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A new species of *Rhizopogon* from Kashmir valley, India

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ABSTRACT

A new species of *Rhizopogon* Fr. & Nordholm, *R. cashmerianus* associated with *Pinus wallichiana* was collected and identified for the first time from Kashmir Himalaya. It was characterized and identified on morpho-anatomical and molecular basis by analysis of ITS sequences. The fungal molecular marker (ITS-rDNA) was amplified using universal fungal primers (ITS1F and ITS4R). Bioinformatic data retrieved for its molecular identification and the rDNA sequence, when aligned in GenBank by performing BLAST indicates that *R. cashmerianus* is a new species. The rDNA sequence of this species forms a distinct clade from the rest of species of the same genus. Therefore, this species is being described for the first time from Kashmir Valley.

KEYWORDS: Molecular identification, morpho-anatomical characterization, phylogenetic analysis, *Rhizopogon*.

INTRODUCTION

The genus, *Rhizopogon* Fr. & Nordholm belonging to Division: *Basidiomycota*, order: *Boletales*, and family: *Rhizopogonaceae* which contains approximately 150 species of hypogeous or subepigeous ectomycorrhizal fungi (Kirk *et al*., 2008). They are reported to form ectomycorrhizal association with members of family *Pinaceae* and are found growing throughout the world in natural habitats (Molina *et al*., 1999). The epigeous mushroom genera such as *Suillus*, *Gomphidius* and *Chroogomphus* are thought to be possible ancestors of *Rhizopogon*. However, it is believed to be evolved from Suillus like ancestor because of its reduced morphological and other characteristics (Bruns *et al*., 1989). It is considered a difficult genus but Smith and Zeller (1966) provided good account of the species of *Rhizopogon* and Grubisha *et al*. (2002) re-examined the infrageneric relationships based on phylogenetic analysis of ITS sequences. Phylogenetic analysis of ITS sequences also revealed that ectomycorrhizal associates of key conifer genera such as *Pinus* and *Abies* were scattered across numerous subgenera of *Rhizopogon* (Smith and Zeller, 1966; Grubisha *et al*.,2002). Till now, about 280 species of mushrooms have been reported from Kashmir Valley (Dar *et al*., 2009, 2010; Pala *et al*., 2011, 2012; Wani *et al*., 2010, 2015; Malik *et al*., 2018).The mushroom diversity of Kashmir Himalaya is still at its infancy and needs further exploration as major portion has remained unexplored. The present study was carried out to explore the mushroom diversity of Kashmir Valley. To date some species of *Rhizopogon* have been reported from the Kashmir Himalaya, *viz. R. vulgaris* (Vittad) M. Lange (1956), *Rhizopogon villosulus* Zeller (1941), *Rhizopogon roseolus* (Corda) Th. Fr. (1909) and *Rhizopogon* sp. (Beig *et al*., 2011; Malik *et al*., 2017). During the present mushroom survey, a new species of *Rhizopogon* associated with *Pinus wallichiana* was identified and is being described here.

MATERIALS AND METHODS

Sampling of mushrooms: The various sites explored during the present study were Lolab, Handwara, Wadpora and Bungus Valley of Kupwara District in J&K UT **(Fig.1)**.

The method given by (Halling, 1996) was followed for the field trips. Standard methods were followed for the collection of sporocarps as given by (Atri *et al*., 2005). Sporocarps were carefully dugout with the help of a knife and photographed in the field using Sony SLR Digital field camera and Sony cyber shot 12.1 megapixel Camera. Field characteristics such as distribution, shape, size and colour were recorded from fresh specimens. Wet preservation was carried out in FAA (Formaldehyde acetic acid) solution for Herbarium purpose. The specimen with the voucher specimen No. KASH-2922 have been deposited, in the Mycological Section of KASH Herbarium, Centre of Plant Taxonomy, Division of Botany, University of Kashmir, Srinagar, Jammu and Kashmir, India (190006).

