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Characterization and identification of *Russula firmula* and *Russula postiana* from Himalayan moist temperate forests of Kashmir

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Two ectomycorrhizal species of genus *Russula*: *Russula postiana* and *Russula firmula* (Basidiomycota, Agaricales) have been characterized and identified from Kashmir Himalaya using morpho-anatomical and molecular methods targeting its rDNA. The target internal transcribe spacer (ITS)-rDNA of both species was amplified using polymerase chain reaction (PCR) with universal fungal primers (ITS1 and ITS4), which generated 700 bp fragments. After sequencing of amplified product, the initial blast analysis revealed and confirmed the identification of both species by comparing the sequences of these respective species present in GenBank. Further, in phylogenetic analysis both species distinctly clustered with their respective groups. Morphological characteristics like shape, size and colour of pileus, stipe and gills, basidiospore size of both the species was measured and compared with data given in literature.

Key words: Ectomycorrhizal, morpho-anatomical, sequencing, phylogenetic.

INTRODUCTION

The genus is cosmopolitan and is among the most diverse genera of Agaricomycetes, represented by hundreds of species worldwide (Kirk et al., 2008). All species are thought to be obligatorily symbiotic and form ectomycorrhizal symbiotic relationships with many species of Gymnosperms and Angiosperms (Beenken, 2004). The members of this fungal genus occur in both the Northern and Southern Hemispheres, in a wide range of climatic regions including boreal, temperate, Mediterranean, subtropical and tropical areas (Buyck, 2007).

The internal transcribe spacer (ITS) region of the nuclear ribosomal RNA has been extensively used in molecular systematics of fungi (Kõljalg et al., 2005; Naumann et al., 2007; Nilsson et al., 2008) and it has become one of the most widely used genomic regions for the identification of the biodiversity in various diverse fungal groups, such as shitake mushroom (*Lentinula, Tricholomataceae*), *Ganoderma lucidum* complex, and *Suillus sensu lato* (Kretzer et al., 1996; Nicholson et al., 1997). The genus *Russula* is most diverse and worldwide is represented by more than 750 ECM species (Miller et al., 2006) and all species show varied morphological characters; therefore the application of molecular tools plays an important role in defining species boundaries in genus *Russula*. Phylogenetic analysis of the internal transcribed spacer (ITS) region and nuclear large subunit rRNA (LSU) in *Russula* has also helped to clarify the identity of different species (Hibbett et al., 1997). Based on rDNA sequence analyses family Russulaceae were

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excluded from the order Agaricales along with Boletaceae (Kretzer et al., 1996). Moreover, species of *Russula* were also excluded from gilled mushrooms in the euagaries clade by phylogenetic analysis of nuclear and mitochondrial DNA sequences (Hibbett et al., 1997).

The forests of the Kashmir Himalaya are diverse and contain many families of ectomycorrhizal trees such as Betulaceae, Fagaceae, and Pinaceae. In the present study, we identified and characterized *Russula postiana* and *Russula firmula* from Kashmir Himalaya by the analysis of their ITS region. These species were found in mixed evergreen forests under *Abies pindrow* and *Pinus wallichiana*, which normally support a wide range of ectomycorrhizal fungi.

MATERIALS AND METHODS

Morphological descriptions

Fruit bodies were collected under conifer trees in Daksum forest strands of Kashmir Himalaya, India, latitude 33.36'N and longitude 75.26'E. The searches for sporocarps were conducted in the months of August to October 2012. Sporocarps were carefully dugout with the help of a knife and photographed in the field. The sporocarps were characterized morphologically, microscopically and molecularly. Macroscopic morphological details of fresh specimens, such as size, shape, colour, and texture, were recorded in the field before preservation. Microscopic features were determined from rehydrated sections of basidiomata mounted in 3% KOH and stained with Melzer's reagent. The spores were studied from the spore deposits and from fresh material as well. The spore prints were taken according to the guidelines given by Kuo (2001), then the spore morphology such as shape and size were recorded and photographed with trinocular microscope in University Scientific Instrumentation Centre. Cotton blue, 3% KOH, lactophenol and Melzer's reagent were used for preparation of spore slides. The specimens have been preserved in formaldehyde acetic acid (FAA), and formaline for herbarium purposes and have been deposited in the Kashmir University Herbarium KUB), Botany Department.

