhizal fungi from the coniferous forests of Kashmir Himalaya using both morphological and molecular methods. Herein we report on the identification and characterization of three potential ectomycorrhizal *Cortinarius* fungal species, namely, *Cortinarius flexipes* (Pers. Ex Fr.), *Cortinarius fulvoconicus* M. M. Moser, and *Cortinarius infractus* (Pers.) Fr., from Kashmir Himalaya, India, on the basis of their morphological and molecular characterization. Morphological characteristics of all species were measured and compared with standard taxonomic literature. ITS-rDNA (the fungal molecular marker) was used for molecular analysis. The target region of rDNA (ITS1 5.8s ITS2) of these species was amplified using universal fungal primers (ITS1 and ITS4). The sequencing of amplified products and their subsequent blast analysis confirmed the identification of species by comparing the sequences of these species with respective species sequences present in GenBank. Phylogenetic analysis also confirmed the identification of species.

1. Introduction

Ectomycorrhizas are mutualistic symbiotic associations formed between roots of higher plants with certain soil fungi. The mycorrhizal fungi benefit associated host trees in a number of ways although the most important is by enhancing nutrient mobilization particularly for elements with low mobility in the soil (N and P) and several micronutrients, release of nutrients from mineral particles or rock surfaces via weathering, effects on carbon cycling, interactions with mycoheterotrophic plants, mediation of plant responses to stress factors such as drought, soil acidification, toxic metals, and plant pathogens, and rehabilitation and regeneration of degraded forest ecosystems, as well as a range of possible interactions with groups of other soil microorganisms [1–4]. The fungi in return get benefited by receiving 30–60% of the net photosynthate produced by the host [5]. Worldwide ectomycorrhizal fungi are represented by 343 genera including 11,950 species, of which 252 genera belong to Basidiomycota, 84 to Ascomycota, and 5 to Zygomycota [6].

Cortinarius is one of the largest genera of mushrooms and is represented by more than 2000 ECM species all over the world [7]. Though the genus *Cortinarius* is very diverse, the species of *Cortinarius* are usually pretty easy to figure out from other mushroom species due to presence of diagnostic macroscopic characters. Firstly their gills are covered with cortina when young; the tiny fibers of the cortina also form ring zone on the stem. Secondly, the mature gills of *Cortinarius* mushrooms are usually rusty brown due

to the presence of rusty brown spores. Though the genus is more or less easy to identify from other genera, the identification of species is one of the most difficult challenges in mushroom mycology, compounded by the fact that there are few comprehensive, current, and reliable keys available for the genus.

The Jammu and Kashmir state located in the far north of India is a mountainous area in the northwest Himalayas that shares international boundaries with Pakistan in the west and China in the northeast. Punjab and Himachal Pradesh are its neighbouring states within the country. The state has a geographic area of 101387 sq. km. It lies between 32°17′ and 37°05′ north latitude and 72°31′ and 80°20′ east longitude. The state is divided into three geographic regions: Ladakh, Kashmir Valley, and Jammu. The Kashmir Valley lies between 33°20′ and 34°54′ N latitude and 73°55′ and 75°35′ E longitudes covering an area of 15,948 sq. km and harbors diverse coniferous forests. The coniferous forests of Kashmir valley support diverse populations of *Pinus*, *Picea*, *Cedrus*, and *Abies* which are well known hosts for ectomycorrhizal fungi. The forests in Kashmir support diverse ectomycorrhizal communities and varied ectomycorrhizal sporocarps are found fruiting in these forests during growing season. Earlier workers like Watling and Abraham [8] have reported the occurrence of numerous ectomycorrhizal species from this region. But until now from this region, taxonomic and phylogenetic studies of ectomycorrhizal fungal sporocarps have been based mainly on the analysis and comparison of macroscopic morphological characters like the shape, size, and colour of caps, stalk, and gills. Though morphological characters are of use in identification and characterization of mushroom species but the number of species that can be identified through morphological methods is relatively few and limited. Morphological methods are of no use in discriminating between closely related species and mostly they are helpful in taxonomic identification only up to the genus level in most cases. Molecular methods are currently available to overcome this problem as they exhibit high sensitivity and specificity for identifying fungi at diverse hierarchical taxonomic levels [9].

