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MOLECULAR SYSTEMATICS AND EVOLUTIONARY RELATIONSHIPS OF SOME INLAND GILLED BASIDIOMYCETES FROM THE HIMALAYAN MOIST TEMPERATE FORESTS OF PAKISTAN BASED ON rDNA MARKER

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Abstract

Molecular systematics based on molecular markers has revolutionized traditional taxonomy because of its comprehensive identification, precise methodology and reliable evolutionary affinities. In this study, the phylogeographical history data based on DNA analysis of eleven (11) basidiomycetous fungal species belonging to four different genera of four families from Pakistani Himalayan forests has been presented. In the phylogenetic analysis based on ITS-rDNA marker, five species of *Lepiota* (Agaricaceae), *L. acutesquamosa*, *L. cristata*, *L. brunneoincarnata L. subincarnata* and *L. himalayensis*, two species of *Hebeloma* (Hymenogasteraceae), *H. mesophaeum* and *H. theobrominum* showed genetic similarity and evolutionary affinities with European species while two (2) *Inocybe* (Inocybaceae), *Inocybe* sp. SR19 and *I. cf. rimosa* and two (2) species of *Gymnopus* (Marasmiaceae), *Gymnopus* sp. 1and *Gymnopus* sp. 2 showed more symplesiomorphic characters with western and Australian species. Close affinities of Pakistani species (endemic and non-endemic) with European and American species can be related with common evolutionary history in Eurasia and African continents. The sympatric and allopatric isolations due to continental drift and climate change, generated genetic differences among local mycoflora from the western hemisphere and therefore the Pakistani species mostly showed close affinities with western species as both have common origin.

Key words: Agaricales, ITS, Phylogeny, Evolution.

Introduction

Pakistani forests, especially the forests of northern Pakistan, are regarded as a hot spot of biodiversity. Nearly 600 fungal species have been recorded from Pakistani forests khan 1962; Tulloss *et al*., 2001; Sultana *et al*., 2011; Razaq *et al*., 2012a, 2018, Ullah *et al*., 2018). Almost all species bear European or North American names (Ahmad *et al*., 1997). Niazi (2008) compiled a report of forty ectomycorrhizal species using morphological characters and most of his taxa carried previous names. Razaq *et al*., (2012b) described and identified first species using a phylogenetic species concept (ITS-rDNA marker) along with morphological characters and this study resulted in the increase of endemic taxa in Pakistani mycota. Razaq (2013) reported 85 species of gilled fungi based on ITS-rDNA and all were subjected to their evolutionary context which proved that almost 50% of the species were endemic. After that report, the fungal diversity picture of Pakistani forests was totally changed from what we had from our previous workers who reported nearly 100% European or North American species from the Pakistani territory (Sultana *et al*., 2011). The work of Razaq *et al*., (2013) signified the need for molecular systematics for the description of the Pakistani mycota using ITS-rDNA, a good marker for species level resolution (Riviere *et al*., 2007).

In the current work, we present the phylogenetic analysis of members of 4 different families of gilled basidiomycetes based on ITS-rDNA sequences. The phylogenetic resemblances and taxonomic affinities of 11 species of 4 different genera of these families were also compared with European and North American species. The purpose of this paper is to introduce the need for, and significance of, molecular markers for taxonomy and classification of fungi to see the true picture of Pakistani biodiversity and to estimate the close relationship of the local mycota with European, American and some Australian species.

Material and Methods

Site description: The Himalayan moist temperate forests lie in northern areas of Pakistan between the dry temperate and sub alpine zone along the Himalaya range with an elevation of 600m to 8100m (Khan, 2006). These consist of Muree-Hazara hills, upper Dir, Swat and some areas of Gilgit-Baltistan. These forests are made up of conifers mixed with deciduous trees (Champion *et al*., 1968; Saima *et al*., 2010). The annual rainfall in these forests ranges from 650-1200mm and experience humidity up to 57% and temperature 10 to 25^{o}C on rainy days (Anon., 2005-2014) (Fig. 1).