Molecular characterization

For molecular characterization, the genomic DNA was extracted from the dried fruit body by placing it in liquid nitrogen and grinding into a fine powder with a mortar and pestle and placing in 2% CTAB buffer [1M Tris HCl, 5 M NaCl, 0.5 M EDTA, 0.2% β-Mercaptoethanol (Sigma Aldrich)]. The ITS region of the rDNA was amplified by PCR with the primers ITS1 and ITS4 as described by White et al. (1990). The 50 µl reaction mixture for PCR amplification contained [5 µl PCR buffer, 5 µl of 2 mM DNTps, 3 µl of each primer, 0.4 µl of Taq polymerase (Sigma Aldrich) and 2 µl template DNA]. Amplifications were performed in a thermal cycler (Applied Biosystems) 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 product of the ITS amplified region was directly sequenced in both directions using the ITS1 and ITS4 pair of amplification primers (Scigenome). For initial comparison and alignment of the sequence, basic local alignment search tool (BLAST) analysis was performed using the National Center for Biotechnology Information (NCBI), USA database. For further phylogenetic analysis and alignment of sequence, closely related sequences were retrieved from GenBank. The sequence alignments and phylogenetic analysis were performed by a neighbor joining (NJ) method using Molecular Evolutionary Genetics Analysis (MEGA) software (Tamura et al., 2011). For phylogenetic analysis 1000 bootstrap replicates were performed to assess the statistical support for the tree.

RESULTS

Two potential ECM macrofunal species of genus *Russula* viz *Russula firmula* and *Russula postiana* was characterized and identified based on their physico-morphological and molecular characteristics from Kashmir Himalaya, India.

Russula formula: Jul. Schaf.

Morphological description

Sporocarp pileus: Cap 2 to 8 cm in diameter, convex when young with age flattens becoming plano-convex; umbo present at apex; surface smooth, colour variable but mostly light brown with age changes to dark brown, colour deep at center than at edges, colour fades quickly; cuticle is fully peeling from cap; margin entire, splitting at maturity, older specimens have furrowed margin; flesh white, fragile, changes colour on bruising or on exposure. Lamellae: gill attachment adnexed, gill space moderate, gills not in series, ventricles broad; white coloured, fragile, changing colour on bruising. Stipe: 4 to 6 cm long, 2 to 3 cm wide, centrally attached with pileus, club shaped, surface smooth, sub viscid, stem fragile, flesh white, changing colour on bruising, chalky, annuls absent, volva absent. Spores: spore print white, spores covered with spines. Season: summer to autumn.

Habit: Tricholomatoid

Growth type: Scattered to gregarious under conifers.

Molecular analysis

The rDNA ITS region amplified with ITS1 and ITS4 primers was approximately 700 bp (Figure 1) which includes ITS1, 5.8S and ITS2 regions. The ITS region was sequenced and the sequence data was submitted to GenBank nucleotide database. The sequence was blast searched and compared with the related published *Russula* sequences available in the NCBI database. In blast our sequence accession (KC797152) showed maximum similarity with *Russula firmula* (GenBank accession # DQ422017.1). Alignment of the sequence with the existing sequences of *Russula* revealed that our sequence showed maximum similarity with *Russula firmula* accession (DQ422017.1) and other accessions of the same species.

A phylogenetic dendrogram was drawn in a neighbor

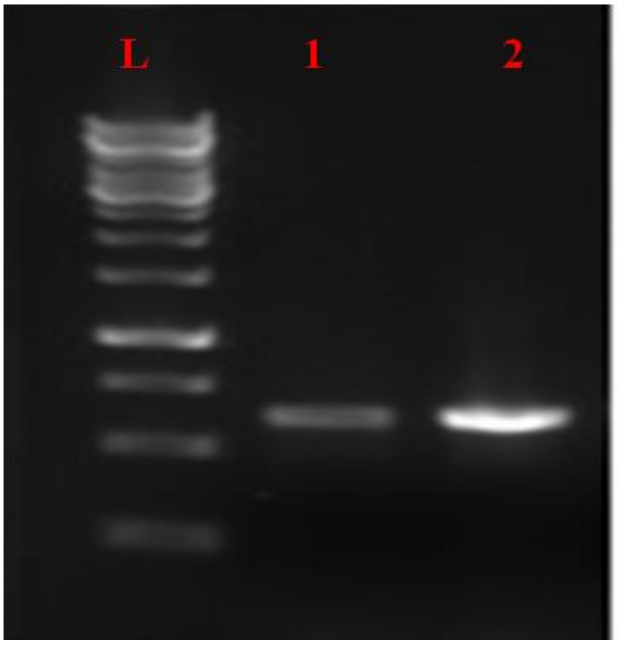

Figure 1. 1.5 % Agarose gel showing amplified ITS rDNA PCR products. Lane (L): Marker (100 bp ladder); lane (1): *Russula firmula* Lane (2): *Russula postiana.*

joining mode with the present study ITS sequence and those registered in the database for confirmation of its identity (Figure 2). Sequences of other species of the genus were also analyzed for confirmation of its identity. The present isolate showed maximum homology with *Russula firmula* and was found very close to *Russula firmula* in the same clade under a significant bootstrap value.

