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Molecular Identification of *Lepiota acutesquamosa* and *L. cristata* (Basidiomycota, Agaricales) Based on ITS-rDNA Barcoding from Himalayan Moist Temperate Forests of Pakistan

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Abstract

Lepiota acutesquamosa and *L. cristata* (Basidiomycota, Agaricales) collected from Himalayan moist temperate forests of Pakistan were characterized using internal transcribed spacers (ITS) of rNDA, a fungal molecular marker. The ITS-rDNA of both species was analyzed using polymerase chain reaction (PCR) and DNA sequencing. The target region when amplified using universal fungal primers (ITS1F and ITS4) generated 650-650bp fragments. Consensus sequences of both species were submitted for initial blast analysis which revealed and confirmed the identification of both species by comparing the sequences of these respective species already present in the GenBank. Sequence of Pakistani collection of *L. acutesquamosa* matched 99% with sequences of same species (FJ998400) and Pakistani *L. cristata* matched 97% with its sequences (EU081956, U85327, AJ237628). Further, in phylogenetic analysis both species distinctly clustered with their respective groups. Morphological characters like shape, size and color of basidiomata, basidiospore size, basidial lengths, shape and size of cheilocystidia of both collections were measured and compared. Both these species have been described first time from Pakistan on morph-anatomical and molecular basis.

Keywords: Internal transcribed spacers; Lepiotaceous fungi; Molecular marker; Phylogeny

Introduction

The Himalayan moist temperate forests of Pakistan are characterized by vigorous and thick vegetation of conifers mixed with some deciduous trees and located at an elevation of 1373 to 3050 m. These forests receive average rainfall 59.3cm, humidity up to 57%; and experience maximum temperature during summer, which varies from 10.7°C to 18°C (Champion *et al.*, 1968). Most of the mushrooms are still to be reported even though Himalaya is included in one of the twenty-five hotspots for biodiversity (Myers *et al*., 2000).

Lepiota (Pers.) Gray (Agaricales, Basidiomycota) is widely distributed and diversified genus having more than 400 species worldwide (Kirk *et al*., 2008; Kumar and Manimohan, 2009). This genus has some diagnostic characters like scaly pileus, free lamellae, annulus, a universal veil; white to colorless, smooth, dextrinoid spores; clamp connections but not always (Vellinga, 2001; Kumar and Manimohan, 2009). Along morphological, molecular characterization has revolutionized the identification of higher fungi (Lian *et al*., 2008; Razaq *et al*., 2012). Genetic variation in ITS region of the rDNA enables reliable identification of fungi up to species level (Riviere *et al.*, 2007). This genetic variation is also used for the identification and phylogenetic analysis of different species of lepiotaceous fungi (Vellinga, 2001, 2003, 2006).

During field work in the Himalayan moist temperate forests, two species of *Lepiota* were collected, which have been identified on molecular basis by extracting and analyzing rDNA. Phylogenetic analysis of both also confirms their identification by clustering with respective sequences submitted from Europe.

In Pakistan only checklist of lepiotaceous fungi is available without any technical descriptions and diagnostic features (Ahmad *et al*., 1997). This paper provides the basic information and molecular data for reliable identification of these two species from this region. In addition, their sequences submitted in the GenBank would be reference sequences for future collections.

Materials and Methods

Morpho-anatomical Characterization

All the basidiocarps after being photographed in the field were carefully dug with the help of a sharp knife. Collected material was characterized morpho-anatomic and molecular basis. For microscopic observation, sections were stained with Congo Red and Melzer's reagent. Size ranges for 25 basidiospores, 20 basidia, 20 cheilocystidia were determined. Following abbreviations are used: avl for

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average length of basidiospores, avw for average width of basidiospores, Q for length/width of basidiospores. Drawings were made using a camera lucida attached to compound microscope. Dried specimens were deposited in the LAH Herbarium, Department of Botany, University of the Punjab, Lahore.