Morphological characterization: Basidiomata were collected from the sampling sites from 2010-2011. Morphological characters, smell, taste and colour were noted and photographed in the field (Fig. 2). After labeling and packing, specimens were brought to the laboratory for microscopic examination.

DNA Extraction and ITS amplification: Dried tissue from gill part of each specimen was macerated in ice-cooled pestle mortar adding liquid nitrogen. The DNA was extracted using 2%CTAB buffer protocol (Porebski *et al*., 1997). ITS-rDNA region of all specimens amplified in 20 µl reaction volume using ITS1F and ITS4 following the Brun's lab protocol (White *et al*., 1990; Gardes & Bruns, 1993). The PCR product after purification was directly sequenced by using both primers (Macrogen, Korea).

Fig. 1. Map of Pakistan showing sampling sites.

Sequence analysis and phylogeny: The consensus sequences of newly generated sequences were produced after manual editing by using the BioEdit ver. 7.0.9.0 (Tom Hall, Ibis Biosciences, Carlsbad, California). Sequences used for alignment and phylogenetic analysis were retrieved from GenBank databse. The BLAST (Basic Local Alignment Search Tool) analysis helped in sorting out most suitable and relevant sequences with their origin. The newly generated and retrieved sequences were aligned using the ClustalW program built in MEGA ver.6. (Molecular Evolutionary Genetics Analysis) tool and the same software has been also used for construction of phylogeny (Tamura *et al*., 2011). All sequences were trimmed with the conserved motifs to produce complete ITS sequences (Dentinger *et al*., 2011). Phylogeny was developed following Razaq *et al*., (2012b).

Results

Molecular description of internal transcribed spacer (ITS) of rDNA: The PCR products of all 11 Pakistani species produced fragments of approximately 600–750bp when Internal Transcribed Spacers (ITS) region of rDNA was amplified with universal primers. DNA extraction and PCR amplification of old collections was difficult compared to relatively fresh specimens. Consensus sequences of ribosomal DNA were queried in the GenBank database using BLAST. All sequences of local fungi are presented with 100 closely related sequences from Europe and North America available in the database. Five species of *Lepiota* (Agaricaceae) were subjected to a BLAST search which showed close matches with Asian, European and North American isolates of species with the same name. These 5 species, *Lepiota acutesquamosa*, *L. brunneoincarnata*, *L. cristata*, *L. subincarnata* and *L. himalayensis* are listed in Table 1 with their matches. Two species sequences of genus *Hebeloma* of the Hymenogastraceae family were compared in GenBank which gave perfect matches with their European isotypes (Table 1). The BLAST match results of two species of *Inocybe* (Inocybaceae) showed that these species had genetic similarities with their close European species (Table 1) but also that these are endemic to Pakistani forests. *Gymnopus*, a genus of Marasmiaceae, has 2 species that when matched with those available in GenBank, had more than 3% difference. The species of *Gymnopus* reported in this work showed close matches with North American and Australian species but their DNA was sufficiently different indicating that these Pakistani species were endemic to Pakistan on molecular basis. The four best BLAST matches of all species used in this study with voucher, accession number and origin are presented in Table1. Furthermore, the evolutionary histories and close relationships of the Pakistani species were determined by phylogenetic analysis. From a phylogenetic perspective, the closely related species clustered with their close relatives.

Phylogenetic analysis and taxonomic affinities: The phylogenetic analysis of members of 4 families of gilled fungi was carried out using the maximum likelihood method. Species from Agaricaceae, Hymenogastraceae and Inocybaceae were analyzed in a single dataset (Fig. 3) while those from Marasmiaceae was analyzed separately (Fig. 4). The phylogenetic tree (Fig. 3) comprised four major clades according to the family members; species of one family lie in one clade with the exception of Inocybaceae whose species were further segregated into two further sub-clades (Fig. 3; Inocybe I & Inocybe II).

Lepiota species (Agaricaeae) were further recovered in four sub-clades. The first sub-clade had very bold branches in the uppermost part of the tree had species with spores that are ellipsoidal to oblong and a pileus covering of a trichoderm nature with one type of pileal elements, the longer type. Three species from Pakistan, *L. brunneoincarnata*, *L. subincarnata* and *L. himalayensis* clustered in this clade. All these species clustered with their known European counterparts with significance statistical values (98% bootstrap).

