INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
12–1293/2013/15–5–857–863
http://www.fspublishers.org

Full Length Article

Molecular Investigations to Determine the Ectomycorrhizal Habit of *Lactarius sanguifluus* Associated with Coniferous and Deciduous Vegetation of Galyat, Khyber Pakhtunkhwa, Pakistan

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Abstract

This is the first detailed report on molecular identification of aboveground fruiting body as well as its ectomycorrhiza, *Lactarius* species, associated with wide host range. *Lactarius sanguifluus* growing in association with different host trees: *Juglans*, *Populus* and *Quercus* were collected from Galyat, Pakistan along with soil blocks containing plant roots. The genomic DNA was extracted from basidiocarps and roots of different trees. The internal transcribed spacers (ITS) region of rDNA was amplified using forward primer ITS1F and reverse primer ITS4, which generated fragments of 700-750bp. The aboveground sequences showed base similarity more than 99% with *L. sanguifluus* submitted from Europe (FJ858746, AF249289). The phylogenetic analysis of above and belowground parts of *L. sanguifluus* showed their clustering in same clade with same species. Sequence data of seven specimens is being published from Pakistan; two belong to epigeous *L. sanguifluus* (HF559378.1, HF559379.1), four ectomycorrhizal roots; two associated with *Quercus incana* (HF559374.1, HF559375.1), one associated with *Pinus wallichiana* (HF559376.1), one associated with *Juglans regia* (HE615155) and one ectomycorrhizal sequence of *L. semisanguifluus* (HF559377.1) associated with *Q. incana*. © 2013 Friends Science Publishers

Keywords: Ectomycorrhiza; ITS-rDNA; *Quercus incana*; *Lactarius semisanguifluus*

Introduction

Ectomycorrhizae (ECM) is mutualistic association between mycelia of higher fungi and roots of plants by which many plants cope with infertile soil in north temperate and boreal conditions (Trocha *et al.,* 2012). A huge network of fungal mycelia even broader than the canopy of the plant below the soil conserves water and nutrients like nitrogen and phosphorus for plant growth and life cycle (Hanif *et al.,* 2012). This network sprouts out in the form of fruiting body mostly in rainy season and these fruiting bodies add more spores in the soil for continuation of this association (Dunk *et al.,* 2011). These fruiting bodies are good indicators of the total number of ectomycorrhizal fungi of a forest floor. This is not the measure of total ectomycorrhizal fungal diversity of a forest because fruiting season for different fungi is different and all fungi not necessarily form an above ground fruiting body (Jabeen *et al.,* 2012). Some fungi have no host specificity while some are host specific (Mikola, 1973). The forest fungal community exclusively not comprises of ectomycorrhizal fungi but also the saprophytic, wood decaying and pathogentic species which make the situation complex for identification of ectomycorrhizal fungus. Therefore, it is necessary to identify both above ground and below grown partner of an ectomycorrhizal fungus.

Previously ectomycorrhizal fungi were identified by methods develop by Agerer and his group (1987-2002) based on ramification, hyphal mantle, color, structure etc. These investigations are time consuming and show difficulty in tracing the connection between above and belowground part of ectomycorrhizal fungi but now molecular analysis of both components fairly establishes confirmed identity (Godbold, 2005).

Pakistan also has a variety of forest types and ecosystems which are characterized by different mycoflora including ectomycorrhizal and saprophytic fungi (Niazi *et al.,* 2009; Razaq *et al.,* 2012a, b). Galyat area of Himalayan moist temperate forests Pakistan is dominated with coniferous vegetation with some mixed deciduous plants. A detail description of 24 species of ectomycorrhizal fungi associated with coniferous vegetation is available in which both above and belowground parts were matched using morpho-anatomical and ecological tools like tracing method, morphotyping etc. (Kazmi *et al.,* 2004; Niazi *et al.,* 2009, 2010). Jabeen *et al.* (2012) provided a detail description of 15 mycorrhizal species from root tips of deciduous vegetation based on morphotyping method. Hanif *et al.* (2012) added three more operational taxonomic units (OTUs) with coniferous vegetation using molecular method without matching aboveground parts.