Together with classical taxonomical methods, molecular methods may be useful and helpful for the correct identification of mushroom species. Through molecular methods most of the fungi have been identified by comparative analyses of the ribosomal DNA sequences, especially the ITS region. For example, Peintner et al. [10] first recorded ectomycorrhizal *Cortinarius* species from tropical India and established their phylogenetic position using ITS sequences. Itoo et al. [11] characterized and identified *Russula firmula* and *R. postiana* from Kashmir Himalaya using analysis of ITS sequence.

In this study, the combined morphological and molecular nucleotide analysis of various *Cortinarius* species collected from different forest areas of Kashmir Himalaya was used as a tool for their characterization and identification. DNA sequence analysis of the ITS region was performed using the whole ITS region, including ITS1, 5.8S, and ITS2 helping us to identify the *Cortinarius* species. We used nuclear ribosomal DNA sequences of the internal transcribed spacers (ITS-rDNA) to identify and to investigate the taxonomic and phylogenetic position of these taxa. These sequences have proved to be most useful for molecular systematics of *Cortinarius* [10, 12] and related genera [13]. For the phylogenetic placement of these species, numerous sequences are available for comparison in GenBank. Our aim was to identify and characterize the field collected *Cortinarius* species and to address the question of the phylogenetic relationships between these and morphologically similar *Cortinarius* species.

2. Materials and Methods

2.1. Specimen Collection. The ectomycorrhizal sporocarps were collected from different coniferous forest areas of Kashmir Himalaya, like Gulmarg, Kokernag, Daksum, Drang, Mammer, and so forth. The colour of the sporocarps was recorded at the time of collection. Colour of sporocarps was described based on the codes of Kornerup and Wanscher (1978). Standard methods were followed for the collection of sporocarps [14]. Sporocarps were carefully dug out with the help of a knife and photographed in the field. Detailed macromorphological characters of sporocarps, such as colour, shape, size, and odour of sporocarps, were recorded in the field and sample specimens of each type were carried to laboratory in a flat wicker basket for detailed examination. The specimens were examined by standard microscopic techniques in 3% KOH. Habit and habitat, association pattern, altitude, and forest status were recorded in the field.

2.2. Molecular Characterization. The molecular characterization of sporocarps involved sequencing of internal transcribed spacer (ITS) region of the nuclear ribosomal genes (rDNA). For this, genomic DNA was isolated from sporocarps and roots of collected species.

2.2.1. DNA Extraction. Genomic DNA was isolated from fresh sporocarps by CTAB method. For this 200–250 mg of material was taken and grinded into fine powder with the aid of liquid nitrogen. The powder was then taken in 15 mL centrifuge tube and to this 5 mL prewarmed CTAB buffer (1 M Tris HCl pH 8.0, 5 M NaCl, 0.5 M EDTA pH 8.0, CTAB, 2% β-mercaptoethanol) was added. This was then subjected to various steps like addition of chloroform, isopropyl alcohol, phenol, chloroform: isoamyl alcohol, and ribonuclease, and finally extracted DNA pellet was kept in 50 μL TE buffer at −20°C. Purified DNA was separated in a 1% agarose gel stained with ethidium bromide and concentration was estimated by comparison with known length standards.

2.2.2. PCR Analysis. The ITS region of rDNA was amplified by polymerase chain reaction (PCR) with ITS1 and ITS4 primers in Applied Biosystems 2720 Thermal Cycler. The amplified fragment includes ITS1, 5.8S, and the ITS2 of rDNA. The 50 μL reaction mixture for PCR amplification contained 2 μL template DNA, 5 μL PCR buffer, 5 μL of 2 mM DNTps, 3 μL of each primer, and 0.4 μL of Taq polymerase. Amplifications were performed in a thermal cycler with an initial denaturation step of 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 54°C for 1 min, and 72°C for

(a) *Cortinarius flexipes*

(b) *Cortinarius fulviconicus*

(c) *Cortinarius intractus*

Figure 1: Field photograps of *Cortinarius* species: (a) *Cortinarius flexipes*, (b) *Cortinarius fulviconicus*, and (c) *Cortinarius intractus*.