The second sub-clade, with less bold branches, was characterized by having spurred spores and a hymeniform pileal covering. *L. cristata* from Pakistan lies in this sub-clade forming a separate branch from the rest of its isolates but this species showed 98% base similarity in BLAST analysis with *L. cristata* from China and the Netherlands. This species is noteworthy for its intraspecific variation in the ITS-rDNA region (Liang *et al*., 2009). The third sub-clade had penguin-shaped spores and a trichoderm pileus covering with two types of pileal elements, longer ones and shorter basal ones. Another closely related sub-clade, with only bold branches, contained the species which had larger basidiomata, heavily dextrinoid larger spores with a suprahillar depression and a broad germ pore. One Pakistani species, *Lepiota acutesquamosa*, was recovered with its counterparts and all the species present in this sub-clade are incorrectly named or synonyms of validly described species. Therefore, there is no reason to consider the Pakistani species as endemic. The *Lepiota* species have very close evolutionary affinities with European species. Two species of *Hebeloma* (*H. theobrominum* and *H. mesophaeum* were recovered with their respective sequences from European countries.

Fig. 2. 1. *Lepiota acutesquamosa*, 2. *Lepiota brunneoincarnata*, 3 & 6. *Lepiota cristata*, 4. *Lepiota himalayensis*, 5. *Lepiota subincarnata*, 7. *Inocybe* sp.19, 8. *Inocybe cf. rimosa*, 9. *Hebeloma theobrominum*, 10. *Hebeloma mesophaeum*, 11. *Gymnopus* sp.1, 12. *Gymnopus* sp. 2.

Table 1. Pakistani species of gilled fungi showing collection information, nucleotide sequences similarities with European, North American, Asian species according to evolutionary relatedness.