***Russula postiana*:** Romell

Morphological description

Sporocarp Pileus: Cap 5 to 8 cm broad; shape convex, with age becomes plane with shallow central depression; apex shallowly depressed, umbo absent; margin incurved when young, with age becomes plane, striate, splitting at maturity; surface usually viscid, smooth, colour variable white when young, with age yellow coloured, colour slightly darker on the disc and paler on the margin; scales absent, cutical fully peeling, flesh white, firm, changes colour on bruising. Lamellae: gill attachment sinuate or notched; gill space moderate; gills yellow, broad, 0.5 0.7 cm thick; gill margin serrate. Stipe: 4to 6 cm long, 1 to 3 cm thick; solid, cylindrical, more or less equal in diameter; surface smooth, colour white; flesh brittle easily broken into pieces (chalky), white colored, no colour change on bruising; attachment central; annulus and volva absent. Spores: spore print yellow. Season: summer to autumn.

Habit: Tricholomatoid

Growth type: Solitary occasionally in groups under conifers.

Molecular analysis

The rDNA ITS region amplified with ITS1 and ITS4 primers was approximately700 bp (Figure 1) which includes ITS1, 5.8S and ITS2 regions. The ITS region was sequenced and the sequence data was submitted to GenBank nucleotide database. The sequence was blast searched and compared with the related published *Russula* sequences available in the NCBI database. In BLAST, our sequence accession (KC797156) showed maximum similarity with *Russula postiana* (GenBank accession # AF230898.1). Alignment of the sequence with the existing sequences of *Russula* revealed that our sequence showed maximum similarity with *Russula postiana* accession (AF230898.1) and other accessions of the same species.

A phylogenetic dendrogram was drawn in a neighbor joining mode with the present study ITS sequence and those registered in the database for confirmation of its identity (Figure 2). Sequences of other species of the genus were also analyzed for confirmation of its identity. The present isolate showed maximum homology with *Russula postiana* and was found very close to *Russula postiana* in the same clade under a significant bootstrap value. *Russula postiana* is a new report of ECM fungi from Kashmir Himalaya.

DISCUSSION

The coniferous forests of Kashmir Himalaya due to its varied and diverse climatic conditions support diverse mycorrhizal host trees which provide a congenial habitat for the growth of diverse macro fungal species which in turn gives it the status of ‘hub’ of macro-fungal species. Two ECM species of genus *Russula* have been identified and characterized from Kashmir Himalaya during the present study. Earlier workers have also reported the occurrence of several species of *Russula* from Kashmir Himalaya. Watling and Abraham (1992) reported 9 species of *Russula* from Kashmir Himalaya. Dar et al. (2010) have reported *Russula aurea* and *Russula atropurpurea* from Kashmir Himalaya. The genus *Russula* is represented by more than 750 ectomycorrhizal species worldwide (Miller et al., 2006).

Russula firmula was reported from Daksum forests and was found growing in association with *Pinus wallichiana*. Earlier Watling and Abraham (1992) have reported *Russula firmula* from Tangmarg and Pahalgam forests of Kashmir Himalaya. *Russula postiana* was collected from Daksum and Mammer forests under *Pinus wallichiana*