Morpho-anatomical examination: Macroscopic characteristics such as colour, shape and size of basidiocarp; colour of gleba; colour and texture of periderm; presence or absence of columella and nature of rhizomorphs were described from fresh specimens. Hand cut sections of mushroom specimen was mounted in water, Congo red, Melzer's reagent, and 3% KOH and examined under Trinocular Microscope at the University Scientific Instrumentation Centre for morphological identification (Castellano *et al*., 2012).The identification was carried out by consulting authentic literature (Smith and Zeller, 1966; Arora, 1986; Castellano *et al*., 1989; Trappe *et al*., 2009; Mujic *et al*., 2019).

DNA extraction and Agarose Gel electrophoresis: The total genomic DNA was extracted from dried fruiting body tissue using NucleoSpin® Plant II Kit (Macherey-Nagel).The quality of isolated DNA was analyzed by agarose gel electrophoresis. 1µL of 6X gel-loading buffer was added to 5µL of isolated DNA. The samples were loaded to 0.8% agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg/mL ET Br. Electrophoresis was performed at 0.5X TBE as electrophoresis buffer at 75 V until the migration of bromophenol dye to the bottom of the gel was observed. The UV transilluminator was used to visualize the gels and Gel documentation system was used to capture images.

PCR amplification: PCR reactions were carried in a 20 µL reaction mixture containing 1X Phire PCR buffer

