Molecular phylogeny and morphological characterization of *Russula livescens* and its ectomycorrhiza from mixed coniferous forests of Pakistan

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Abstract: Basidiomata of *Russula livescens* and its ectomycorrhiza were collected and described morphologically and molecular phylogenetically from different coniferous forests of Pakistan. The species is characterized by creamy white lamellae, fusoid pleurocystidia, ellipsoid basidiospores with reticulations and warts as wall ornamentation. Ectomycorrhiza of the taxon is dichotomous to coralloid with spiny appearance, pseudoparenchymatous mantle layers; branched emanating hyphae have frequent clamp connections and bottle shaped cystidia with a septum. Phylogenetic studies revealed that sequences generated from Pakistani specimens are closely related to sequences from China. Occurrence of *R. livescens* in the form of basidiomata is an addition to the macrofungi of Pakistan.

Zusammenfassung: Fruchtkörper von *Russula livescens* und ihre Ektomykorrhiza wurden in verschiedenen Nadelwäldern Pakistans gesammelt sowie morphologisch und molekular phylogenetisch charakterisiert. Die Art ist durch cremefarbene Lamellen, fusoide Pleurozystiden, ellipsoide Basidiosporen mit Netzstrukturen und Warzen als Wandornamente gekennzeichnet. Ihre Ektomykorrhiza ist dichotom bis koralloid mit stacheligem Aussehen, pseudoparenchymatösen Mantelschichten, verzweigten abgehenden Hyphen mit häufigen Schnallen und flaschenförmigen Zystiden mit einem Septum. Phylogenetische Studien zeigten, dass Sequenzen der pakistanischen Belege weitgehend mit Sequenzen aus China übereinstimmen. Das Auftreten von *R. livescens* in Form von Fruchtkörpern ist eine Ergänzung der Funga von Pakistan.

Russula (*Basidiomycetes*, *Russulales*, *Russulaceae*) is considered a large and widely distributed agaric genus with an estimated 750 species worldwide (KIRK & al. 2008, DAS & al. 2013, ARORA & NGUYEN 2014, RAZAQ & al. 2014). From Pakistan 24 species of *Russula* have been reported until today (AHMAD & al. 1997, NIAZI & al. 2006, RAZAQ & al. 2014). As ectomycorrhizal symbionts with the species of the coniferous tree genera *Abies*, *Cedrus*, *Larix*, *Picea*, *Pinus* and *Tsuga,* their importance is well

documented (BILLS & al. 1986; VILLENEUVE & al. 1989, 1991; GARDES & BRUNS 1996; BUYCK & al. 1996; HANIF & al. 2012). They are important dietary elements for insects and larger animals (FOGEL 1975, FOGEL & TRAPPE 1978). Many species are harvested worldwide for human consumption (HU & ZENG 1992, GUO 1992, RAMMELOO & WALLEYN 1993, BUYCK 1994). Monographs giving descriptions of *Russula* have been published by many researchers (SINGER 1932, HEIM 1937, ROMAGNESI 1967, BON 1988, SARNARI 1998). However their phylogenetic relationships have not been fully determined. The present study aims to use the ribosomal DNA sequences from the ITS1, 5.8S and ITS2 regions to provide the information about the phylogeny and to give detailed morphological description of fruit body and ectomycorrhiza of *Russula livescens*.

Materials and methods

Sampling site description

Sampling was carried out during monsoon season from 2009 to 2013. Mansehra district and Hazara district were selected as sampling sites. Mansehra district is located at the eastern border of the Khyber Pakhtunkhwa (KP) province. The climate of the district is dry temperate to moist temperate. Tree species are well represented by deciduous and evergreen plants. *Pinus wallichana* A. B. JACKS. and *Pinus roxburghii* SARG. are dominant tree species while *Juglans regia* L. is the most common broad-leaved tree (MUSTAFA 2003). Hazara district is located at the northern side of KP province. The climate of the district is moist temperate. Mean annual rainfall is comparatively high. Among conifers *Cedrus*, *Picea*, *Pinus* and *Taxus* are dominants along with some deciduous tree species (SHEIKH 1993).