In this paper a new ectomycorrhiza of *Lactarius sanguifluus* is being described associated with coniferous

To cite this paper: Ilyas, S., A. Razaq and A.N. Khalid, 2013. Molecular investigations to determine the ectomycorrhizal habit of *Lactarius sanguifluus* associated with coniferous and deciduous vegetation of Galyat, Khyber Pakhtunkhwa, Pakistan. *Int. J. Agric. Biol*., 15: 857–863

and deciduous vegetation. Both above and belowground part of *L. sanguifluus* were collected with *Quercus incana* Bartram, *Juglans regia* L. and *Pinus wallichiana* A. B. Jacks., and described morpho-anatomically and molecularly. ITS-rDNA sequences obtained from mantle sheath of each host root were compared with those obtained from local basidiocarps. A further phylogenetic analysis was also used for the identification of both parts. This genus is well known for its ectomycorrhizal association with conifers and broad leaves trees (Eberhardt *et al.,* 2000). Previously, only 43 species of *Lactarius* are described having ectomycorrhizal nature out of total 500 species (Roman *et al.,* 2005). This study will add four ectomycorrhizal sequences of one new ectomycorrhizal species.

Materials and Methods

Description of Site

Galyat is a narrow strip or area roughly 50–80 km north-east of Islamabad, Pakistan, extending on both sides of the Khyber Pakhtunkhwa-Punjab border, between Abbottabad and Murree. The area is dominated by conifers along with patches of deciduous trees like *Alnus nitida*, *J. regia*, *Populus* spp., *Salix* spp. *and Quercus* spp. In this region maximum rainfall of 600 mm occurs from July to September with an average humidity of 57% that is the peak season for the growth of mushrooms (Hussain, 1995).

Collection of Basidiocarps and ECM Root Tips

Sampling sites were visited during 2008-2012. Sporocarps were collected, dried carefully and brought to laboratory. Beneath basidiocarps, soil blocks of 10 cm^2 along with root system of selected trees of *Q. incana*, *J. regia* and *P. wallichiana* were dugout with the help of a digger and packed in polythene bags to avoid evaporation and crumbling. Each sample isolated from a single core was given an individual collection number. The soil blocks containing morphotypes were washed in water and after removing soil particles by sieving through mesh of 2 mm, morphotypes were stored in McCartney bottles filled with distilled, sterilized water and some of them were stored in 2% CTAB buffer for DNA analysis. Fresh mycorrhizas were photographed under stereomicroscope.

Morpho-anatomical Characters

For morphoanatomical characterization of ectomycorrhizae, terminologies of Agerer (1991, 2006) were followed and sporocarps were analyzed macroscopically (color, lamellae, shape etc.) and microscopically (basidia, basidiospores, cystidia etc.) following Reid (1984) methodology.

Molecular Identification

The protocol of Extract N-Amp (Sigma, St Louis, MO, USA) was followed. Dried material of sporocarp (approx. 1 mg) and subsequently root tip (approx. 1 mm) was taken in small PCR tubes and 10 uL of extraction solution (XNAP-2) was added and incubated at 65ºC for 10 min and later on at 94ºC for 10 min as well. After that 10 µL of dilution solution (XNAP-2) was added and incubated at 4ºC for 1h. Fungal specific primers ITS-1F and ITS-4 were used to amplify part of 18S rDNA, the ITS1 region, 5.8S region, ITS2 region and part of 28 S region. The negative control (no DNA) was included in each set of amplification. The following profile was used for PCR: initial denaturation step at 95°C for 2 min followed by 35 cycles, each consisting of 95°C for 40s, annealing temperature 53°C for 30s and 72°C for 40s, with a final extension at 72°C for 5 min. PCR products were loaded in a 1% agarose gel immersed in 1 x TAE buffer and electrophoresis was run for half an hour at 70 V. The gel was stained with ethidium bromide (0.5 µg mL^{-1}) and photographed on a UV transilluminator with a Polaroid camera. PCR product was sequenced in both directions using the same pair of primers.