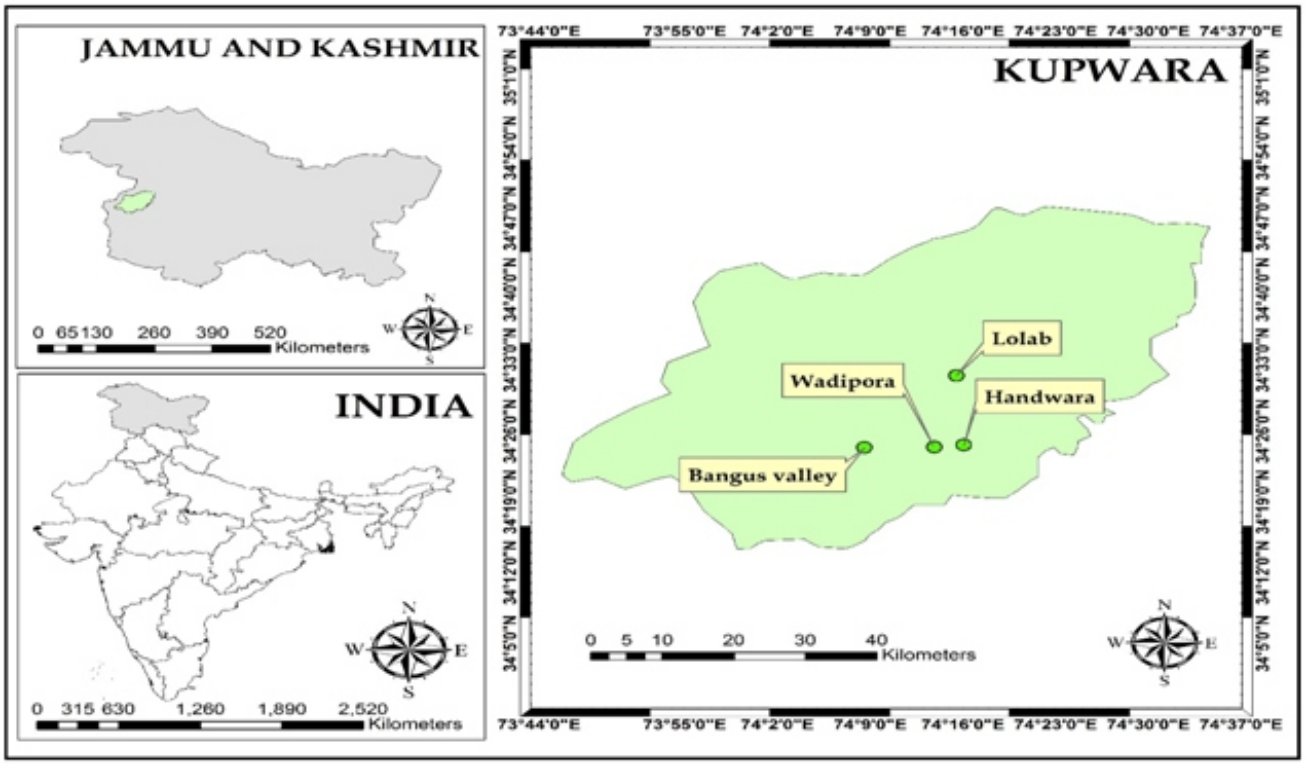

Fig. 1. Map showing the surveyed area of Kupwara District of Jammu and Kashmir, India.

(comprising 1.5 mM $MgCl_2$), 0.2 mM each dNTPs (dATP, dGTP, dCTP and dTTP), 1µLDNA, 0.2 µL Phire Hotstart II DNA polymerase enzyme, 0.1 mg/mL BSA and 3%DMSO,0.5M Betaine, 5pM of forward and reverse primers using PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) (White *et al*., 1990).

PCR amplification profile

98 °C	-	30 sec	
98 °C	-	5 sec	
58 °C	-	10 sec	40 cycles
72 °C	-	15 sec	
72 °C	-	60 sec	
. °C	-	∞	

To 5µL of PCR product, 1µL of 6X loading dye was added and loaded to 1.2% agarose gel which was made in 0.5X TBE buffer comprising 0.5 µg/mL ET Br. Electrophoresis was performed at 75 V for 1-2 hours till the migration of bromophenol dye to the bottom of the gel was observed. The UV transilluminator was used to visualize the gels and Gel documentation system was used to capture images **(Fig.2**).

Sequencing: The PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) was used to perform the sequencing reaction using BigDye Terminator v3.1Cycle sequencing Kit concomitant to manufactures protocol. The sequence quality was assessed using Sequence Scanner Software v1 (Applied Biosystems). The isolated ITS sequence was with an ORF of 405 bps. The sequence was submitted to National Center for Biotechnology Information

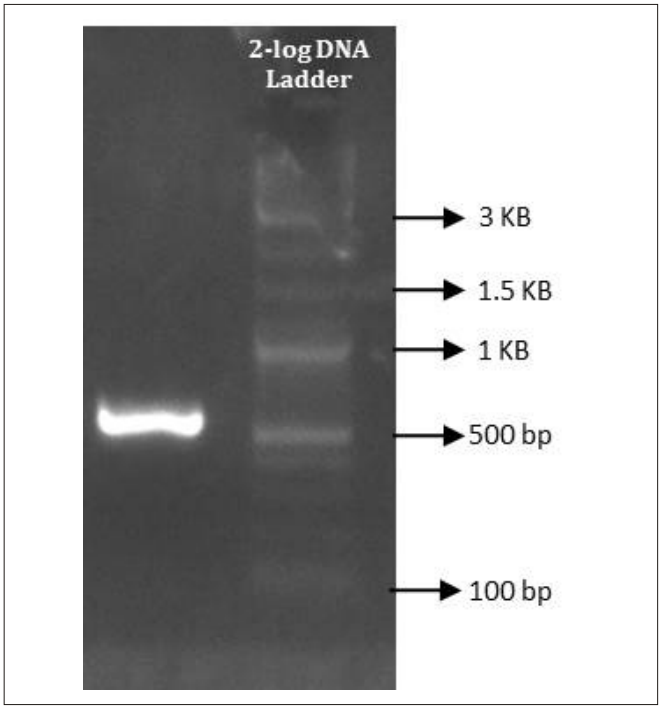

Fig. 2 1. 2% Agarose gel showing amplified ITS rDNA PCR Product against the molecular standard of 2-log DNA ladder ranging from 100 bp to 3kb.