Collection, morphological and anatomical study of basidiomata

Specimens were photographed using Nikon D70S digital camera in the field and carefully dugged out. Morphological features of fresh specimens were recorded in the field. Color codes were designated using MUNSELL Soil Color Charts (1975). Specimens were then dried in hot air. For anatomical study; tissues from lamallae, pileipellis and stipitipellis were mounted on slide in 5% KOH. Anatomical features were noted using microscope (MX4300H, Meiji Techno Co., Ltd., Japan). Measurements were recorded using Carl Zeiss Jena ocular micrometer and line drawing were made using LEITZ Wetzlar Camera Lucida.

Collection, isolation, morphological and anatomical study of ectomycorrhiza

Sampling procedure was carried out very carefully to make sure that the fine roots in the soil block belong to the selected tree species. A soil block of 15 cm^3 was dug a few centimetres away from the tree trunk with the help of digger. Five soil cores were taken from each sampling site. In laboratory, each soil core was soaked in water for few hours to loosen the soil particles and then put on a 2 mm sieve under shower to separate the roots from the soil. The ectomycorrhizae were carefully sorted out into morphotypes under incandescent light. The morphotypes were cleaned under stereomicroscope (EMZ-5TR, Meiji Techno Co., Ltd., Japan) using a fine brush. Morphologicaly identical morphotypes were kept in McCartany bottles in distilled water for further morphological and anatomical studies. Replicates of these morphotypes were kept at 8 °C in eppendorf tubes containing 1 ml of 2% CTAB buffer for molecular analysis. Morphological characters such as color, size, ramification, presence or absence of radiating elements were noted under the stereomicroscope. For anatomical details, mantle layers (inner and outer) and radiating hyphae were mounted in trypan blue stain (AGERER 1991). Measurements of hyphae were made using ocular micrometer and drawn using camera lucida.

DNA extraction, amplification and sequencing

For molecular analysis 2 mg of fungal tissue was taken from the gills in an eppendorf tube, 2–4 ectomycorrhizal root tips were taken in a separate eppendorf tube. DNA was extracted using modified CTAB method (BRUNS 1995). For PCR amplification, ITS1F (5'-CTTGGTCATTTAGAGGAAGT-

3') and ITS4 (5'- TCCTCCGCTTATTGATATGC-3') primers were used to amplify internal transcribed spacer (ITS) regions of nuclear ribosomal DNA following GARDES and BRUNS (1993). The PCR products were sent to Macrogen Inc. (Korea) for sequencing.