Sequence Data Analysis

Initially, the sequence was analyzed and compared in the GenBank through Basic Local Alignment Search Tool (BLAST) network services using National Center for Biotechnology Information (NCBI), USA database. From GenBank, the closely related sequences were selected and extracted for comparison and alignment. Closely related sequences were retrieved from the GenBank and aligned by Clustal W using default setting in Molecular Evolutionary Genetics Analysis (MEGA) software (Tamura *et al.,* 2011). For only a complete ITS sequences in analysis, all sequences were trimmed with the conserved motifs 5'-(...GAT) CATTA— and —GACCT (CAAA...)-3' and the alignment portion between them was included in analysis (Dentinger *et al.,* 2011). Maximum likelihood (ML) analysis was performed in the MEGA.5 software using Jukes-Cantor model of nrITS sequences and Nearest-Neighbor-Interchange (NNI) as ML heuristic search method to determine the relationship between above and below ground parts. Nucleotide sequences of *L. sanguifluus* and *L. semisanguifluus* were submitted to European Molecular Biology Laboratory (EMBL) database and are available in the GenBank (Table 1).

Results

Molecular Identification of Above- and Belowground Parts of *L. sanguifluus*

ITS-rDNA sequences from basidiocarps and mantle sheath of ectomycorrhizal roots of *Quercus*, *Juglans* and *Pinus* were compared in the GenBank database using Basic Local Alignment Search Tool (BLAST). All these sequences matched more than 96% with *L. sanguifluus* (FJ858746, AF249289**)** (Table 1) with Query coverage more than 98%. Later on, both sequences of roots and basidiocarps were

Table 1: Specimens used in this study

Species	Origin	Host	Source	Collector	GenBank
Lactarius sanguifluus	Spain	Unknown	MC	Vila et al., 2009	FJ858746
Lactarius sanguifluus	France	Unknown	BS	Guerin-Laguette, 2000	AF249289
Lactarius sanguifluus	Belgium	*Pinus*	BS	Nuytinck and Verbeken, 2003	AY332547
Lactarius sanguifluus	Belgium	Cni +dec	BS	Nuytinck, 2003	AY332545
Lactarius sanguifluus	Belgium	*Pinus*	BS	Nuytinck, 2003	AY332544
Lactarius rubrozonatus	Italy	Unknown	BS	Lalli and Pacioni, 2002	AY292987
Lactarius sanguifluus	Spain	*Pinus*	BS	Hortal et al.,2005	DQ116906
Lactarius sanguifluus	Spain	Unknown	BS	Marin et al., 1998	AF096981
Lactarius vinosus	Belgium	Unknown	BS	Nuytinck, 2003	AY332552
Lactarius sanguifluus	Belgium	*Pinus*	BS	Nuytinck, 2003	AY332548
Lactarius sanguifluus	Spain	*Pinus*	BS	Hortal and Parlade, 2008	EU423921
Lactarius sanguifluus	France	Unknown	BS	Guerin-Laguette et al., 2000	AF249290
Lactarius vinosus	Belgium	Unknown	BS	Nuytinck, 2003	AY332551
Lactarius vinosus	Belgium	Unknown	BS	Nuytinck and Verbeken, 2003	AY332549
Lactarius deliciosus	Canada	Unknown		Kranabetter et al, 2009	FJ845418
Lactarius sanguifluus	Pakistan	*Juglans*	ECM	Ilyas et al., 2011	HE615155
Lactarius sanguifluus	Pakistan	*Quercus*	ECM	Ilyas et al., 2012	HF559374
Lactarius sanguifluus	Pakistan	*Quercus*	ECM	Ilyas et al., 2012	HF559375
Lactarius sanguifluus	Pakistan	*Pinus*	ECM	Ilyas et al., 2012	HF559376
Lactarius semisanguifluus	Pakistan	*Quercus*	ECM	Ilyas et al., 2012	HF559377
Lactarius sanguifluus	Pakistan	*Pinus*	BS	Ilyas et al., 2012	HF559378
Lactarius sanguifluus	Pakistan	*Quercus*	BS	Ilyas et al., 2012	HF559379

MC= Mycelial strand, BS= Basidiocarp, ECM= Ectomycorhiza, Coni+Deci=Coniferous and deciduous trees
All bold taxa are newly sequenced in this study

aligned in a single file and the trimmed region between two motifs results showed 100% similarity. One sequence (HF559377.1) from the *Quercus* roots matches with *L. semisanguifluus* that is very closely related species to *L. sanguifluus.* Its local fruiting data is not available in herbaria or literature and therefore this data is only based on only its ectomycorrhiza. As far as the intraspecific variability of *L. sanguifluus* is concerned, all sequences (HF559374.1, HF559375.1, HF559376.1, HF559378.1, HF559379.1) reported from Pakistan showed almost 100% similarity and two sequences (AY33254, AY332547) submitted from Belgium showed no variation, in the same way two sequences (FJ858746, AF249289) submitted from Europe are identical. Pakistani sequences showed similarity with European and American collections of the same species.

