RESEARCH PAPERS

# MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF ECTOMYCORRHIZAL *INOCYBE* SPECIES FROM TEMPERATE FORESTS OF KASHMIR HIMALAYA, INDIA

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**Abstract**

Two ectomycorrhizal mushroom species of genus *Inocybe* namely *Inocybe geophylla* and *Inocybe mimica* have been identified and characterized from temperate forests of Kashmir Himalaya, India. The species were identified on the basis of detailed morphological description of sporocarps and by performing molecular characterization of rDNA ITS region. Morphological characteristics like shape, size and colour of pileus, stipe and gills, basidiospore size, basidial lengths of the two species were measured and compared with data available in the literature. ITS-rDNA (the fungal molecular marker) was used for molecular analysis. The target region of rDNA (ITS1 5.8s ITS2) was amplified using polymerase chain reaction (PCR) with universal fungal primers (ITS1 and ITS4), which generated 700 to 750bp fragments. The BLAST search of amplified sequenced product confirmed the identification of species by comparing the sequences of these species with respective species present in GenBank. Phylogenetic analysis of ITS sequence was performed in neighbour joining method and confirmed the identity of species.

***Keywords:*** *ectomycorrhizal; coniferous; sporocarp; phylogenetic; GenBank.*

## Introduction

The genus *Inocybe* (Fr.) Fr. is a highly diverse monophyletic group of ectomycorrhizal (ECM) fungi that includes between 500 and 700 species worldwide (Matheny et al., 2009; Alvarado et al., 2010). The species of *Inocybe* form ectomycorrhizal associations primarily with members of various plant families, such as Pinaceae, Fagaceae, and Salicaceae and more than 80% species of the genus have been described from the North Temperate Zone (Matheny et al., 2012).

The Kashmir Valley, that lies between 33° 20′and 34° 54′ N latitude and 73° 55′ and 75° 35′ E longitudes covering an area of 15,948 sq. kms, harbours diverse temperate coniferous forests 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 (1992) have reported the occurrence of numerous ectomycorrhizal species from this region. Based on the surveys carried out by these authors, three ectomycorrhizal *Inocybe* species have been reported to be associated with *Salix alba* in the Kashmir Himalaya. But until now taxonomic and phylogenetic studies of ectomycorrhizal fungal sporocarps in Kashmir Himalayan 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 approaches have resulted in beautifully illustrated manuals (Agerer, 1987; Ingleby et al., 1990), but the number of species described and identified based on morphological characteristics is relatively few and limited and many common types are essentially described as imperfect states with unknown affinities (Ingleby et al., 1990; Agerer, 1994). Molecular methods are currently available to overcome this problem as they exhibit high sensitivity and specificity for identifying fungi at diverse hierarchical taxonomic levels (Sette et al., 2006).

Together with classical taxonomical methods, molecular methods are 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. (2003) first recorded ectomycorrhizal *Cortinarius* species from tropical India and established their phylogenetic position using ITS sequences. Huang et al. (2009) identified 34 endophytic fungi isolated from three *Artemisia* species using molecular phylogenetic analysis of ITS region. Itoo et al. (2013) characterized and identified *Russula firmula* and *R. postiana* from Kashmir Himalaya using analysis of ITS sequence.

In the present study, the combined morphological and molecular nucleotide analysis of *Inocybe* 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 and helped us in the identification of *Inocybe* species.

## Materials and Methods

### Specimen collection

The ectomycorrhizal sporocarps were collected from different coniferous forest areas of Kashmir Himalaya, like Gulmarg, Kokernag, Daksum, Drang, Mammer etc. Standard methods were followed for the collection of sporocarps (Atri et al., 2003). Sporocarps were carefully dugout with the help of a knife and photographed in the field. Detailed macro-morphological characters of sporocarps, such as colour, shape, size, 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, forest status were recorded in the field.

### Molecular characterization

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 of collected species.

### DNA extraction

Genomic DNA was isolated from fresh sporocarps by CTAB method. For this 200-250mg of material was taken and ground into fine powder in liquid nitrogen. The powder was then taken in 15ml centrifuge tube and to this 5 ml pre-warmed CTAB buffer (1M TrisHCl pH 8.0, 5M NaCl, 0.5M 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, 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.

### 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 50µl reaction mixture for PCR amplification contained 2µl template DNA, 5µl PCR buffer, 5µl of 2mM DNTps, 3µl of each primer, and 0.4µl 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 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 (Scigenome).