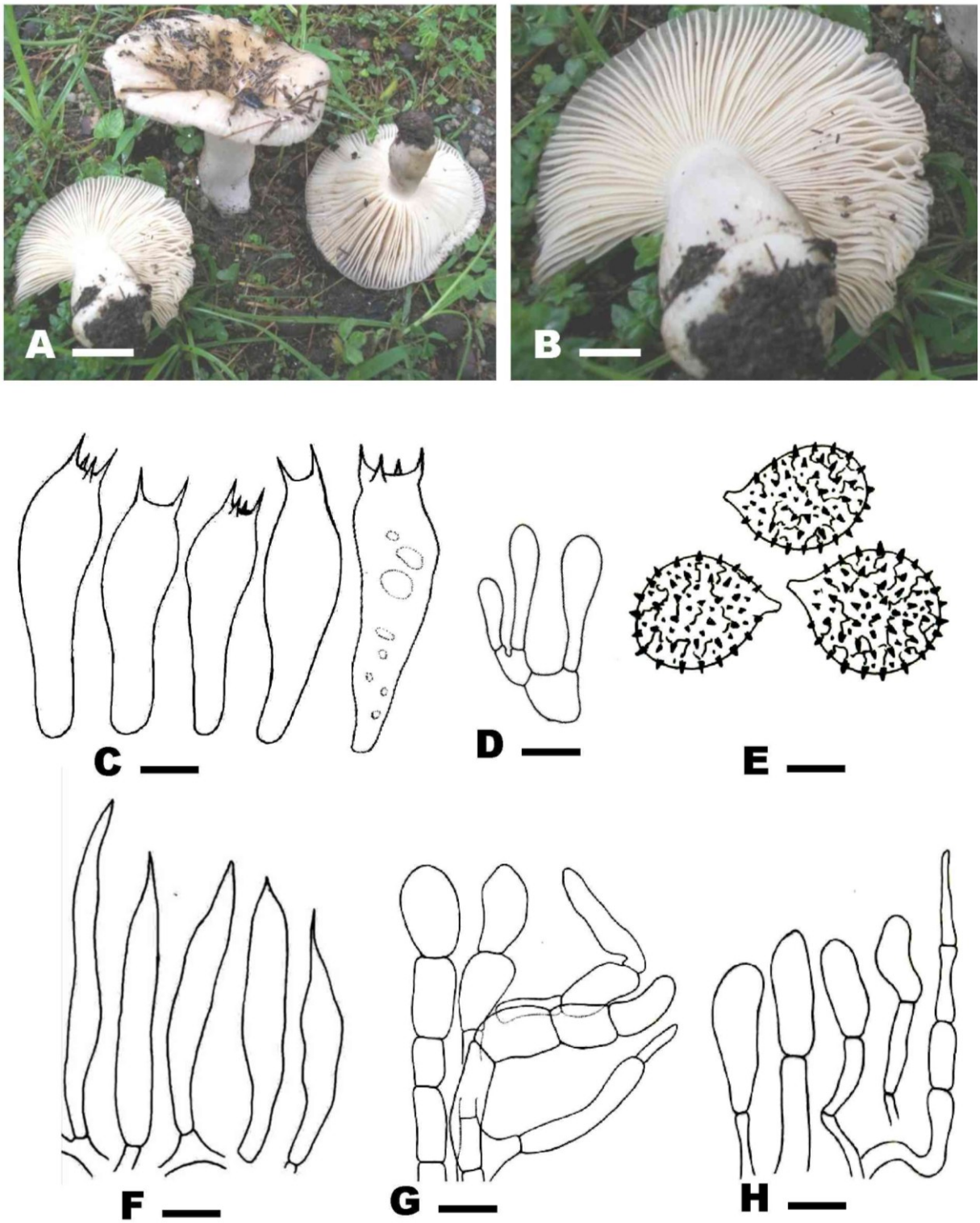

Fig. 1. *Russula livescens, A* and *B* basidioma, *C* basidia, *D* subhymenium, *E* basidiospores, *F* pleurocystidia, *G* pileus elements, *H* stipitipellis elements. Scale bar: A 2.4 cm, B 1.3 cm, C 7 µm, D 13 µm, E 5 µm, F 15 µm, G 22 µm, H 17 µm.

Molecular phylogenetic analysis

Consensus sequences were generated from the sequences obtained by using both forward (ITS1F) and reverse (ITS4) primers in BioEdit software and then BLAST searched at NCBI (http://www.ncbi.nlm.nih.gov/). Sequences with closest match were selected from GenBank to reconstruct phylogeny. The sequences showing less query cover were left aside. Published sequences of the closest relatives of the species were also included in the final data set. *Boletus reticuloceps* (M. ZANG, M. S. YUAN & M. Q. GONG) Q. B. WANG & Y. J. YAO (EU231968) was chosen as outgroup. Multiple sequences were aligned using online MUSCLE tool at EMBL-EBI (http://www.ebi.ac.uk/). The sequences were trimmed with the conserved motifs 5'-(…GAT) CATTA…and…GACCT (CAAA…)-3' and the alignment portions between them were used to reconstruct phylogeny. Maximum likelihood (ML) analysis was performed using Jukes-Cantor model in MEGA6 software to test the phylogeny at 1000 bootstraps. Sequences generated in this study were submitted to GenBank.

Results

Russula livescens (BATSCH) BATAILLE, Fl. Monogr. Astérosporales: 76, 1908. – Figs. 1–3.

Morphological characterization of basidiomata (Fig. 1)

P i l e u s : up to 8.5 cm broad, almost flat to depressed, creamy white near the margins, central depressed region somewhat darker, turning to dull yellow, surface sticky or gelatinized initially, covered by decomposed plant debris, sticked onto the surface, becoming dry and unpolished very soon, striated from mid pileus radius to the margins; Context white.

L a m e l l a e : 2–6 mm broad, adnexed, creamy white, thin, subdistant to minutely crowded or close, entire.

L a m e l l u l a e : infrequent, of diverse length.

S t i p e : up to 5 cm long, 2.3 cm thick, central, tapering to the apex, with angular bulbous base, white to offwhite, glabrous.