Both the above and belowground parts of *L. sanguifluus* were further analyzed using phylogenetic analysis approach. All those *Lactarius* species, which lack epithelial pileal covering without rosette formation in their pileal coverings are clustered in, *Lactarius* sect. *Deliciosi* (Fr.) Redeuilh, while those which can be distinguished on this diagnostic character are clustered in *Russularia* sect. *Olentes* Bon. *Lactarius* sect. *Deliciosi* clade has four sub-clades and almost each sub-clade represents one species isolates from different regions of the world. They cluster together as non-coding DNA like ITS-rDNA is least effected by environmental changes. *L. sanguifluus* sequences clustered together in a single clade (Fig. 1, *Lactarius* sect. *Deliciosi* Clade (I). Ectomycorrhizal sequences dispersed among fruiting bodies sequences from Pakistan or the rest of the world. This clade collectively forms a sub-clade with other very closely related *L. vinosus* isolates (Fig. 1, *Lactarius* sect. *Deliciosi* Clade (II). *L. semisanuifluus* also distinctly separated with sequences of the same species (Fig. 1, *Lactarius* sect. *Deliciosi* Clade (III).

Taxonomy

Lactarius sanguifluus (Paulet) Fr. in Epicr. Syst. Mycol.: 341, 1838 (Fig. 2A–B), (Fig. 3A–C).

Hypophyllum sanguifluum Paulet, Traité des Champignons, Vol. 2, 9th ed., pl. 81, Fig. 3-5, 1811.

Pileus 6.2–7.5 cm in diameter, plano-convex, obtuse broad umbo with depressed circular zone around, vinaceos red, orange buff, pinkish buff in an irregular pattern, surface smooth, viscid to semi viscid when wet, margins entire to minutely dentate, decurved, pale brown. Context: firm, thick, fresh red vinaceous in color. Lamellae vinaceous red, margins pale brown, entire, slightly decurrent, crowded, forked or anastomosing near stipe and margins. Stipe 7×1.3 cm, vinaceous red color, whitish toward base, a white ring (ribbon shaped) very prominent and uniform under the gills attachment region, more or less cylindrical, surface smooth and shiny. Stipe hollow with brick red context.

Basidiospores 8–9.5 × 6.5–7.5 µm, globose to subglobose, ornamented, verricose, ridges prominent, thick reticulate. Basidia, 44–53×7–8.5 µm, four spores, clavate to club shaped with oil droplets in 5% KOH. Pleuromacrocystidia 37.8–47.5 × 8.7–9.4 µm, hyaline to light olive green, smooth, transparent, clavate to sub clavate, fusiform to ventricose, apices very narrow.