Pakistani species	Reference sequence	% Age identity
Lepiota acutesquamosa	*L. acutesquamosa* (MCVE:7527) FJ998400 (Italy)	99
Accession # (HE974767.)	*Echinoderma asperum* (UBCF20356) KC581336 (Canada)	99
Abies pindrow, 13/8/2010, Himalayan Moist Temperate, Khanspur, 2250 m a.s.l, Khyber	*L. aff. aspera* (HKAS7645) EU681782 (China)	99
Pakhtunkhwa (KPK), Pakistan (PK).	*L. cf. aspera* (MFLU 09-0181) JN224820 (Thailand)	99
Lepiota brunneoincarnata	*L. brunneoincarnata* (xsd08106) FJ481017 (China)	99
Accession # (HE863668.1)	*L. brunneoincarnata* (HMAS 63488) EU416302 (China)	99
Ground floor near mixed vegetation, 13/8/2010, Ayubia -Khanspur, at 2400-2580 m a. s. l.,	*L. brunneoincarnata* (MCVE:1402) FJ998395 (Italy)	99
KPK, PK.	*L. brunneoincarnata* (7-X-1998) AY176355 (Netherlands)	99
Lepiota cristata	*L. cristata* (22-IX-1993) AF391042 (Netherlands)	98
Accession # (HF542115.1)	*L. cristata* (HKAS7547) EU081958 (China)	98
Abies pindrow, 23/8/2010, 27/07/2012 Himalayan Moist Temperate, Khanspur, 2250 m a.s.l,	*L. cristata* (HKAS49367) EU081960 (China)	98
(KPK), (PK).	*L. cristata* (HKAS46345) EU081943 (China)	98
Lepiota himalayensis	*L. revelata* (HKAS50115) GU199359 (China)	91
Accession # (HE614898.1)	*Lepiota* sp. Vellinga (9-VIII-2000) AY176478 (USA)	86
Ground floor 13/8/2010, Himalayan Moist Temperate, Ayubia -Khanspur, at 2400-2580 m a.	*L. tomentella* (H.A.Huijser) EF080868 (Netherlands)	84
s. l, (KPK), (PK).	*L. brunneoincarnata* (7-X-1998) AY176355 (Netherlands)	84
Lepiota subincarnata	*Galeropsis desertorum* (PR 154181) AY194534 (USA)	99
Accession # (HE863669.1)	*L. subincarnata* (ecv3929 (UC)) KC556779 (USA)	99
Abies pindrow, 13/8/2010, Himalayan Moist Temperate, Khanspur, 2250 m a.s.l, (KPK),	*L. subincarnata* (19-IX-1998) AY176491 (Netherlands)	99
(PK).	*Lepiota* sp. MFLU 09-0192 JN224828 (Thailand)	91
Hebeloma theobrominum	*H. theobrominum* (ROHB925LQ) NR120177 (Netherlands)	99
Accession # (HE649368.1)	*H. theobrominum* (GC06 10 08 01) JQ751214 France	99
Abies pindrow, 23/8/2010, Himalayan Moist Temperate, Ayubia -Khanspur, at 2400-2580 m	*H. theobrominum* FJ816623 (Belgium)	99
a. s. l, (KPK), (PK).	*H. theobrominum* FJ816621 (Denmark)	99
Hebeloma mesophaeum	*H. mesophaeum* (UU_3b_I) JQ724062 (Sweden)	100
Accession # (HE649369.1)	*H. mesophaeum* (IB2005309) EF644106 (Austria)	100
Thick coniferous vegetation, 06/07/2010, Himalayan Moist Temperate, Ayubia -Khanspur, at	*H. mesophaeum* EF451057 (Netherlands)	99
3300 m a. s. l, Fairy Meadows, Nanga Purbat, Gigit-Baltistan, (PK).	*Inocybe abjecta* (UBC F19514) HQ604751 (Netherlands)	99
Inocybe cf. rimosa	*Inocybe cf. sororia* (src60) DQ974802 (USA)	91
Accession # (Submitted, Awaiting)	*I. rimosa* (UBCF 19519)HQ604622 (Canada)	89
Abies pindrow, Pinus wallichiana, 23/8/2010, Himalayan Moist Temperate, Ayubia -	*I. bulbosissima* (EL88_06) FJ904159 (Sweden)	89
Khanspur, at 2400-2580 m a. s. l, (KPK), (PK).	*I. cf. fastigiata* (26_N2F2_1) HQ445610 (Sweden)	89
Inocybe sp 19	*I. nitidiuscula* (UCBSB73) HG796970 (Pakistan)	99
Accession # (Submitted, Awaiting)	*I. nitidiuscula* (UBC F19797) HQ604090 (Canada)	99
Thick coniferous vegetation, 06/07/2010, Himalayan Moist Temperate, Ayubia -Khanspur, at	*Inocybe* sp. SB78 HG796986 (Pakistan)	89
3300 m a. s. l, Fairy Meadows, Nanga Purbat, Gigit-Baltistan, (PK).	*Inocybe* sp. SB76 HG796984 (Pakistan)	89
Gymnopus sp. 1	*G. confluens* (FB7219) AF505773(USA)	78
Accession # (Submitted, Awaiting)	*G. confluens* (TENN61147) FJ596784 (USA)	78
Among dead logs of pine vegetation, 23/8/2010, Himalayan Moist Temperate, Khanspur,	*G. dryophilus* (TENN59500) AY256698 (USA)	78
2250 m a.s.l, (KPK), (PK).	*G. confluens* (TFB5824 DQ450053 (USA)	78
Gymnopus sp. 2	*G. readiae* (TFB7571)DQ450034(New Zealand)	95
Accession # (Submitted, Awaiting)	*G. readiae* (PDD:95844)HQ533036 (New Zealand)	94
Among dead logs of pine vegetation, 23/8/2010, Himalayan Moist Temperate, Khanspur,	*Gymnopus* sp. H21 JX029935 (Czech Republic)	93
2250 m a.s.l, (KPK), (PK).	*G. confluens* (TFB10977) DQ450047(Russia)	93