B a s i d i a : 7.3–8.5 × 29.2–33.3 µm, clavate, somewhat inflated near the middle, becoming fusoid, 2–4 sterigmate, hyaline, smooth-walled, sometime with oily contents.

P l e u r o c y s t i d i a : 5.9–9.7 × 30.7–51.9 µm, cylindrical to narrowly fusoid, attenuating, with acute apex, hyaline, smooth-walled.

S u b h y m e n i u m : composed of medium to large, hyaline and comparatively elongated cells.

T r a m a : composed of interconnected hyphae and sphaerocytes of comparatively medium size.

B a s i d i o s p o r e s : (4.7–)5.4–6.6(–7.3) × (5.9–)6.8–7.8(–8.7) µm, ellipsoid to broadly ellipsoid to ovoid, sub-reticulate with a few isolated low warts, incompletely amyloid, apiculus prominent, up to 2.1 µm high.

P i l e i p e l l i s : two layered, subpellis comprised of gelatinized, less frequently septate, thin walled, hyaline, smooth and comparatively thick hyphae, 3.5–10.6 µm in diam, mostly running parallel, close to the underlying trama.

S u p r a p e l l i s : composed of 3–7(–9) µm cylindrical to slightly inflated, hyaline, smooth, thin walled cells, 9.4–23.1 × 30.9–49.8 µm, gradually smaller to the tip; sometimes the terminal cell inflated and larger than the lower cells.