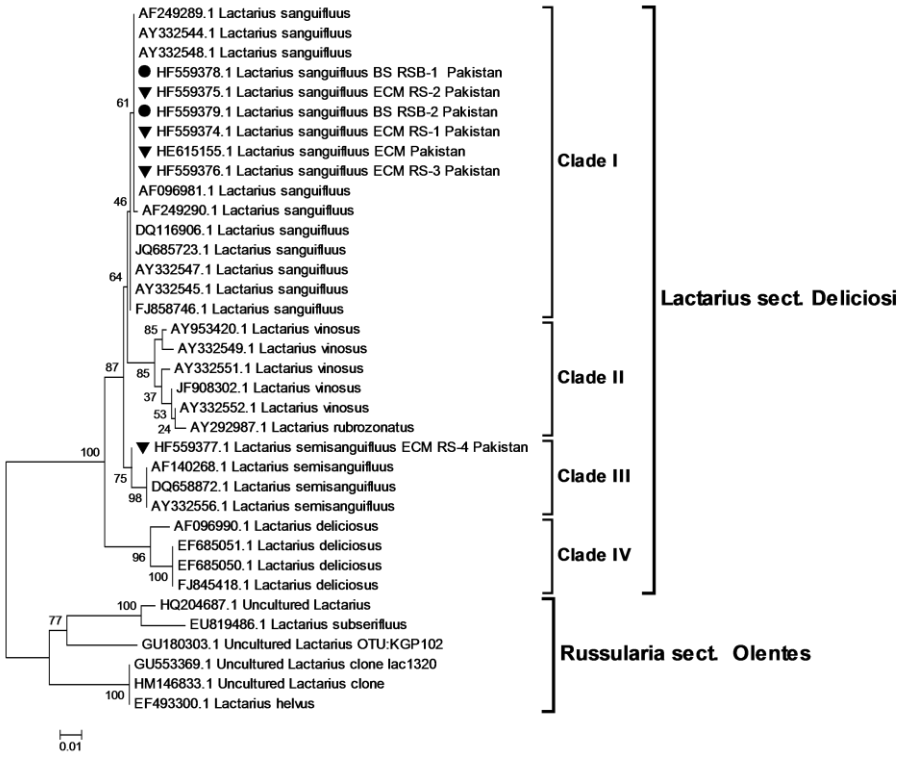

Fig. 1: Phylogenetic relationship and general clustering of *Lactarius sanguifluus* basidiocarps and its ectomycorrhizal parts with other members of *Lactarius* and *L. sanguifluus* sequences from rest of the world based on maximum likelihood method inferred from nrITS sequences. Ectomycorrhiza of *L. semisanguifluus* is also described with respective sequences.Bootstrap values based on 100 replicates are shown above the branches and below 50 are not shown. The analysis involved 26 sequences. All positions containing gaps are treated as data. ECM= ectomycorrhiza, BS = Basidiocarp

Habitat and Distribution

Pakistan: Khyber Pakhtunkhwa (KPK), Abbotabad, Galyat, Khanspur, at 2200 to 2500 m a.s.l. associated with *P. wallichiana* gregarious mostly near the tree trunks, 23. 08. 2012, RBS.1, LAH 2308101, accession # HF559378.1. Pakistan: Khyber Pakhtunkhwa (KPK), Abbotabad, Galyat, Kozagali, associated with *J. regia* and *Q. incana* gregarious mostly near the tree trunks, 25. 12. 2010, RBS.2, LAH 2508102, accession # HF559379.1.

Description of Ectomycorrhizae of *Lactarius sanguifluus*

Morphological characteristics (Fig. 2A–C): As shown Fig. 2A–C, ectomycorrhizal system frequently found, hydrophilic, dichotomously branched, (1.5–) 4–5 mm long, main axis very short up to 0.5 mm wide, chestnut brown; non-ramified ends straight, 0.8 to 2 mm long and 0.3 to 0.5 mm wide uniform, young tips orange-brown, older tips chestnut brown. Mantle surface wooly due to thick hyphal growth and with shiny luster, host tissue visible under mantle surface. Rhizomorphs frequent, covering whole mycorrhizal system, connecting distinctly to mantle surface, forming hyphal fans, light honey brown in young ECM turning chocolate brown when gets older, branched frequently, flat in cross section, entangled to each other, difficult to separate. Emanating hyphae common, straight, branched, white to light yellow on young ECM and honey brown on getting old, very common at tips and light brown foreign hyphae intermixed with it. Sclerotia not observed.

Anatomical characteristics of mantle in plane views (Fig. 4B–C): The mycorrhiza in plane view shows pseudoparenchymatous mantle with few hyphae running parallel Outer mantle layer not gelatinous, transparent in color, pseudoparenchymatous arrangement with few hyphae of 2 µm running parallel (Type K, Agerer, 1987-2002), cells oval to rounded 3–5 µm long and 2–3.5 µm wide, unique organization, matrix granular, cell contents granular and oily droplets, hyphal junction not seen, hyphal anastomoses absent.