1 min, and a final extension of 72°C for 8 min. The purified PCR products of the ITS amplified region were directly sequenced in both directions using the ITS1 and ITS4 pair of amplification primers (SciGenom).

2.2.3. DNA Sequence Assembly and Alignment. The sequenced PCR amplicons were BLAST (Basic Local Alignment Search Tool) searched using the National Center for Biotechnology Information (NCBI), USA, database for comparison of sequences. The initial alignment of all sequences was directly made with the ClustalX multiple alignment program [15]. The alignment was examined and adjusted manually using Microsoft Word on a computer. Manual alignment was facilitated by the use of a colour font.

2.3. Phylogenetic Analysis. For phylogenetic analysis closely related sequences were retrieved from GenBank. The sequence alignments were performed using Molecular Evolutionary Genetics Analysis (MEGA) software [16]. Phylogenetic analysis was conducted on both the ITS and 5.8S gene data in neighbor-joining (NJ) method using Clustalx and PHYLIP 3.69 programmes. The programs DNADIST and NEIGHBOR from PHYLIP 3.69 [17] were used to generate the distance matrix and to produce the tree. Confidence in the branches of the neighbour-joining tree was assessed by bootstrap analysis [18] using 1000 replicates. The programs SEQBOOT, DNADIST, NEIGHBOR, and CONSENSE in the PHYLIP package [17] were used for this purpose.

3. Results

Three ectomycorrhizal species from genus *Cortinarius* were characterized and identified by using morphoanatomical and molecular characterization of sporocarps. The species characterized and identified are *Cortinarius flexipes* (Pers. ex Fr.), *Cortinarius fulvoconicus* M. M. Moser, and *Cortinarius infractus* (Pers.) Fr.

3.1. Taxonomy

3.1.1. Cortinarius flexipes (Figure 1(a)). Pileus (Pers. ex Fr.): cap 2–5 cm broad, shape convex or conical when young, then expanded and umbonate with age, surface subviscid, silky,

dark brown when moist, especially at the centre, drying pale fawn, covered with minute white fibrous scales; flesh pale orange coloured, no colour change on bruising. Lamellae: gills close and crowded; attachment adnate; dark brown often with violet tinge. Stipe: stem 3–8 cm long, 1-2 cm thick at the base, clavate; solid and firm when young, viscid; brownish, covered at first with the white cottony veil which forms a distinct but short-lived ring and cottony scales below. Spores: spores broadly elliptic, spore print rust brown. Habit: naucorioid; growth type: solitary occasionally scattered in coniferous forests.

3.1.2. Cortinarius fulvoconicus (Figure 1(b)). Pileus: cap 5–10 cm broad; convex or conical when young, with age becomes broadly convex; surface sub viscid, silky, smooth with appressed fibrils over the entire surface, fibrils more towards margin; colour light yellow to orange, with age colour faints; margin incurved, entire, not splitting at maturity; flesh pale orange coloured, no colour change on bruising. Lamellae: gills attached with stem, attachment adnate, space moderate, coloured like the cap, covered with orange coloured cortina when young. Stipe: 2–8 cm long, 1-2 cm thick; attachment central; more or less equal; dry; silky; pale orangish above, cream white colored below; fibrillar orange coloured cortina at stipe apex; flesh pale orangish; odor: mild. Habit: naucorioid; growth type: solitary occasionally scattered.

3.1.3. Cortinarius infractus (Pers.) Fr. (Figure 1(c)). Pileus: cap 5–10 cm broad, shape convex, with age becomes broadly convex to flat, or sometimes bell-shaped; surface moist and sticky; margin entire, incurved, splitting at maturity; colour generally grayish when young, with age becomes brown with deep dark brown shades; flesh white, no colour change on bruising or on exposure to air. Lamellae: gills attached with stem, attachment adnate; close; colour light brow to gray, with age becoming rusty brown. Stipe: stem 5–8 cm long, 2-3 cm thick; club shaped; surface dry; colour whitish, with age discolouring brownish, occasionally purplish scales preset at apex when young; apex remains adhering with rusty cortina remnants; flesh white and firm. Spores: spore print rusty brown. Habit: naucorioid; growth type: solitary occasionally scattered.