Molecular Characterization and Phylogenetic Analysis

The protocol of Extract-N-Amp (XNAP-2) (Sigma, St Louis, MO, USA) was followed with some modifications. A piece of dried lamellae (approx. 1 mg) was taken in small PCR tubes and 10 µL of extraction solution was added. These tubes were incubated at 65ºC and then at 94ºC for 10 min each and subsequently 10 µL of dilution solution (XNAP-2) was added and left for one hour at room temperature. ITS regions along with 5.8S of rDNA were amplified using universal primer pair ITS1F and ITS4 (White *et al*., 1990; Gardes and Bruns, 1993). PCR was performed in 25 µL reaction volume in PCR mix provided with Extract-N-Amp (XNAP-2) kit, following the PCR conditions given by Gardes and Bruns (1993). PCR product of the ITS-amplified was directly sequenced in both directions using the same pair of amplification primers (Macrogen, Korea). Initial Basic Local Alignment Search Tool (BLAST) was used to compare sequences in National Center for Biotechnology Information (NCBI), USA database. For further phylogenetic analysis and alignment of sequences, closely related published sequences, which have query coverage more than 99% and E-value =0 were retrieved from GenBank database. The sequence alignments and phylogenetic analysis were performed using Molecular Evolutionary Genetics Analysis (MEGA) software (Tamura *et al*., 2011). Maximum Likelihood (ML) method was based on the Jukes-Cantor model of nrITS sequences using Nearest-Neighbor-Interchange (NNI) as ML heuristic search method. Phylogeny was tested by bootstrap value of 1000 replicates. Nucleotide sequences of *L. acutesquamosa* (Accession # HF542114) and *L. cristata* (Accession # HF542115) were submitted to European Molecular Biology Laboratory (EMBL) database and are available in the GenBank.

Results and Discussion

Taxonomy

***L. acutesquamosa*:** Weinm., Syll. Pl. Nov. Ratisb. 1: 70. 1824 (Fig. 1 A-E). =Lepiota aspera (Pers.) Quél., Enchir. Fung. (Paris): 5 (1886).

Pileus 6 cm across, fleshy, broadly convex, brittle, slightly umbonate to obtuse, fibrillose; fibrils brown, pyramidal, larger and denser in the centre, while lighter in colour toward margins, brown scales on white ground; margins entire; context white, soft and up to 0.5 cm thick at disc and thin towards margins. Lamellae white to cream, free, crowded, edges entire, thin; lamellulae absent. Stipe 7.0 × 1 cm, hollow, equal, dry, smooth, white above the veil with brown warts on light brown ground, girdles surface; annulus membranous, white, entirely covering the gills in premature stages, drooping down when mature.

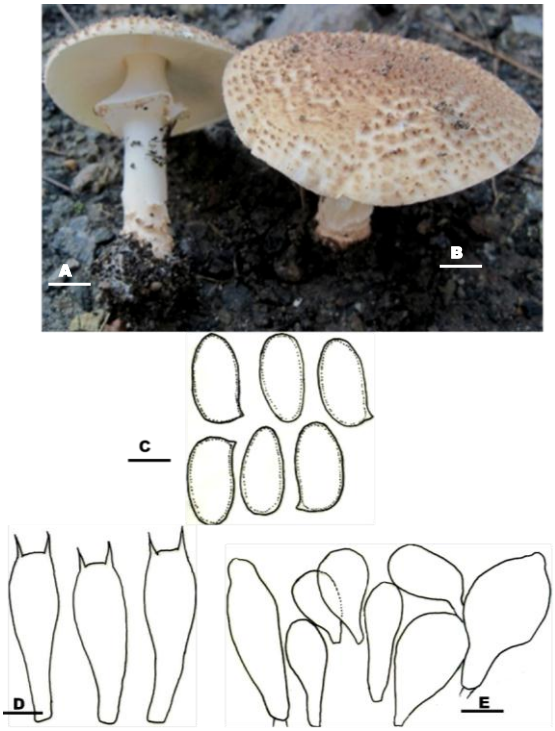

Fig. 1A-E: *Lepiota acutesquamosa* (A) Photograph of basidioma showing lamellae (B) Photograph of basidioma showing scaly pileus (C) Basidiospores (D) Basidia (E) Chielocystidia. Bars = A–B = 2.0 cm; C=3.45 µm; D= 4.5 µm; E= 11.0 µm