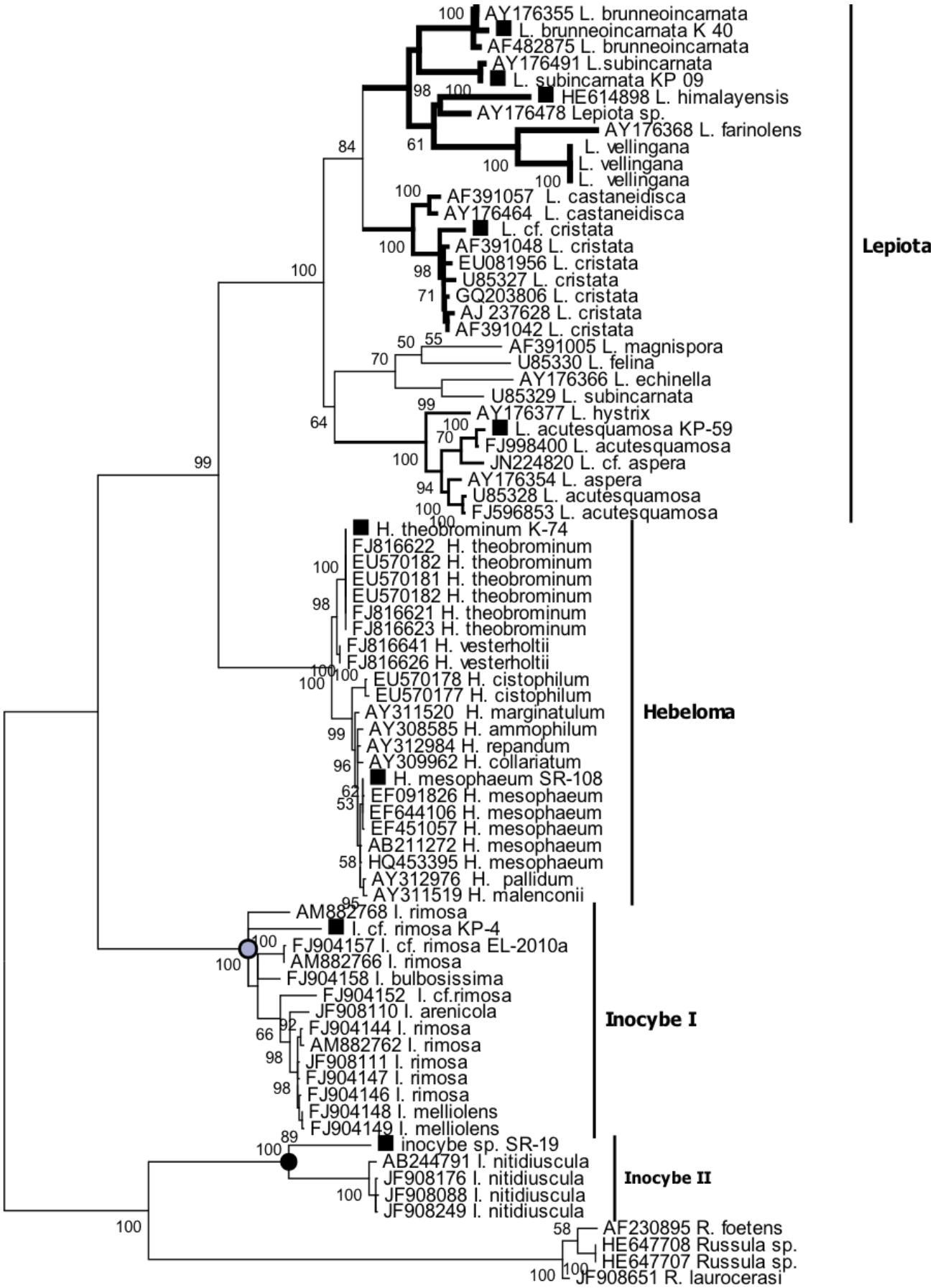

Fig. 3. Phylogenetic relationship of Pakistani species of *Lepiota*, *Hebeloma* and *Inocybe* with their allies inferred by using the maximum likelihood analysis based on the Tamura-Nei model. The analysis involved 79 nucleotide sequences. Species collected from Pakistan have been labeled with a box (■).