Inner mantle layer pseudoparenchymatous with few hyphae extending outward (Type P, Agerer 1987-2002), cells with smooth surface, colorless, up to 5–8 µm long and 3–4.7 µm wide, matrix material with light green granules, septa and clamped septa are insignificant, hyphal junction common at an angle of 45º, anastomoses not observed. Every tip was with the same structures as in the lateral parts.

Fig. 2: *L. sanguifluus* and its ectomycorrhiza. A. Lower view of Sporocarp exposing Lamellae B. Upper view of sporocarp showing pileus, C. Morphotypes associated with *J. regia*, D. *Pinus wallichiana* E. *Quercus incana*,. Bar. A. 1.5 cm, B. 2 cm, C and D. 1.3, E. 0.8 cm

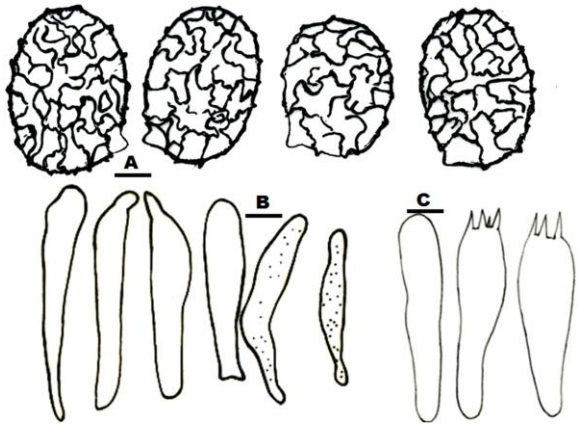

Fig. 3: *Lactarius sanguifluus* A. Basidiospores B. Basidia C. Macropleurocystidia, Bars= A: 4 µm, B: 10 µm, C: 11 µm

Anatomical characteristics of emanating elements (Fig. 4D-F): Rhizomorphs undifferentiated (Type B, Agerer 1987-2002); margins rather smooth, thick, hyphae compactly arranged and thickly interwoven which are very difficult to separate, cells upto 3.5 µm in diameter, septate

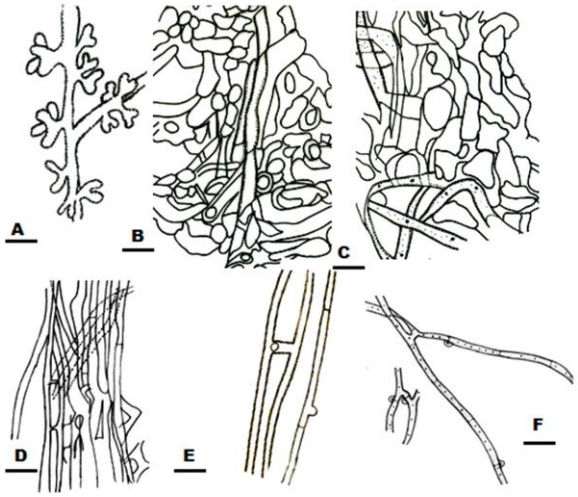

Fig. 4: Morphology and Anatomical features of ectomycorrhiza of *Lactarius sanguifluus* A-Habit of ectomycorrhiza illustrating the dichotomously ramified ends B- Pseudoparenchymatous outer mantle C- Pseudoparenchymatous Inner mantle D- Rhizomorph E- Emanating hyphe with c.c, old F- Emanating hyphae with c.c, young. Bar. A. 1 mm, B. 0.67 µm, C. 3.6 µm, D17.5 mm, E. 10 µm, F. 13.7 µm

infrequently, clamps not observed, hyphae sometimes fuses with eachother forming H-shaped anastomoses without clamp, hyphal junction not observed.

Emanating hyphae smooth, 2.5–3.0 µm wide and 22–51 µm long, septa and clamped septa commonly found, cell contents granular, small oily droplets present, sometimes transparent cells and clear contents also observed, thick walled, anastomoses common often H-shaped without clamp, hyphal junction rare with an angle of 45º cystidia absent, chlamydospores not observed.