### 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 (Higgins et al., 1992). The alignment was examined and adjusted manually using Microsoft Word. Manual alignment was facilitated by the use of a colour font.

### Phylogenetic analysis

For phylogenetic analysis closely related sequences were retrieved from GenBank. The sequence alignments were performed using Molecular Evolutionary Genetics Analysis (MEGA) software (Tamura et al., 2011). 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 (Felsenstein, 1995) 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 (Felsenstein, 1985) using 1000 replicates. The programs SEQBOOT, DNADIST, NEIGHBOR, and CONSENSE in the PHYLIP package (Felsenstein, 1995) were used for this purpose.

### Results

Two ectomycorrhizal species of genus *Inocybe*, namely *Inocybe geophylla* and *Inocybe mimica* collected from Kashmir Himalaya, India were characterized and identified based on their physico-morphological and molecular characteristics.

### Morphological characterization

***Inocybe geophylla*** (Pers.) Kummer (Fig. 1a): **Pileus:** Cap 1.5-2.0 cm broad at maturity, ovoid in button stage, becoming conic to campanulate, plane to plano-depressed with age, umbonate; immature margin adherent to stipe, fibrillose, incurved, then decurved, eventually plane to slightly raised with age; surface at first pale lavender, mature caps appressed-fibrillose, straw-brown; context thin, flesh unchanging on bruising; taste mild. **Lamellae:** Gills adnexed, close, pale-lavender when young, fading to whitish, with age brown to pale grey; gill edges fringed. **Stipe:** 2.0-4.5 cm long, 2.0-3.0 mm thick, equal in diameter, round, stuffed to hollow at maturity; surface pale lavender, pruinose to minutely hairy, in age nearly glabrous or with scattered appressed hairs; partial veil evanescent, fibrillose, best seen in young sporocarps, leaving sparse fibrils in an annular zone high on the

stipe; volva absent. **Habit:** Naucorioid. **Growth type:** Solitary.

***Inocybe mimica*** Massee (Fig. 1b): **Pileus:** Cap 2-5 cm broad, shape conic when young, with age becomes flat; surface dry, steaky radial fibers on cap, with age and in dry weather fibers split radially towards the edge of cap; margin entire, incurved, with age becomes slightly uplifted and radially splitted; flesh white, no colour change on bruising or on exposure to air. **Lamellae:** Gills attached with stipe, attachment varies from adnexed to adnate; crowded; creamy grey coloured with white edges, with spore maturation becomes olive-grey coloured. **Stipe:** 3-8 cm tall, 1-2 cm thick; surface smooth, silky, fibrillose towards the base; apex pale, base straw-yellow coloured; equal in diameter, cylindrical; flesh white, no colour change on bruising; attachment central; veil absent. Spore print dull brown. **Habit:** Naucorioid**. Growth type:** Solitary, often scattered or gregarious.

### 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 700-750p in the two species (Fig. 2). The ITS region was 700bp in *Inocybe geophylla* and 750bp in *Inocybe mimica.* The amplified ITS region was sequenced with the same set of primers in both the species and the sequences have been deposited in the GenBank under various accession numbers (Table 1). The ITS sequences were then 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 two species were identified as follows: Accession KF835446 showed 99% identity with *Inocybe geophylla* (JQ888171.1, 99% coverage) and accession KF056319 showed 98% identity with *Inocybe mimica* (FJ904124.1, 100% coverage).

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. The phylogenetic analysis of the large dataset including 20 top matched ITS sequences of *Inocybe* species together with two present study accessions resulted in formation of cladogram (Fig. 3). The phylogenetic cladogram revealed a close relationship between KF835446 and JQ888171.1 (*Inocybe geophylla*) and between KF056319 and FJ904124.1 (*Inocybe mimica*). The identity of species was further confirmed by computing mean pair-wise distance. The two accessions of present study *Inocybe* species showed below

0.004 distances with respective top matched BLAST searched accession and with which they clustered on parsimony analysis thus confirming their identity (Table 2). Accession KF835446 showed 0.002 pair wise distance with JQ888171 (*I. geophylla*) (Table 2a) and accession KF056319 showed 0.003 pair wise distance with FJ904124.1 (*I. mimica*) (Table 2b).