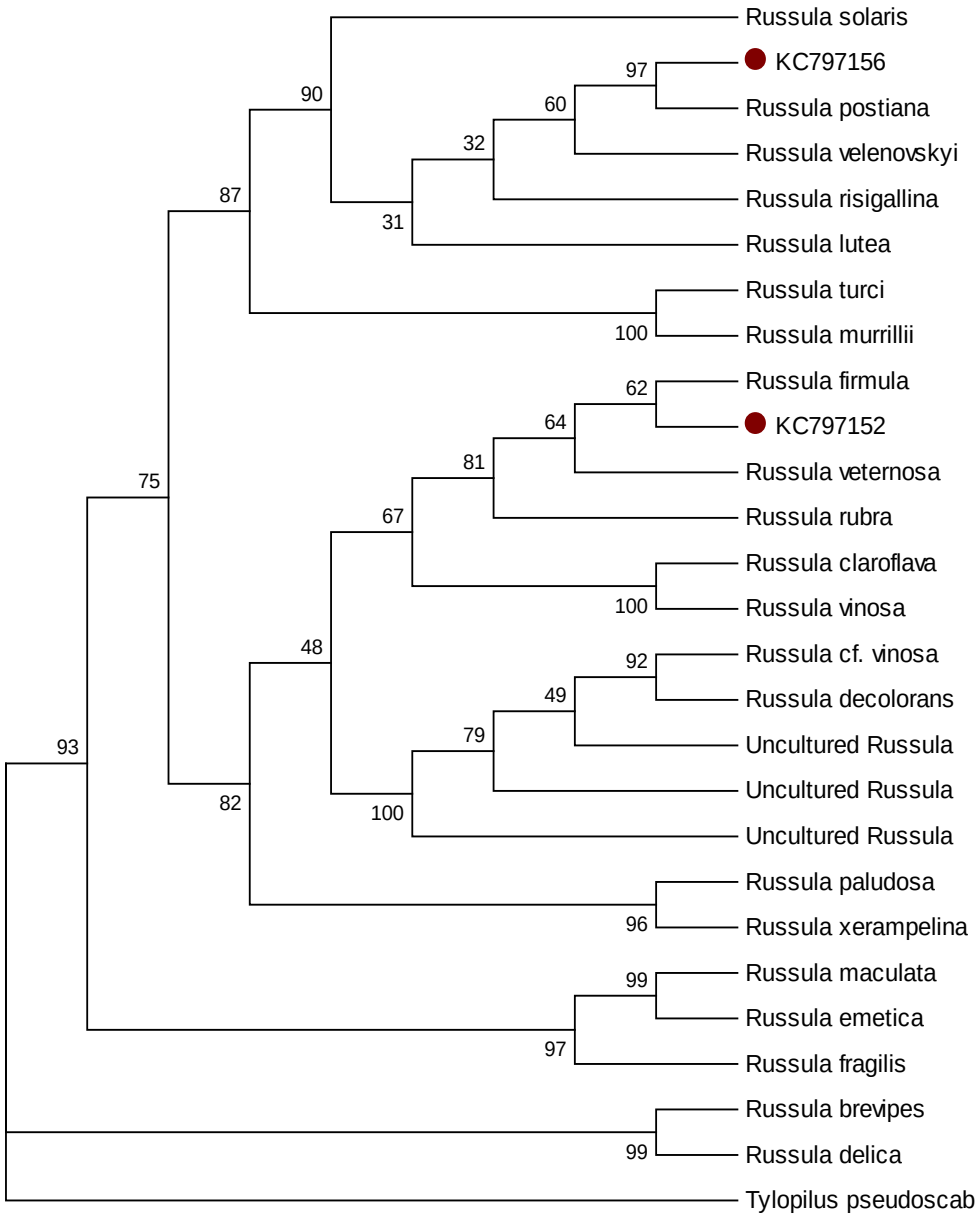

Figure 2. Phylogenetic relationship of our two isolates (● accession KC717956 and KC797152) with other related members based on Maximum Likelihood method inferred from ITS sequences**.** *Tylopilus pseudoscaber* was used as outgroup. Numerical values on branches are the bootstrap values as percentage of bootstrap replication from 1000 replicate analysis.

and *Abies pindrow*. There was no earlier report of this species from Kashmir. Both morphological and molecular studies showed differences of *Russula postiana* from other species of *Russula* reported previously from Kashmir Himalaya by various workers, it was concluded that this is a new species from Kashmir and was identified as *Russula postiana* by both morphological and molecular analysis, which form ectomycorrhizal association with *Pinus wallichiana* and *Abies pindrow*. Phylogenetic analysis based on ITS sequences also clearly separated the present collection from the other species of *Russula* reported previously from Kashmir. Both species were described morpho-anatomically and identified molecularly using rDNA sequences. Sequences from the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA are commonly used for the identification of fungi (Kõljalg et al., 2005; Naumann et al., 2007; Nilsson et al., 2008). We also found this technique quite effective in the correct identification of fungi. Reddy et al. (2005) identified *Pisolithus indicus,* a

new species of ectomycorrhizal fungus associated with Dipetrocarps in India on the basis of similar studies. Manassila et al. (2005) used similar technique while describing phylogenetic diversity of wild edible *Russula* from Northeastern Thailand. Our findings are also in agreement with the studies conducted by Hortal et al. (2006), Iotti and Zambonelli (2006) and Hanif et al. (2012). They used the similar technique for identification of ECM species. Garay-Serrano et al. (2012) identified ectomycorrhizal association of *Lactarius fumosibrunneus* and *Fagus grandifolia* var. *Mexicana* trees in eastern Mexico by using morphological and molecular characterization. ITS-rDNA the fungal molecular marker in combination with morpho-anatomical characters and illustrations is thus a valuable tool for correct identification of ECM species.

Conclusion

The detailed literature survey and the present study investigation emphasize the use of rDNA based technology in combination with morpho-anatomical descriptions for the precise and accurate identification of ectomycorrhizal fungi. The ECM host trees of this region are still unexplored and comprehensive study could add the macro-fungi associated with them. The use of ITS-rDNA the fungal molecular marker in combination with morpho-anatomical characters and illustrations can lead not to only correct identification but also discovery of many new species from this diverse region.

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