Basidiospores, 7.5–10 × 3–4.5 µm, avl × avw = 8.5×4 µm Q=2.1, ellipsoidal to oblong, thin walled, hyaline to colouless, smooth, reddish brown in Melzer ‘s reagent, dextrinoid. Basidia 8–15 × 5–7 µm, 2–spored, clavate to broadly club shaped, thin walled, hyaline to light brown to pale yellow in 5% KOH. Cheilocystidia clavate to sub-clavate, tapering toward base, some sub-cylidrucal and capitates, normally, size varies 29.5–39.5 × 8.5–16.5 µm, thin walled, hyaline to pale yellow in 5% KOH, with no contents. Macrochemical Tests spores in Melzer‘s reagent darkly brown.

Material Examined

Pakistan, Khyber Pakhtunkhwa, Himalayan Moist Temperate Forests, Khanspur, (34.0171° N, 73.4167° E) at 2250 m a.s.l., solitary, on moist ground under *Abies pindrow* (Royle ex D. Don) Royle, 13 August 2010, Abdul Razaq (KP-59) LAH.No.13081059’. GenBank Accsession # HF542114.

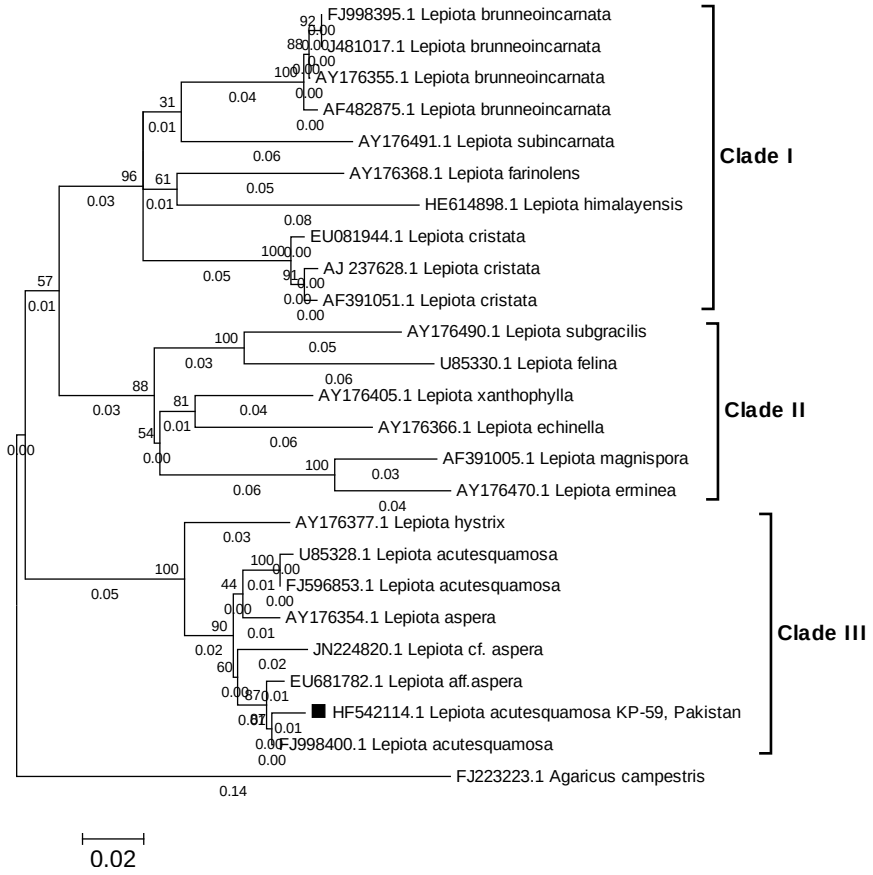

Fig. 2: Phylogenetic relationship of *Lepiota acutesequamosa* (■) with other members of *Lepiota* based on Maximum Likelihood method inferred from nrITS sequences. Bootstrap values based on 100 replicates are shown above the branches and below 50 are not shown. Branch length is shown below each branch. The topology of the tree based on Maximum Parsimony is same for *Lepiota acutesequamosa*. The analysis involved 25 sequences. All positions containing gaps and missing data were eliminated. There were a total of 464 positions in the final dataset