3.2. Molecular Characterization. The molecular characterization was performed by carrying out sequencing of rDNA ITS region. The ITS region amplified with ITS1 and ITS4 pair of primers varied in length from 650 to 750 bp in the three species (Figure 2). The ITS region was 650 bp in *Cortinarius flexipes* and 750 bp in *Cortinarius fulvoconicus* and *Cortinarius infractus*. The ITS sequences were subjected to nucleotide sequence alignments using the software ClustalX and sequence comparisons were performed with BLAST network services using National Center for Biotechnology Information (NCBI), USA database for confirmation of their identity. The BLAST result is presented in Table 1. Based on percentage identity and query coverage, the three mushroom species may be identified as follows: accession KC859462 showed 92% identity with *Cortinarius flexipes* (AY669683.1; 90% coverage); KF023073 showed 99% identity with *Cortinarius fulvoconicus* (AY669677.1; 100% coverage); and KF727563 showed 99% identity with *Cortinarius* cf. *infractus* (FJ039612.1; 100% coverage). All the three species were identified up to species level from the available GenBank database.

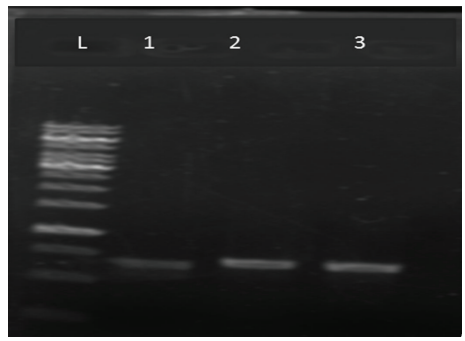

Figure 2: 1% agarose gel showing amplified ITS rDNA PCR products of 3 different ECM *Cortinarius* species. Samples in each lane are as follows: L, 1 kb DNA ladder; 1, *Cortinarius flexipes*; 2, *Cortinarius fulvoconicus*; and 3, *Cortinarius infractus*.

The identity of species was further confirmed by performing phylogenetic analysis of the species in neighbour-joining method. The closely related top matched BLAST sequences with which the present study isolates showed maximum identity were retrieved from GenBank for phylogenetic analysis with present study species accessions. The phylogenetic analysis of the large dataset including 38 ITS sequences of *Cortinarius* spp. treated resulted in formation of cladogram (Figure 3). The phylogenetic cladogram revealed a close relationship between KC859462 and AY669683.1 (*Cortinarius flexipes*), KF023073.1 and AY669677.1 (*Cortinarius fulvoconicus*), and KF727563 and FJ039612.1 (*Cortinarius infractus*). The identity of species was further confirmed by computing mean pairwise distance. All the three accessions of the present study *Cortinarius* species showed below 0.004 distances with top matched BLAST searched accession and with which they clustered on parsimony analysis thus confirming their identity (Table 2). Accession KC859462.1 showed 0.000 pairwise distance with AY669683.1 (*C. flexipes*) (Table 2(a)); accession KF023073.1 showed 0.001 pairwise distance with AY669677.1 (*C. fulvoconicus*) (Table 2(b)), and accession KF727563.1 showed 0.001 distance with FJ039612.1 (*Cortinarius* cf. *infractus*) (Table 2(c)).

4. Discussion

The coniferous forests of Kashmir Himalaya support diverse ectomycorrhizal communities due to varied and diverse climatic conditions. The ECM species richness of the region is directly related to its diverse forest communities and weather patterns, but all the regions of the state have not been extensively surveyed and explored for ectomycorrhizal fungi fruiting in different seasons and associated with different host trees till now. Most of the species reported from Kashmir Himalaya have been identified solely on the basis

Table 1: GenBank accession numbers and top BLAST match sequences of the mushroom isolates along with maximum identity, query coverage.