(NCBI) data base with accession submission R_KMK692540.

Phylogenetic analysis: The nucleic acid sequences were aligned using Clustal Omega Multiple Sequence Alignment tool and the phylogenetic analysis was performed using the Clustal W program and MEGA 7 software (Jukes and Cantor, 1969; Kumar *et al*., 2016).

About 20 nucleic acid sequences were selected from different species submitted to NCBI database**(Table-1)**.

Table-1: Database of materials used for molecular analyses

Species Name	*GeneBank Number of ITS*	*References*
Rhizopogon sp.	MH878753.1	From Genebank
Rhizopogon sp.	MH878765.1	From Genebank
Rhizopogon guzmanii	KC152201.1	From Gene bank
Rhizopogon milleri	MH819344.1	From Gene bank
Rhizopogon subpurpurascens	MH298904.1	From Gene bank
Rhizopogon atroviolaceus	KT968584.1	From Gene bank
Rhizopogon chamaleontinus	KP859270.1	From Gene bank
Rhizopogon semitectus	KP859275.1	From Gene bank
Rhizopogon subbadius	NR_121274.1	From Gene bank
Rhizopogon salebrosus	HQ914337.1	From Gene bank
Rhizopogon sp. Siskiyou	AF377172.1	From Gene bank
Rhizopogon milleri	NR_119445.1	From Gene bank
Rhizopogon kauffmanii	NR_119444.1	From Gene bank
Uncultured ectomycorrhizal fungus	KU861489.1	From Gene bank
Rhizopogon salebrosus	KC170120.1	From Gene bank
Rhizopogon fallax	KC152199.1	From Gene bank
Rhizopogon atroviolaceus	AF377131.1	From Gene bank
Rhizopogon semireticulatus	AF058307.1	From Gene bank
Uncultured *Rhizopogon*	KP403084.1	From Gene bank

RESULTS

Taxonomic description

Rhizopogon cashmerianus M.D. Talie & A.H. Wani sp. nov.

Fig. 3 (a-h)

Mycobank number: MB834836

Diagonosis: Basidiocarp globose to subglobose or irregular sometimes, smooth, black to brown in colour, on reaction with KOH changes to black immediately. Basidiospores subglobose or round, measuring about 10-12 µm, dark brown, thick walled, ornamented with raised reticulation, dextrinoid, cystidia, basidioles and clamp connections present.

Etymology: In Latin *cashmerianus*, refers to Kashmir from where the specimen was collected for the first time.

Holotype: INDIA. Jammu and Kashmir UT: Kupwara Forest, found to be associated with *Pinus wallichiana* A.B. Jakes. 34.4319 ºN, 74.1240 ºE, 1600–1657 m, May 17, 2018, M.D. Talie & A.H. Wani, KASH-2922-Holotype (GenBank RKMK 692540, Mycological Section of KASH Herbarium, Centre of Plant Taxonomy, Division of Botany, University of Kashmir Hazratbal, Srinagar -190006. Jammu and Kashmir, India.

Taxonomy

Basidiomata (sporocarp) of *Rhizopogon cashmerianus* is hypogeous to subepigeous, globose to subglobose or sometimes irregular with a basal point of attachment. It reaches up to 5.8-7.7 × 4.6-6.3 cm in size. Peridium dry, smooth, black to brown in colour. On reaction with KOH it changes to black immediately. Gleba loculate, white in colour when young and becomes brown at maturity, columella absent (**Fig.3 a-b**). Rhizomorphs originate from the base of sporocarp singly, thick at the base and tapering towards lower end, dark brown to black in colour. Odor pleasant, tasteless, edibility unknown. Periderm thin, measuring about 200-450 µm, composed of thick walled, double layered, hyaline, hollow, septate, and loosely interwoven hyphae about 1.5-2.5 µm in diameter. Trama composed of hyaline, loosely interwoven, irregular, septate hyphae measuring about 3.5-5.2 µm in diameter. Basidia 13.6-16.6 × 9.2-12.3 µm, 02-04 spored, sterigamtal attachment evident.