**Discussion**

The temperate coniferous forests of Kashmir Himalaya support diverse ectomycorrhizal communities due to varied climatic conditions. The ECM species richness in the region is directly related to its diverse forest communities and weather patterns. Despite rich ECM diversity, very few studies have been carried out with an objective of documenting the diversity of such mutualists. Most of the species reported from Kashmir Himalaya have been identified solely on the basis of morphological 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 (Avin et al., 2012). Molecular data provides more precise information of the genetic variability of individuals than the phenotypic characters (Siddiquee et al., 2012). A range of studies have been carried throughout the world by many researchers based on morphological characteristics of sporocarps, characteristics of mycelia and mating type tests (Gonzalez and Labare`re, 2000; Shnyreva and Shtaer, 2006), but such studies are unreliable and not useful for distinguishing between different species of Basidiomycetes (Choi et al., 2007). For example, *Pleurotus pulmonarius* was misidentified as *P. ostreatus* in a study based on the morphological information conducted in Patagonia (Shnyreva and Shtaer, 2006). The combined approach employing morphological and molecular biology helped us to identify and characterize the field collected ectomycorrhizal sporocarps of *Inocybe* species into two species, namely *Inocybe geophylla* and *Inocybe mimica*. We used sequences of ITS as an efficient taxonomic tool to identify ectomycorrhizal fungi from basidiocarps collected directly in the field. This was an initial attempt to solve some of the many ambiguities related to the taxonomic classification of *Inocybe* species, especially with regard to discriminating among closely related species. The ITS region of fungal ribosomal DNA (rDNA) is highly variable sequence of great importance in distinguishing fungal species by PCR analysis (Nauman et al., 2007; Nilsson et al., 2008). 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 (White et al., 1990), which 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 (Kulmann et al., 2003; Lee et al., 2006). The ITS (ITS1, 5.8S, ITS2) region of the species sequenced varied considerably in length from 700-750bp. Phylogenetic analysis of the ITS sequence with other related sequences retrieved from GenBank confirmed their identity. Out of the two species characterized *Inocybe mimica*, is a new report from Kashmir Himalaya. Peintner et al. (2003) recorded ectomycorrhizal *Cortinarius* species from tropical India and established their phylogenetic position using ITS sequences. Reddy et al. (2005) identified *Pisolithus indicus,* a new species of ectomycorrhizal fungus associated with Dipetrocarps in India on the basis of similar studies. Our findings are also in agreement with the studies conducted by Hortal et al. (2006); Iotti and Zambonelli (2006); Huang et al. (2009) as they also used the similar approach for identification of ECM species. ITS-rDNA the fungal molecular marker in combination with morpho-anatomical characters is thus a valuable tool for correct identification of ECM species.

## Conclusion

The combined approach of morphological and molecular analysis can provide valuable and additional information for precise documentation of mushroom biodiversity.

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**Table 1: GenBank accession numbers and top BLAST match sequences of the two *Inocybe* species along with maximum identity, query coverage.**

| GeneBank accession No | BLAST match sequence | | |
|---|---|---|---|
| | Reference accession No. | Coverage | Maximum identity |
| KF835446 | JQ888171.1 *Inocybe geophylla* | 99 | 99 |
| | JF908236.1 *Inocybe lilacina* | 97 | 98 |
| | AM882873.2 *Inocybe lilacina* | 99 | 96 |
| | AM882869.1 *Inocybe geophylla var. lilacina* | 94 | 97 |
| | EU525981.1 *Inocybe geophylla* | 98 | 96 |
| | HQ604295.1 *Inocybe fuscidula var. fuscidula* | 98 | 95 |
| | EU523556.1 *Inocybe aff. Lilacina* | 95 | 95 |
| | HQ201357.1 *Inocybe lilacina* | 93 | 94 |
| | HQ604288.1 *Inocybe cf. sambucina* | 98 | 87 |
| | EF559265.1 *Inocybe armeniaca* | 94 | 87 |
| KF056319.1 | FJ904124.1 *Inocybe mimica* | 100 | 98 |
| | AM882781.2 *Inocybe mimica* | 95 | 97 |
| | HQ604626.1 *Inocybe sororia* | 99 | 91 |
| | FJ904125.1 *Inocybe cf. rimosa* | 99 | 90 |
| | FJ904139.1 *Inocybe spuria* | 99 | 90 |
| | FJ904129.1 *Inocybe cf. flavella* | 99 | 90 |
| | AM882785.2 *Inocybe squamata* | 99 | 89 |
| | AM882782.2 *Inocybe flavella* | 99 | 89 |
| | AM882783.2 *Inocybe squamata* | 98 | 89 |
| | FJ904130.1 *Inocybe xanthocephala* | 88 | 89 |