Accession number	BLAST match sequence		
	Reference accession number	Coverage	Maximum identity
KC859462	AY669683.1 *Cortinarius flexipes*	90	92
	AF430262.1 *Cortinarius* sp.	89	90
	JF907921.1 *Cortinarius traganus*	88	90
	FJ827156.1 *Cortinarius* clone 3	89	90
	GQ159816.1 *Cortinarius vernus*	86	89
	FN428982.1 *Cortinarius gallurae*	85	89
	AF389156.1 *Cortinarius paleaceus*	84	85
	EU259284.1 *Cortinarius brunneifolius*	84	89
	FJ039542.1 *Cortinarius sertipes*	80	85
	HQ604727.1 *Cortinarius fulvescens*	80	84
KF023073.1	AY669677.1 *Cortinarius fulvoconicus*	100	99
	FJ039534.1 *Cortinarius alnetorum*	99	97
	HQ604701.1 *Cortinarius* cf. *umbrinolens*	100	97
	HQ604714.1 *Cortinarius casimiri*	100	97
	FJ039552.1 *Cortinarius subsertipes*	100	97
	FJ039562.1 *Cortinarius canabarba*	100	97
	HQ604706.1 *Cortinarius* cf. *subsertipes*	100	97
	FJ039551.1 *Cortinarius saturninus*	100	97
	AY669672.1 *Cortinarius psammocephalus*	100	97
	AY669658.1 *Cortinarius umbrinolens*	100	97
KF727563	FJ039612.1 *Cortinarius* cf. *infractus*	100	99
	HQ604687.1 *Cortinarius infractus*	100	98
	HQ604688.1 *Cortinarius infractus*	100	98
	AY669536.1 *Cortinarius infractus*	96	97
	FJ717596.1 *Cortinarius variosimilis*	99	94
	HQ604653.1 *Cortinarius cinnamomeus*	99	94
	FJ717595.1 *Cortinarius badiolatus*	99	94
	DQ384593.1 *Cortinarius phoeniceus*	99	94
	FJ039602.1 *Cortinarius illumines*	99	94
	FJ039601.1 *Cortinarius olivaceopictus*	99	94

of morphological and microscopic characters. Morphological identification of ECM sporocarps is difficult and requires profound knowledge and experience; it is prone to mistakes due to the frequent homoplasy of phenetic characters. In addition phenotypic variation in fungi can be affected by substrate and environmental factors limiting the application of morphological characters in the identification of ECM sporocarps [19]. Molecular data provides more precise information of the genetic variability of individuals than the phenotypic characters [20].

The combined approach of morphological and molecular biology lets us identify and characterize the field collected ectomycorrhizal sporocarps of three *Cortinarius* species, namely, *Cortinarius flexipes*, *Cortinarius fulvoconicus*, and *Cortinarius infractus*. We used sequences of ITS as an efficient taxonomic tool to identify ectomycorrhizal fungi from pure basidiocarps collected directly in the field.

The species of *Cortinarius* were found associated with *Pinus wallichiana* as the sporocarps were collected under the *Pinus wallichiana* trees and were found associated with the host roots by tracing out the root system. The association of various *Cortinarius* species with conifers has also been reported earlier by Watling and Abraham [8]. This was an initial attempt to solve some of the many ambiguities related to the taxonomic classification of *Cortinarius* species, especially with regard to discriminating among closely related species from this region. Morphological characters are of no use in discriminating between closely related *Cortinarius* species. The ITS region of fungal ribosomal DNA (rDNA) is highly variable sequence of great importance in distinguishing fungal species by PCR analysis [21, 22]. The highly conserved nature of the ITS sequences makes them an efficient DNA marker for taxonomic and phylogenetic evaluations of the Basidiomycetes. The advantages of using ITS sequences as tool for identification are demonstrated by distinct sequences with high levels of divergence and differentiation, easy amplification, ample type data, and their location between highly conserved regions [23], which