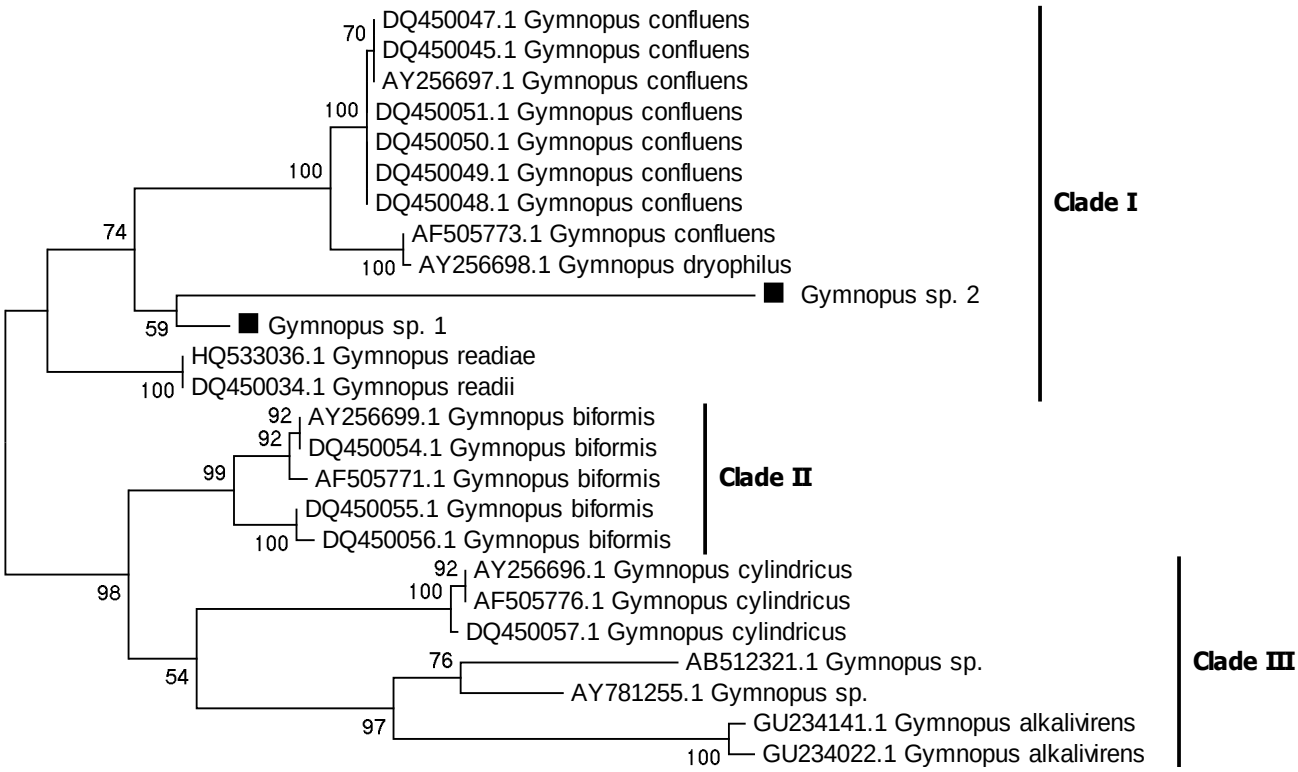

Fig. 4. Phylogenetic analysis of *Gymnopus* species collected from Pakistan based on nrITS-rDNA regions. This tree used the maximum likelihood method. The bootstrap value is given on the node of each clade. Species collected from Pakistan have been labeled with a box (■).

In the phylogenetic analysis of *Hebeloma*, the species recovered in two sub-clades (Fig. 3) and each Pakistani species was located in each sub-clade among different European and North American collections. In one sub-clade, only the species, *H. theobrominum* of section *Theobromina* Vesterholt. Pakistani collection was recovered with European collections of *H. theobrominum* Quadr. In the second sub-clade, all the sequences of *H. mesophaeum* (Pers.) Quél. were present, also containing the Pakistani collection.