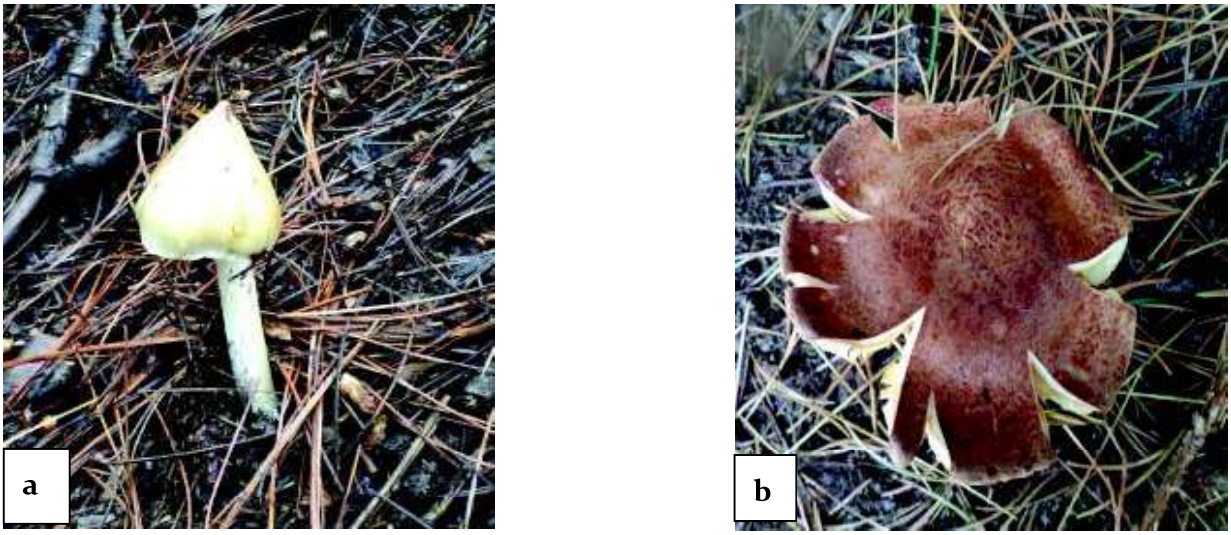


**Fig. 1: Sporocarps of *Inocybe* species. (a) *Inocybe geophylla* (b) *Inocybe mimica***

**Fig. 2: 1.5 % Agarose gel showing amplified rDNA ITS region. Samples in each lane are as follows: (A) L, 100 bp ladder, 1, *Inocybe geophylla* 2, *Inocybe mimica*.**