Basidiole hyaline, clavate, measuring about 20.2-23.4 × 9.5-11.3 µm (**Figs.3 g-h**). Basidiospores subglobose or round in shape measuring about 09-12 µm, dark brown, hyaline in KOH, dextronoid, thick walled, ornamented with raised reticulation (**Fig.3 c**). Cystidia clavate, with a long stalk, measuring about 70-75 µm in length, 2.5-4.2 µm in breadth at the base and 14.3-16.4 µm at the apex (**Fig.3 g**). Clamp connections present.

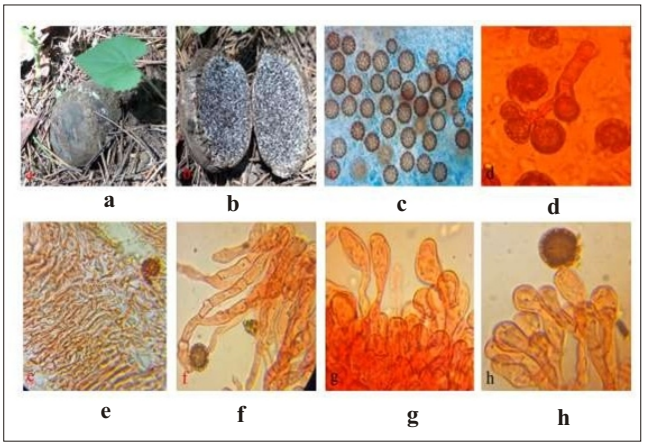

Fig. 3 (a-h): *Rhizopogon cashmerianus*: (a) Globose fruiting body; (b) Cross section of basidiocarp; (c) Basidiospores in Melzer's reagent; (d) 4-spored basidia; (e) Section of peridium in Congo red; (f) Hyphae of trama in Congored; (g) Cystidia and basidioles in Congo red; (h) Basidia with sterigma and single basidiospore in Congo red.

Habit and Habitat: Hypogeous to subepigeous showing putative ectomycorrhizal association with *Pinus wallichiana* at an elev. of 1600-1657 m, fruiting from April-June. So far only known in Kashmir Himalaya, India.

Specimens examined: INDIA. Jammu and Kashmir UT: Kupwara and Handwara Forests, found in association with *Pinus wallichiana* A.B. Jacks. 34.4319 ºN, 27 74.1240 ºE, 1600–1657 m, 17 May 2018, M.D. Talie & A.H. Wani, KASH-2922, KASH- 2923,KASH-2924.

Phylogenetic analysis: To elucidate the phylogenetic relationship of ITS nucleotide sequence of *Rhizopogon cashmerianus* with related nucleotide sequences, a phylogenetic analysis was performed by maximum likelihood method using the MUSCLE program and

MEGA-7 software. The phylogenetic relationship of ITS sequences was determined in order to get insights of evolutionary distance, a phylogenetic analysis of nucleotide sequences with related ITS sequences from BLAST analysis was performed. Pairwise alignment of deduced primary ITS sequences showed that these are highly similar, with great identity at nucleotide level. From the phylogenetic tree analysis, it is clear that *R. cashmerianus* aligned in close proximity with *Rhizopogon himalayensis* (MH878765.1) in comparison to other *Rhizopogon* species (**Fig.4**).