In the present phylogenetic analysis, Pakistani *Inocybe* (Inocybaceae) was recovered in different clades. The Pakistani collections belong to two different clades; one, *I.* cf. *rimosa*, has transparent and thin-walled cystidia and smooth spores while the other species, *Inocybe* sp.19 in sub-clade II has thick-walled cystidia or metuloids and smooth spores. Both these sub-clades, Inocybe I and Inocybe II had split into two separate clades; the former showed affinities with the euagarics while the latter showed a close relationship with non-gilled fungi. In the phylogenetic analysis, some species of *Russula*, (Russulales), were used to root the tree which formed a sister clade with the Inocybe II clade. The Pakistani *I.* cf. *rimosa* clustered with *I. rimosa* (Bull.) P. Kumm. sequences and other closely related species but the clustering pattern showed that the Pakistani species was a more closely related to *I. rimosa* rather than conspecific with it. Actually the European and Asian collections of *I. rimosa* in our phylogenetic tree show a species complex which needs to be re-evaluated to ascertain the status of all sequences. *Inocybe* sp. 19 formed a sister clade with sequences of *I. nitidiuscula* (Britzelm.) indicating its unique identity and may be treated as a new species.

The phylogenetic analysis of the *Gymnopus* (Pers.) Gray, formed three major clades in its maximum likelihood phylogenetic tree, clade I, clade II and clade III (Fig. 3). All local collections were recovered among the members of clade I along the close collections and species from the world forming a sister clade to *G. dryophilus* and *G. confluens*. *Gymnopus* sp. 1 and *Gymnopus* sp. 2 occupied separate positons showing their distinct identities.

Discussion

The identification and naming of gilled basidiomycetes is traditionally based on phenotypical characteristics, however, since 1988, part of rDNA sequences, e.g., non-coding (ITS regions) and coding (LSU& LSS) parts of the ribosomal DNA, have been extensively used as essential taxonomic and phylogenetic tools (Hibbett *et al*., 2007). LSU (Large Subunit) and SSU (Small Subunit) are good phylogenetic markers for higher level taxonomy but ITS regions are best for species level identification and systematics (Nilsson *et al*., 2006; Osmundson *et al*., 2013; Razaq *et al*., 2014). Vellinga (2003) studied the phylogenetic relationships of *Lepiota* based on ribosomal DNA sequences from both the internal transcribed spacer (ITS) region and the large subunit (nLSU) of nuclear ribosomal DNA to shed new light on the taxonomy and biogeography of European species. The *Lepiota* species were grouped into three major clades: the first had a trichoderm pileal covering with longer elements, the second had a hymeniform pileus covering and the third had a cutis pileus covering.

Specimens with longer pileal hyphal elements and mixed longer and shorter pileal hyphal elements were also separated on a molecular basis; previously they were treated together (Vellinga, 2001). In our present study, the Pakistani species of *Lepiota* also lie in the same clades of Vellinga (2003). Section-based morpho-anatomical details of endemic species are similar to European species which may possibly lead to misidentification at species rank but molecular characters of the ITS region of rDNA give a more comprehensive taxonomic resolution at species level. In our results from Pakistan, three *Lepiota* species have longer elements in their pileal coverings, one native to Pakistan, *L. himalayensis* while the other two represent the European species, *L. brunneoincarnata* and *L. subincarnata*. Previously, the Pakistani *L. brunneoincarnata* and *L. subincarnata* have been treated as members of *Lepiota* sect. *Ovisporae* (having longer and shorter mixed trichodermal elements) (Ahmed *et al*., 1997, Sultana *et al*., 2011), and the molecular analysis of Razaq *et al*., (2013) based on rDNA also placed them with the *Ovisporae* clade of Vellinga (2001). On morphological characterization only, the identification of *L. himalayensis* as a new species was not possible as this species, macro and microscopic characters, closely resembled with the European *L. brunneoincarnata*. However, the use of molecular markers along with morpho-anatomical characterizations enabled Razaq *et al*., (2012) to confirm *L. himalayensis* as a new species. The Pakistani *Lepiota* show a close relationship with European and North American species on a rDNA data basis for two possible reasons: 1. this group originated in Pangean time and later on became distributed with separating land mass or 2. crossed the European boundaries via spore dispersal to the Indian plate. Those spores from Europe which landed on sites that had environmental conditions closely similar to their original environment survived and flourished with little phenotypic changes and the remainder which could not find suitable environmental conditions and substrates vanished. The climate of Northern Pakistan is also comparable with that of Europe and the movement of material from one place to another within the Northern Hemisphere is acceptable. To date, the *Lepiota* specimens reported from Pakistan lie in the three clades of European sequences (Razaq *et al*., (2012a, 2013b), Nawaz *et al*., 2013, Qasim *et al*., (2015, 2016).