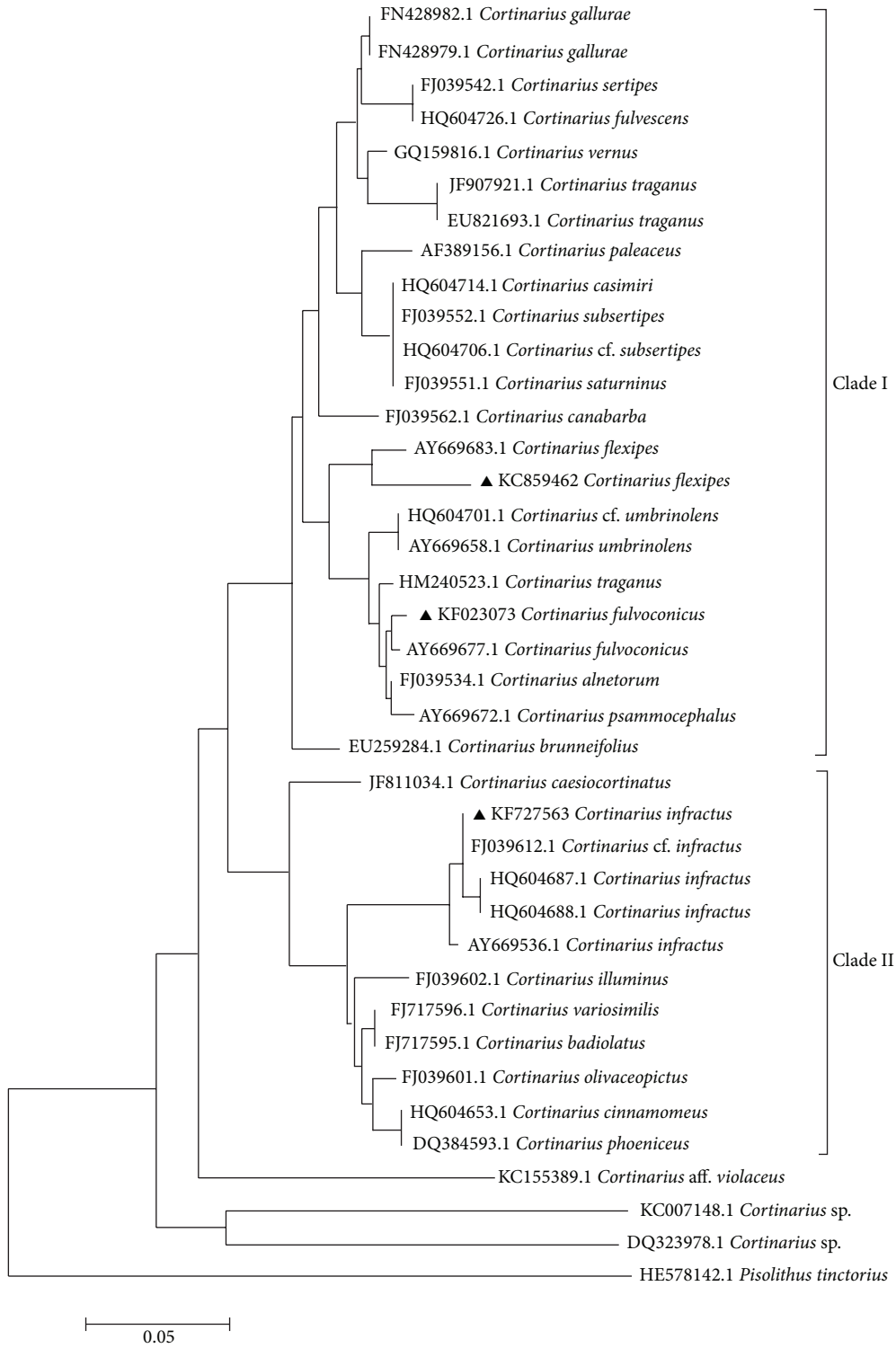

Figure 3: Phylogenetic relationship of present study ITS sequences ▲ with other related members based on maximum likelihood method inferred from ITS sequences. *Pisolithus tinctorius* was used as outgroup.

makes them the superior molecular DNA barcode for the identification of Basidiomycetes at the families and species level.

Analysis of basidiocarps using ITS primers (ITS1 and ITS4 and their derivatives) and PCR analysis had proven very useful and easy method for identifying particular ECM species [24, 25]. The ITS (ITS1, 5.8S, ITS2) region of the species sequenced varied considerably in length from 650 to 700 bp. Phylogenetic analysis of the ITS sequence with other related sequences retrieved from GenBank confirmed their

Table 2: (a) Pairwise nucleotide divergence among the various accessions with which KC859462.1 showed maximum similarity. (b) Pairwise nucleotide divergence among the various accessions with which KF023073.1 showed maximum similarity. (c) Pairwise nucleotide divergence among the various accessions with which KF727563.1 showed maximum similarity.