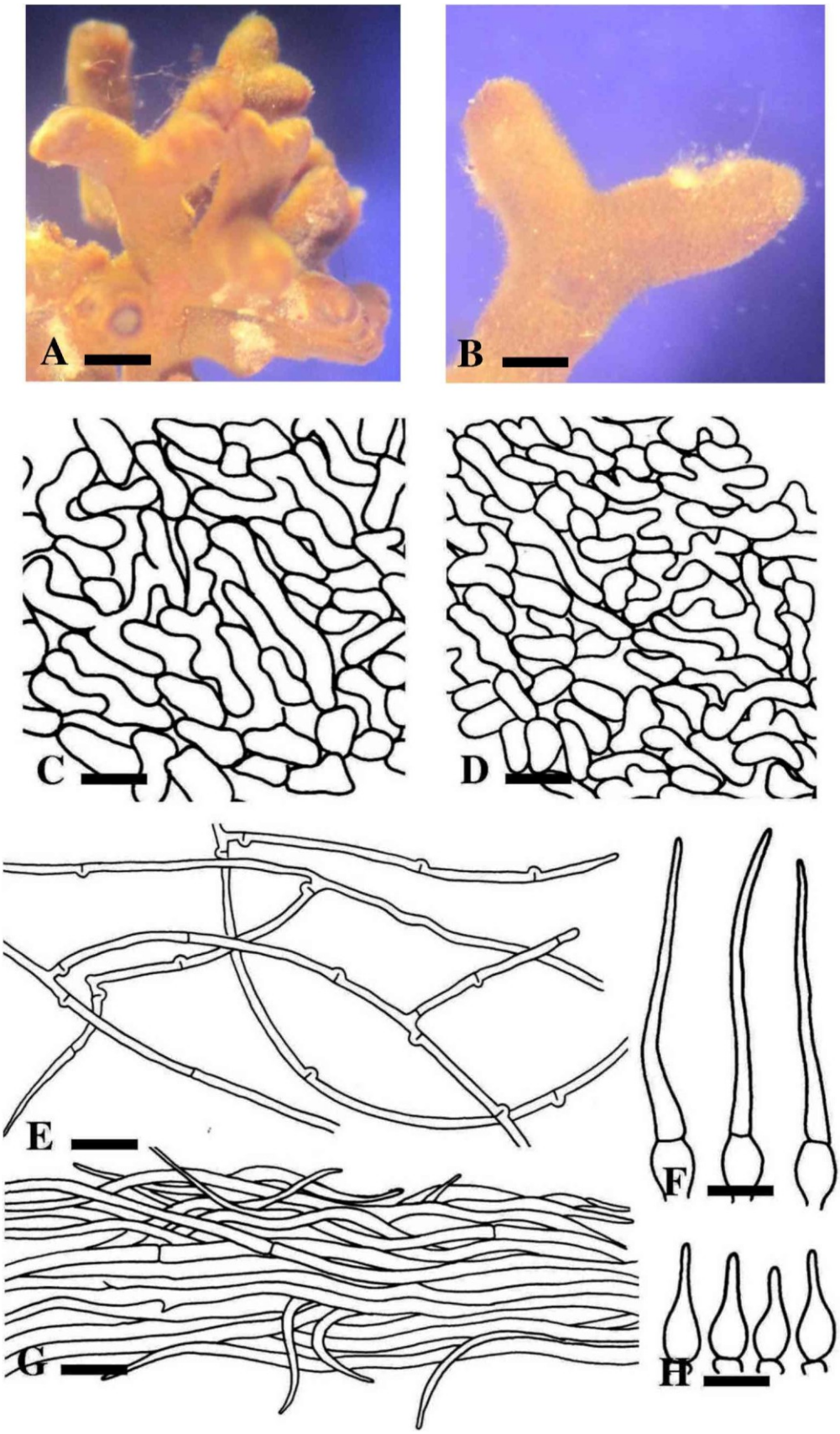

Fig. 2. Ectomycorrhiza of *Russula livescens*, *A* ectomycorrhizal system, *B* ectomycorrhizal root tip, *C* outer mantle layer, *D* inner mantle layer, *E* emanating hyphae, *F* awl-shaped cystidia, *G* rhizomorphs, *H* bottle shaped cystidia. Scale bars: A 0.3 mm, B 0.2 mm, C, D 5.5 µm, E 40 µm, F, G 8 µm, H 20 µm.

P i l e o c y s t i d i a : either absent or not observed**.**

S t i p i t i p e l l i s : composed of comparatively more septate hyphae, 2–10 µm in diam, thin walled, hyaline and smooth, terminal cells sub-cylindrical to broadly fusoid with rounded tips, 8.3–12.50 × 20.2–33.5 µm, occasionally cylindrical and tapering to the apex.

C a u l o c y s t i d i a : not observed.

Material examined: Pakistan, Khyber Pakhtunkhwa Province, District Mansehra, Oghi, Khabbal, Gregarious on ground along with *Pinus wallichiana* A. B. JACKSON, Dub, at 2046 m a. s. l., July 2009, M. FIAZ # FM–225. (HUP Herbarium No. MFM–346; GenBank KR020029; Chattar Plain, 2046 m a. s. l., Gregarious on ground along with *Pinus wallichiana*; 15 Sep 2012. M. SABA M33 (LAH-M33-2013; KM596858).

Morphological characterization of ectomycorrhiza (Fig. 2)

E c t o m y c o r r h i z a l s y s t e m : dichotomously ramified to coralloid with spiny appearance, explored at upper layer of soil; 3–4.5 mm long; tips 0.5–1.5mm long and up to 0.3 mm wide, apex 0.2 mm wide, base up to 0.4 mm wide; main axis 1–1.5 mm in diameter; unramified ends straight, not inflated and cylindrical; yellowish brown (5YR7/10) to dark brown (5YR4/8) or black at maturity.

M a n t l e : yellowish brown (5YR7/10) to transparent, distinct, spiny surface; ; host tissue visible under the mantle surface.

R h i z o m o r p h s : rare, smooth, yellowish brown (5YR7/10) to transparent. Emanating hyphae frequent, sometimes abundant at few tips, straight, cylindrical, frequently branched.

M a n t l e : psedoparenchymatous in all layers, non-gelatinous, lobed cells.

O u t e r m a n t l e l a y e r : pseudoparenchymatous, cells lobed and elongated, tend towards the plectenchymatous state (mantle type H, AGERER 1987–2002; AGERER & RAMBOLD 1998), cells membranaceously and plasmatically yellowish brown, 9.8 ± 3.1 x 5.2 ± 0.7 µm, matrix clear.

I n n e r m a n t l e l a y e r : pseudoparenchymatous, cells lobed (mantle type H, AGERER 1987–2002; AGERER & RAMBOLD 1998), cells membranaceously and plasmatically yellowish brown, 8.6 ± 3.2 x 4.2 ± 0.4 µm, cell contents clear.