Molecular Characterization and Phylogenetic Analysis of *L. acutesquamosa*

The target region copmrising of internal transcibed spacers (ITS1 and ITS2) and 5.8S of rDNA gave fragments of approximately 600bp on amplification in polymerase chian reaction (PCR) using fungal universal primers pairs (ITS1 and ITS4). Initial BLAST analysis of nucleotide sequences revealed that Pakistani collection matches maximum with *L. acutesquamosa* (GenBank accession # FJ998400). Sequences from GenBank database were retrieved for further alignment and phylogenetic analysis. Phylogenetic analysis based on all these sequences showed its clustering with *L. acutesquamosa* and other closely related taxa. In phylogenetic tree three clades were formed, all Lepiotaceous fungi having ellipsoid spores and trichoderm pileus covering having one type of pileal elements clustered in clade I (Fig. 1) and those which have trichodermial pilael covering but having two kinds of pileal elements separated in other clade (Fig. 1 clade II). Pakistani collection lies in clade III in which *L. acutesquamosa* isolates and closely related taxa grouped under a significant bootstrapping value.

Comments

L. acutesquamosa is characterized by pileus having light brown prominent scales which are thick in the centre, while fadded off towards margins, 2-spored basidia, ellipsoid to oblong, dextrinoid spores, calvate to capitate cystidia on the infertile margins of the lamellae. These characteristics are very basic to the *Lepiota* members of section *Echinatae* Fayod in which this species has been treated (Zelený, 2006). Shibata (1992) reported this species from Pakistan without giving any technical description and no herbarium specimen is available for reference. In phylogenetic analysis during this study, *Lepiota* sequences belonging to different sections have been included and they were separated in three clades. Clade I (Fig. 2) has memebers of section *Ovisporae* (J.E. Lange) Kühner in which all members have ellipsoid spores and trichodermial pileal covering. Clade II (Fig. 2) accommodates the members of section *Lepiota* Singer, this clade has trichodermial pileal covering, while pileal

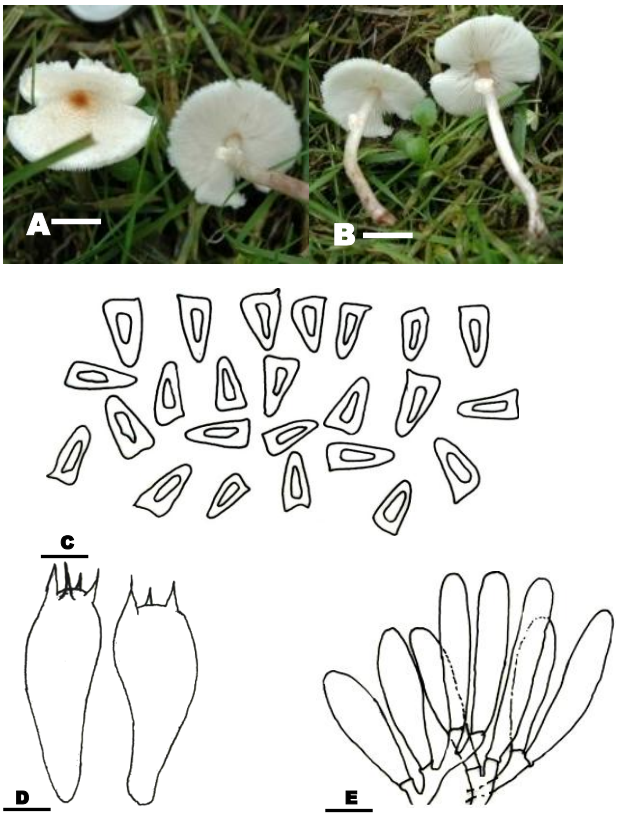

Fig. 3A-E: *Lepiota cristata* (A) Basidiomata (B) Lamellae. (C) Basidiospores (D) Basidia (E) Chielocystidia. Bar = A–B = 1.0 cm; C=2.5 µm; D= 6.0 µm; E= 11.0 µm