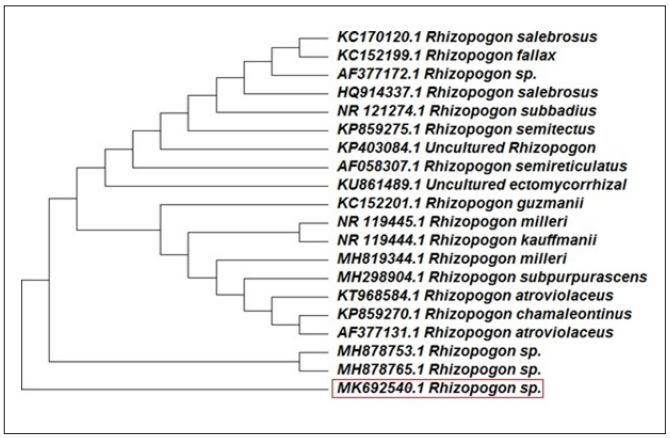

Fig. 4. Phylogenetic analysis of *Rhizopogon cashmerianus*

Fig. 4. Phylogenetic analysis of *Rhizopogon cashmerianus*: The analysis involved alignment of 20 nucleotide sequences which were chosen by BLAST search of *Rhizopogon* from NCBI data-base. The tree with the highest log likelihood (-638.6384) is shown. Initial tree for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. All positions containing gaps and missing data were eliminated. There was a total of 341 positions in the final dataset.

DISCUSSION

From the morphological, anatomical and molecular characters, the examined collections of *Rhizopogon* appear to represent a new species which has been named as *R. cashmerianus* sp. nov. The species was taxonomically characterized and its phylogenetic relationship with gene sequences information available in databases was analyzed using bioinformatics tools. *R. cashmerianus* possesses altogether different basidiospore and rhizomorph morphology in comparison to all other known *Rhizopogon* species. The presently named species was found associated with the *Pinus wallichiana* trees unlike *Rhizopogon himalayensis* which shows association with *Cedrus deodara* (Mujic *et al*., 2019). All previously described species of *Rhizopogon* possess smooth ellipsoid or smooth ellipsoid-truncate basidiospores bearing two prongs along the edge of the basal truncation (Smith, 1964; Smith and Zeller, 1966; Martin, 1996; Visnovsky *et al*., 2010; Walbert *et al*., 2010) except *R. himalayensis* which possess globose to subglobose non dextrinoid basidiospores with irregular reticulations (Mujic *et al*., 2019). However, the basidiospores of *R. cashmerianus* are larger, thick walled, unique because they are globose to subglobose, dextrinoid, dark brown and ornamented having fused ridges forming a complete reticulum as compared to *R. himalayensis* (Mujic *et al*., 2019). Cystidia and clamp connections are also present in *R. cashmerianus* as compared to *R. himalayensis* and some other species of *Rhizopogon* in which they are absent (Mujic *et al*., 2019) (**Table-2**). Furthermore, taxonomic identification is also supported by molecular studies. The rDNA sequence of this species forms a distinct clade from rest of the species of *Rhizopogon*.

Table 2: Comparison between *R. cashmerianus* with its closely allied species *R. himalayensis.*

R. himalayensis	*R. cashmerianus*
1. Associated with *Cedrus deodara*	Associated with *Pinus wallichiana*
2. Basidiomata 0.5-4.0 cm in diameter	Basidiomata 3.0-7.7 cm in diameter
3. Taste of boiled potato	Tasteless
4. No distinctive odor	Pleasant odor
5. Periderm 140-300 µm thick	Periderm 200-450 µm thick
6. Peridermal hyphae thin walled	Peridermal hyphae thick walled
7. Tramal hyphae gelatinized	Tramal hyphae not gelatinized (dry)
8. Clamp connections absent	Clamp connections present
9. Basidia 40-45 x 5-8 µm and 1 or 2 spored spored	Basidia 13-16 x 9-12 µm and 2-4
10. Basidiospores non-dextrinoid	Basidiospores dextrinoid
11. Cystidia absent	Cystidia present

CONCLUSION

A new species of truffle like mushroom *R. cashmerianus* has been described on the basis of morpho-anatomical and molecular sequence studies. The identified species of mushroom was found forming putative association with *Pinus wallichiana*.

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