Material Examined

Pakistan: Khyber Pakhtunkhwa (KPK), Abbotabad, Galyat, Khanspur, at 2200 to 2500 m a.s.l. associated with *P. wallichiana* roots, 23. 10. 2010, RS.1, LAH 2308101, accession # HF559376.1. Pakistan: Khyber Pakhtunkhwa (KPK), Abbotabad, Galyat, Kozagali, associated with *J. regia* and *Q. incana* roots, 25. 10. 2012, J2, RS.2, RS.3 LAH 2508102, LAH 2508113, LAH 2508122, LAH 2508123, accession # (HE615155.1, HF559374.1, HF559375.1) respectively. Pakistan: Khyber Pakhtunkhwa (KPK), Abbotabad, Galyat, Kozagali, associated with *Q. incana* roots, 25. 12. 2012, *L. semisanguifluus* (HF559377.1).

Discussion

Lactarius is a genus of ectomycorrhizal macrofungi with an estimated 500 species worldwide (Le *et al.*, 1997). Most of the species form ectomycorrhiza with coniferous

and deciduous trees. Roman *et al.* (2005) described 43 ectomycorrhizal species of *Lactairus* that form association with deciduous vegetation and coniferous plants. *Lactarius* generally forms simple or monopodial pinnate ramification and with laticiferous cells. Geml *et al.* (2009) also studied community structure of Alaska vegetation of mixed coniferous and deciduous ecosystem. In this study both the operational taxonomic units (OTUs) delimitations and phylogenetic approaches were used to study 918 soil clones libraries and Herbaria specimens of *Lactarius*. An OUT showing more than 97% similarity with fruiting body sequence is usually given a same taxonomic name (O'Brien *et al.*, 2005; Arnold and Lutzoni 2007; Higgins *et al.*, 2007). *Lactarius* species accumulation curves significantly differ at 97, 95 and 90%. Phylogenetic approach is also useful tool for identification of both above and below ground parts as *Lactarius* is a monophyletic group (Miller *et al*., 2006). During present study, both these approaches are used to identify the ectomycorrhizae of *L. sanguifluus* and *L. semisanguifluus*.

The ITS region of basidiocarps and ectomycorrhizae have been compared with GenBank data where these sequences showed 98-99% base similarity with *L. sanguifluus* isolates from different geographical regions. *L. sanguifluus* ectomycorrhizae of *Q. incana*, *J. regia* and *P. wallichiana* have similarity more than any suggested cut value to local basidiocarps sequences. In the phylogenetic analysis of Pakistani sequences, all *L. sanguifluus* and *L. semisanguifluus* clustered with European sequences (Fig. 1). According to Miller *et al.* (2006), *Lactarius* genus has low intraspecific variation in ITS-rDNA region and monophyletic nature of this group makes unambiguous clustering in phylogenetic analysis. Similar sequences clustered in a particular clade in a phylogenetic tree while dissimilar ones were clustered in a separate clade (Ryberg *et al.*, 2008).

Morphologically, the ectomycorrhizae of *L. sanguifluus* shares a number of features with other morphotypes of this genus for example orange-brown color of ectomycorrhizae, lack of macroscopically visible laticifers on surface, pseudoparenchymatous structure of outer mantle that is generally formed by angular cells, open anastomoses among emanating hyphae and lack of cystidia. A few strongly sticky crystalline structures over the surface of morphotype reflects its hydrophilic nature. This feature makes it closer with the ectomycorrhizal morphotype of *L. controversus* associated with *Populus alba* (Jakucs *et al.*, 2000). There are few differences observed in the ectomycorrhizae of *L. sanguifluus* from others. The ectomycorrhizae is dichotomously branched which is different from other reports. The unique feature of newly described ectomycorrhiza is the presence of well-developed clamps in emanating hyphae. This is the distinguishing character not reported in any ECM of *Lactarius* but reported rarely in Russulaceae (Lee *et al.*, 1997; Agerer, 2006). Because mycorrhizal fungi play a key ecological role in the ecosystem for decomposition, mineralization, immobilization and transfer of nutrients to plants, therefore documentation of fungal diversity is crucial for overall functional biodiversity.

Acknowledgments

We are sincerely thankful to Higher Education Commission, Pakistan for providing financial assistance under Indigenous Fellowship IV and International Research Support Initiative Program (IRSIP).

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(Received 03 December 2012; Accepted 22 April 2013)