elements are of two types. Clade III has species, which have ellipsoid to oblong spore and hyminoform pileal covering which means that it consists of interwoven hyphae. *L. acutesquamosa* lies in this clade (Fig. 2, Clade III) with its own sequences, sequences of *L. aspera* (Pers.: Fr.) and sequence of *L. hystrix* F.H. Møller and J.E. Lange. Our phylogenetic results are also in accordace with Kropp *et al.* (2012) in which *L. aspera* and *L. hystrix* formed sister clades. It is noted that *L. hystrix* formed sister clade to sequences of *L. aspera* and *L. acutesquamosa* while both these species formed a single clade in which there is no separation of these species into sub clades. It means that *L. aspera* and *L. acutesquamosa* are sequences of same species or both are varieties of single species or there is conspecific relationship between them. Kuo (2007) and Vellinga (2004) treated both these species as synonym on morphological basis also.

Taxonomy

***L. cristata*:** (Bolton: Fr.) P. Kumm., Fu¨hrer in die Pilzkunde: 137. 1871. (Fig. 3 A-E).

Pileus 2.1 cm wide, fleshy, campanulate when young to broadly plano-convex when mature, umbonate, central umbo, orange-brown to pinkish brown, fibrillose; fibrils orange brown, fine, stretches towards margins, uniformly distributed over white ground around orange brown umbo; context white, soft, slightly thick at disc, margins dentate and fragile; remains unchanging on bruising or cut. Lamellae free, white, crowded, thin, edges entire, lamellulae absent. Stipe 3.8 × 0.5 cm, central, cylindrical, equal, dry, smooth to slightly striated, yellowish brown above the annulus with brown warts and white below annulus, which is membranous, non-persistent, white to light brown. Odor and taste not recorded.

Basidiospores 5.5–7.0 × 3–4.5µm; avl × avw = 6.5×4 Q=1.6, smooth, strongly dextrinoid, dark brown in Melzer‘s reagent, distinctively shaped like a wedge or a bullet. Basidia 22–30×10–12 µm, 4–spored, clavate to broadly clavate, thin walled, hyaline to pale yellow in 5% KOH. Pleurocystidia absent. Cheilocystidia 22–45×10–15 µm, hyaline to pale yellow in 5% KOH, thin walled, broadly clavate, clustered on margins of the lamellae.

Material Examined

Pakistan, Khyber Pakhtunkhwa, Himalayan Moist Temperate Forests, Khanspur (34.0169° N, 73.4167° E), at 2250 m a.s.l., solitary, on moist ground under *Abies pindrow*, 23 August 2010, Abdul Razaq (K-41) LAH.No.230841.’ GenBank Accsession # HF542115.

Molecular characterization and phylogenetic analysis of *L. cristata*

The variable internal transcibed spacers (ITS) conserved 5.8S regiongs of rDNA when amplified using fungal universal primers pairs (ITS1 and ITS4) generated fragments of approximately 650 bp. Initial BLAST analysis of nucleotide sequences revealed that Pakistani collection matches maximum with *L. cristata* (GenBank accession # EU081956, U85327, AJ 237628 etc.). Sequences from GenBank database were retrieved for further alignment and phylogenetic analysis. In alignment, it was noted that ITS region of our sequence is 50 bp shorter to the GenBank sequences. Therefore, extra part of other sequences was removed from the analysis. Phylogenetic analysis based on all these sequences showed its clustering with *L. cristata*. Maximum Likelihood (ML) method was based on the Jukes-Cantor model of nrITS sequences using Nearest-Neighbor-Interchange (NNI) as ML heuristic search method. In phylogenetic tree three clades were formed, all Lepiotaceous fungi having hymniform pileus covering clustered in clade I and those which have trichoderm separated in other two clades (clade IIand III). Pakistani collection lies in clade I.I in which all isolates have bullet shaped spores under a significant clustering value.