(a)

KC859462											
AY669683.1 *Cortinarius flexipes*	0.000										
AF430262.1 *Cortinarius* sp.	0.003	0.032									
JF907921.1 *Cortinarius traganus*	0.004	0.024	0.026								
HM240523.1 *Cortinarius traganus*	0.004	0.030	0.030	0.020							
GQ159816.1 *Cortinarius vernus*	0.034	0.030	0.030	0.020	0.000						
FN428982.1 *Cortinarius gallurae*	0.034	0.034	0.034	0.030	0.026	0.026					
AF389156.1 *Cortinarius paleaceus*	0.034	0.030	0.030	0.020	0.000	0.000	0.026				
EU821693.1 *Cortinarius traganus*	0.034	0.030	0.030	0.020	0.000	0.000	0.026	0.000			
FJ039542.1 *Cortinarius sertipes*	0.037	0.030	0.005	0.024	0.028	0.028	0.032	0.028	0.028		
FN428979.1 *Cortinarius gallurae*	0.032	0.024	0.026	0.000	0.020	0.020	0.030	0.020	0.020	0.024	
HQ604726.1 *Cortinarius fulvescens*	0.395	0.395	0.392	0.384	0.389	0.389	0.391	0.389	0.389	0.398	0.384

(b)

KF023073										
AY669677.1 *Cortinarius fulvoconicus*	0.001									
FJ039534.1 *Cortinarius alnetorum*	0.009	0.032								
HQ604701.1 *Cortinarius* cf. *umbrinolens*	0.032	0.024	0.026							
HQ604714.1 *Cortinarius casimiri*	0.034	0.030	0.030	0.020						
FJ039552.1 *Cortinarius subsertipes*	0.034	0.030	0.030	0.020	0.000					
FJ039562.1 *Cortinarius canabarba*	0.034	0.034	0.034	0.030	0.026	0.026				
HQ604706.1 *Cortinarius* cf. *subsertipes*	0.034	0.030	0.030	0.020	0.000	0.000	0.026			
FJ039551.1 *Cortinarius saturninus*	0.034	0.030	0.030	0.020	0.000	0.000	0.026	0.000		
AY669672.1 *Cortinarius psammocephalus*	0.037	0.030	0.005	0.024	0.028	0.028	0.032	0.028	0.028	
AY669658.1 *Cortinarius umbrinolens*	0.032	0.024	0.026	0.000	0.020	0.020	0.030	0.020	0.020	0.024

(c)

KF727563										
FJ039612.1 *Cortinarius* cf. *infractus*	0.001									
HQ604687.1 *Cortinarius infractus*	0.004	0.004								
HQ604688.1 *Cortinarius infractus*	0.004	0.004	0.000							
AY669536.1 *Cortinarius infractus*	0.011	0.011	0.012	0.012						
FJ717596.1 *Cortinarius variosimilis*	0.049	0.044	0.044	0.044	0.041					
HQ604653.1 *Cortinarius cinnamomeus*	0.044	0.041	0.040	0.040	0.038	0.037				
FJ717595.1 *Cortinarius badiolatus*	0.047	0.043	0.042	0.042	0.039	0.016	0.034			
DQ384593.1 *Cortinarius phoeniceus*	0.044	0.040	0.039	0.039	0.037	0.039	0.006	0.034		
FJ039602.1 *Cortinarius illumines*	0.045	0.043	0.042	0.042	0.040	0.036	0.035	0.035	0.036	
FJ039601.1 *Cortinarius olivaceopictus*	0.046	0.041	0.041	0.041	0.041	0.035	0.020	0.031	0.021	0.034

identity. Out of the three species characterized *Cortinarius flexipes* is a new report from Kashmir Himalaya. Peintner et al. [10] recorded ectomycorrhizal *Cortinarius* species from tropical India and established their phylogenetic position using ITS sequences. Some others have also used a similar technique for identification of ECM species [26, 27]. ITS-rDNA the fungal molecular marker in combination with morphoanatomical characters and illustrations is thus a valuable tool for correct identification of ECM species.

5. Conclusion

The combined approach of morphological and molecular analysis can enrich and provide additional information to

mushroom biodiversity and GenBank database and can also lead to the discovery of many new unidentified species from the region.

Conflict of Interests

The authors declare no financial or nonfinancial conflict of interests.

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