The family Inocybaceae, whose members have been studied in this work, is also well represented in Pakistani forests. In our molecular characterization, two *Inocybe* species: *Inocybe cf. rimosa* and *Inocybe* sp. 19 showed their molecular resemblance with *I. rimosa* and *I. nitidiuscula* respectively (Table 1). The *Inocybe* clade recovered into two sub-clades, Inocybe I and Inocybe II and the latter showed distinctions by forming a sister clade with the out-group *Russula* species. Both *Russula* and metuloidal *Inocybe* have gills or lamellae but the former has already been excluded from euagarics by Hibbett *et al*., (2007) and extensive ITS data of this *Inocybe* group is needed to clarify this result. Pakistani *I.*cf. *rimosa* has a more yellow coloration which makes this species closer to *I. fastigiata* than *I. rimosa*. Many closely related species were clustered together in the *I. rimosa* clade which led to a species complex situation (Wilson & Desjardin, 2005). The two *Hebeloma* species were recovered in their respective clade (Fig. 3). Eberhardt & Beker (2010) also described a new species of *Hebeloma*, closely related to the Pakistani species, using ITS-rDNA markers.

In the phylogenetic analysis of *Gymnopus*, two Pakistani species, *Gymnopus* sp.1 and *Gymnopus* sp.2, are situated on two branches of the same clade, clade I. These two species are also phenotypically different from each other for example in the texture, shape and colour of basidiocarps. The Pakistani collection, *Gymnopus* sp. 2 shows affinity with Australian ones as both may have evolved from a common ancestor while the Indo-Australian plate separated from the African plate.

Molecular data not only strengthen the taxonomy of species but also facilitate the prediction of the evolutionary history. For example, the data predict that Pakistani *Lepiota* species with longer pileal hyphal elements co-evolved with those of European ones with the same characters under the same environment and time. Later on, geographic distribution and plate tectonics have modified some of the characters and new species have evolved. The evolution could possibly be correlated with Pangean time or its early dissociations. Bruns *et al*., (1998) also estimated the divergence time and evolution of fungi before the Cretaceous in the Permian and Jurassic and this is also the time when land plants evolved. Those species which evolved after the creation of the Himalayas are native to Pakistan while the older ones represent the European and North American ones which might have moved with the Indian plate. Generally, it has been observed that the Pakistani species from Northern Pakistan (which experience low temperature and heavy rain throughout the year) show a close resemblance to European species either with morphological or molecular data (Razaq *et al*., 2013). This is due to either similar environmental conditions or the fact that the ancestors of the Pakistani species lie in European territories. Among Asian countries, the Pakistani mycoflora matches with the Indian and the Chinese. Both these countries share the Himalayas but greatest share of Asian sequence data in GenBank is from China (Myer, 2000).

Conclusion

The intraspecific conservation and interspecific genetic variation, high copy number and availability of huge accession numbers in GenBank of ITS regions of ribosomal DNA of basidiomycetes make it a reliable tool for fungal taxonomy and systematics. Phylogenetic analysis also gives a reliable clue to the novel and cosmopolitan nature of local species and their relationship with mycoflora of different geographic regions. Using molecular data, five out of eleven species showed less than 97% base similarity, and one already has been described (*L. himalayensis*). The evolutionary relationships of most of these Pakistani species belonging to four families showed close affinities with the species of the Western Hemisphere. In this work it has been noted that the ratio of European isotypes is higher in Pakistan

compared to the rest of the world. This study also indicated that although the Pakistani species have roots in the western side of the Northern Hemisphere, the level of endemism in the local mycoflora is considerable. The close affinity of one of our species (*Gymnopus* sp. 2) with Australian collections is indicative of evolution on a moving land mass (Indo-Australian plate).

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