E m a n a t i n g h y p h a e : straight, elongated, cylindrical, septate, clamped septa frequent, 3.9 µm in diameter, surface smooth, septa 66.4 µm apart, contents clear, ramification rather frequent, Y-shaped; anastomosing not observed.

C y s t i d i a : frequent, varying in size and structure, awl-shaped cystidia 45.86 µm long, 2.2 µm wide neck, slightly enlarged base separated by a septum, septa 3 µm wide, bottle shaped cystidia with strongly inflated base 3.7 µm wide, 5.09 µm wide at the center and a long straight neck 2.3 µm in diameter.

R h i z o m o r p h s : rare; hyphae uniform and loosely arranged, 4.8 µm in diameter; septa infrequent; yellowish brown to brown pigmented.

Material examined: Pakistan, Khyber Pakhtunkhwa, Himalayan moist temperate forests, Khanspur Halipad, 2250 m a.s.l., associated with *Cedrus deodara* (ROXB. ex D. DON) G. DON, 21 September 2013. S. JABEEN. SA107; HP-6 (LAH-EM2-2013; GenBank KR020028); 21 May 2014 S. JABEEN. SA178; HP14-3c (LAH-EM5-2014; GenBank KT944025); 21 May 2014 S. JABEEN. SA317; HP14-3a (LAH-EM7-2014; GenBank KT944027); associated with *Pinus* sp. 21 May 2014 S. JABEEN. SA206; HP14-4a (LAH-EM6-2014; GenBank KT944026).