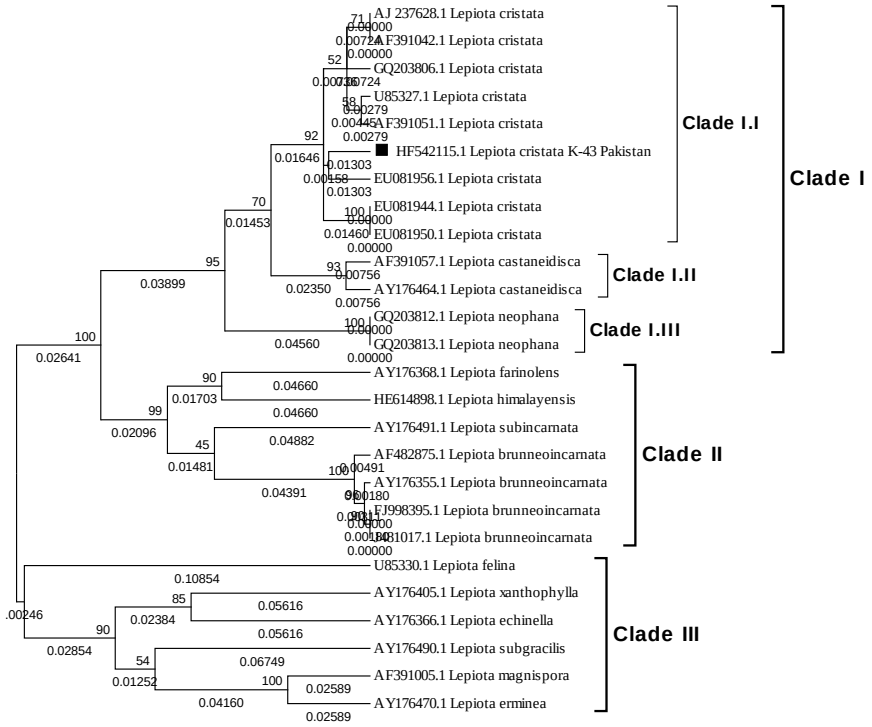

Fig. 4: Phylogenetic relationship of *Lepiota cristata* (■) with other members of *Lepiota* based on Maximum Likelihood method inferred from nrITS sequences. Bootstrap values based on 100 replicates are shown above the branches and below 50 are not shown. Branch length is shown below each branch. The topology of the tree based on Maximum Parsimony is same for *Lepiota cristata*. The analysis involved 26 sequences. All positions containing gaps and missing data were eliminated. There were a total of 492 positions in the final dataset

Comments

L. cristata is widely distributed ecologically and geographically. It is described from North America (Velling, 2001), from Asian contries like China (Liang *et al*., 2009), Iran (Asef and Muradov, 2012) etc. It is commonly collected from Himalayan moist temperate forests of Pakistan and described first time from Pakistan using morpho-anatomical and molecular characters. This species having dextrinoid, bullet shaped spores differs from all other species of genus *Lepiota*. Morpho-anatomically, this species shows best matches with description of Vellinga (2001) but with some variations in dimensions of spores, basidia and chielocystidia. This variation is also evident from its molecular analysis. It lies in *cristata* clade but showing intraspecific variation by forming terminal branch (Fig. 4, clade I.I). In other word it lies among the members of *Lepiota* section *Lilaceae* Bon. Because of this intraspecific variation regardless of geographical isolation, *L. cristata* is known as species complex from temperate areas of northern hemisphere (Vellinga, 2001; Liang *et al*., 2009). This genetic variation cannot be correlated clearly with morphological characters (Vellinga, 2001). Vellinga (2001) tried to explain one possible reason for this genetic variation is its introduction to manmade environment. But Pakistani collection is purely from natural habitat which also depicts intraspecific variation. Careful examination and sequence analysis of all possible collections of *L. cristata* may enable to identify them as separate species as Vellinga (2001) treated *L. castaneidisca* Murill as separate species from *L. cristata* on molecular basis. Liang *et al*. (2009) worked on 47 isolates of *L. cristata* using three loci (ITS of rDNA, IGS and mtSSU rRNA) to resolve this species complex. But the genetic variation and molecular divergence of these three loci is too high that they could not classify this complex into different species. Other possible suggestion to the solution of the problem is to search for other conserved region of DNA for classification of this species complex.

Acknowledgments

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