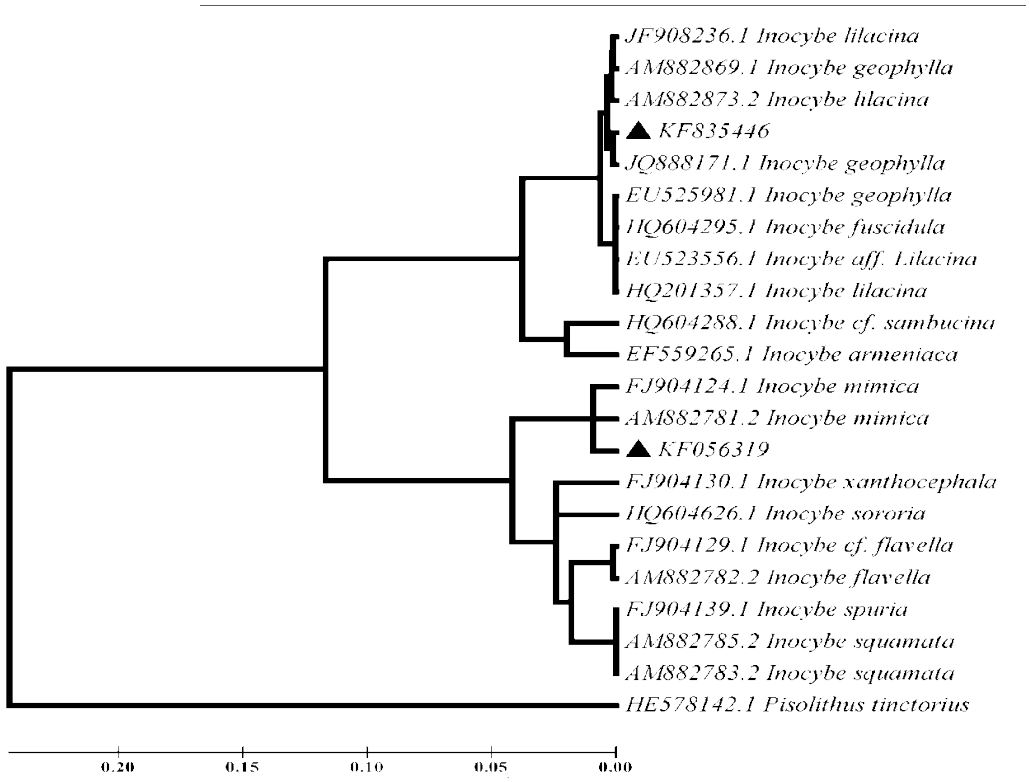


**Fig. 3: Phylogenetic relationship of present study ITS sequences with other related members based on Maximum Likelihood method inferred from ITS sequences. *Pisolithus tinctorius* was used as outgroup.**

**Table 2 (a): Pair-wise nucleotide divergence among the various accessions with which KF835446.1 showed maximum similarity.**

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| KF835446 | | | | | | | | | | |
| JQ888171.1 *Inocybe geophylla* | 0.002 | | | | | | | | | |
| JF908236.1 *Inocybe lilacina* | 0.009 | 0.007 | | | | | | | | |
| AM882873.2 *Inocybe lilacina* | 0.011 | 0.009 | 0.002 | | | | | | | |
| AM882869.1 *Inocybe geophylla* | 0.011 | 0.009 | 0.002 | 0.004 | | | | | | |
| EU525981.1 *Inocybe geophylla* | 0.015 | 0.013 | 0.009 | 0.011 | 0.011 | | | | | |
| HQ604295.1 *Inocybe fuscidula* | 0.017 | 0.015 | 0.011 | 0.013 | 0.013 | 0.002 | | | | |
| EU523556.1 *Inocybe aff. Lilacina* | 0.020 | 0.019 | 0.015 | 0.017 | 0.017 | 0.006 | 0.004 | | | |
| HQ201357.1 *Inocybe lilacina* | 0.019 | 0.017 | 0.009 | 0.011 | 0.011 | 0.004 | 0.006 | 0.009 | | |
| HQ604288.1 *Inocybe cf. sambucina* | 0.084 | 0.082 | 0.082 | 0.084 | 0.084 | 0.082 | 0.084 | 0.088 | 0.080 | |
| EF559265.1 *Inocybe armeniaca* | 0.089 | 0.086 | 0.086 | 0.088 | 0.088 | 0.086 | 0.089 | 0.093 | 0.086 | 0.032 |

**Table 2 (b): Pair-wise nucleotide divergence among the various accessions with which KF056319 showed maximum similarity.**

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| KF056319 | | | | | | | | | |
| FJ904124.1 *Inocybe mimica* | 0.003 | | | | | | | | |
| AM882781.2 *Inocybe mimica* | 0.004 | 0.022 | | | | | | | |
| HQ604626.1 *Inocybe sororia* | 0.009 | 0.097 | 0.116 | | | | | | |
| FJ904139.1 *Inocybe spuria* | 0.009 | 0.086 | 0.100 | 0.066 | | | | | |
| FJ904129.1 *Inocybe cf. flavella* | 0.100 | 0.094 | 0.109 | 0.064 | 0.030 | | | | |
| AM882785.2 *Inocybe squamata* | 0.100 | 0.086 | 0.100 | 0.066 | 0.000 | 0.030 | | | |
| AM882782.2 *Inocybe flavella* | 0.111 | 0.096 | 0.111 | 0.066 | 0.032 | 0.002 | 0.032 | | |
| AM882783.2 *Inocybe squamata* | 0.100 | 0.086 | 0.100 | 0.066 | 0.000 | 0.030 | 0.000 | 0.032 | |
| FJ904130.1 *Inocybe xanthocephala* | 0.126 | 0.115 | 0.131 | 0.097 | 0.053 | 0.057 | 0.053 | 0.059 | 0.053 |