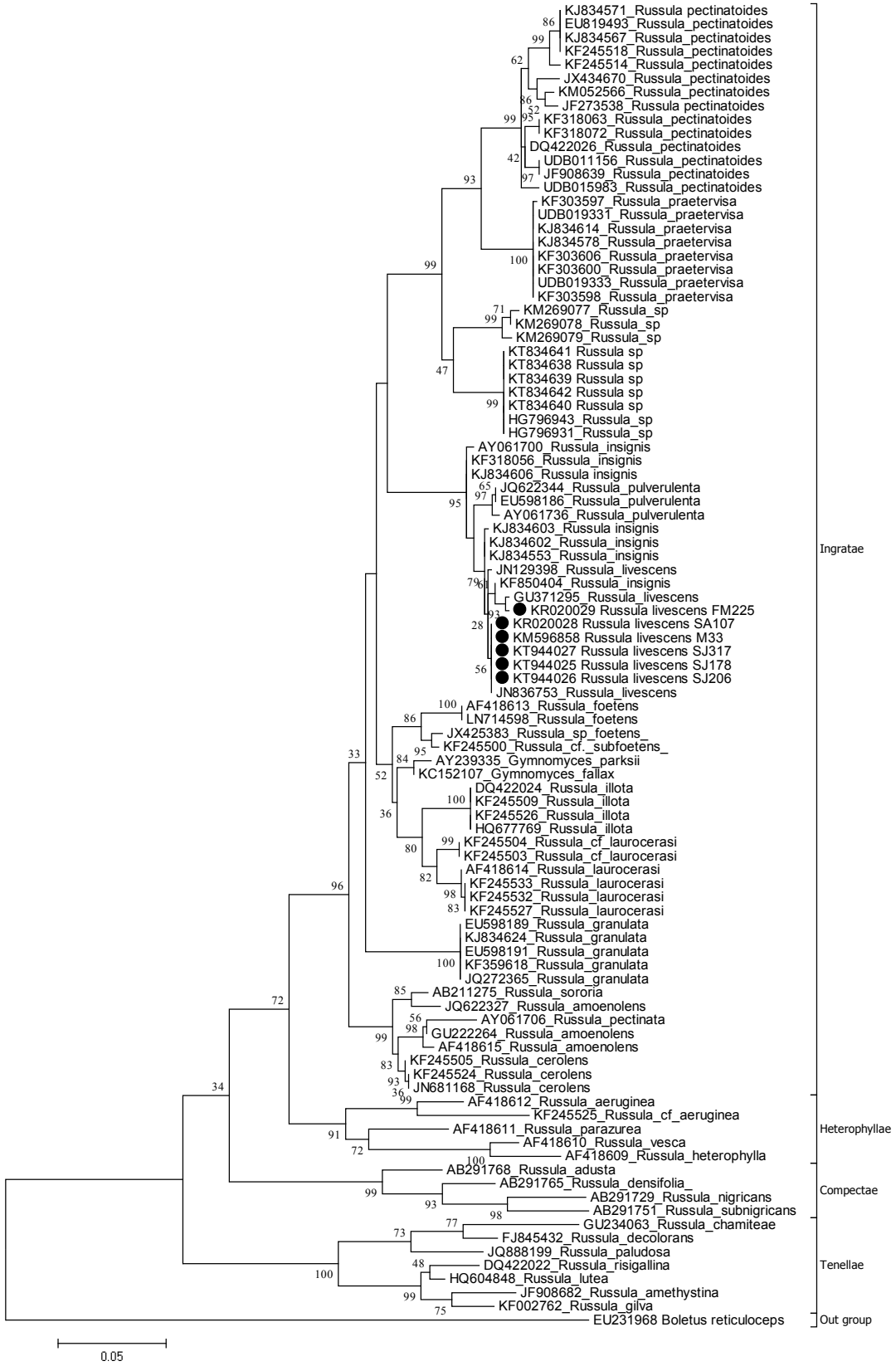

Fig. 3. Molecular phylogenetic analysis inferred by using the Maximum Likelihood method. Bootstrap values shown next to the branches. Initial tree for the heuristic search was obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 97 nucleotide sequences. Sequences generated from Pakistan are marked with ●. There were a total of 827 positions in the final dataset.

Discussion

Russula species are ectomycorrhizal symbionts with a diverse host range (TRAPPE 1962). The species are found among the dominants in the ectomycorrhizal fungal communities (DICKIE & al. 2010). Many taxa belonging to this genus have been reported from Himalayan moist temperate forests of Pakistan (AHMAD & al. 1997; NIAZI & al. 2006; NIAZI 2008; JABEEN & al. 2012, 2014; HANIF & al. 2012; JABEEN & KHALID 2014; RAZAQ & al. 2014). However detailed morphology of these taxa is little studied. In present work, basidiomata of *Russula livescens* were characterized by the presence of creamy white lamellae, fusoid pleurocystidia, ellipsoid basidiospores with reticulations and warts on the wall. Ectomycorrhiza of *R. livescens* was characterized by dichotomous to coralloid ramification with spiny appearance, pseudoparenchymatous mantle, branched emanating hyphae with frequent clamp connections and presence of bottle shaped cystidia. Sequences of the fruit body and ectomycorrhizal tissue along with morphological descriptions were found helpful in identification.

Russula livescens sequences generated from fruit bodies and ectomycorrhizal tissue during this study clustered with the similar taxa in the section *Ingratae* with strong bootstrap support (Fig. 3). It forms a sister clade with *R. insignis* QUÉL and *R. pulverulenta* PECK. Separation of these taxa from *R. livescens* is supported by morphological characters. *Russula insignis* has a stipe base with yellowish veil remnants and encrusted hyphae of pileipelis (ROMAGNESI 1967, SARNARI 1998, KRÄNZLIN 2005). *Russula pulverulenta* has a floccose pileus cuticle, scurfy or minutely fibrillose stipe, and terminal cells of pileipellis hyphae with occasionally sparsely warty walls (SHAFFER 1972). Moreover spores of *Russula livescens* have more connectives and so are more commonly partially reticulate as compared to *R. pulverulenta* (SHAFFER 1972).

In the phylogenetic tree, besides of its own clade, some *Russula insignis* sequences (KJ834603, KJ834602, KJ834553, KF850404) are clustered with the *Russula livescens*. It is worth mentioning that *Russula livescens* sensu LANGE is a synonym of *R. insignis* QUÉL. 1888 (= *R. livescens* var. *depauperata* J. E. LANGE 1926), and *R. livescens* (BATSCH) BATAILLE 1908 is a valid species. So, *Russula insignis* QUél. and *R. livescens* (BATSCH) BATAILLE are two distinct species. *Russula livescens* was originally described from Europe and its occurrence has been recorded in Denmark, Germany, Spain, Uruguay (http://www.gbif.org/species/2551585) and China (GE & al. 2012). Its ectomycorrhizal association has previously been reported in the form of operational taxonomic unit from Pakistan (HANIF & al. 2012). Occurrence of *Russula livescens* in Pakistan is an extension to the known distribution of the taxon.

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