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MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF MACRO FUNGI FROM DISTRICT ASTORE, GILGIT-BALTISTAN, PAKISTAN

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Abstract

Present study is an attempt to estimate the genetic diversity of important macro fungi from district Astore using DNA based markers. The Province Gilgit-Baltistab represents a floristically rich area characterized by the moist and dry temperate forest region with rich macro fungal diversity. Duringthe field visits from March to August selected different localities of Astore were visited and collection was doneand subsequently identified macro fungal species on the basis of morphological and molecular characteristics viz., *Bjerkandra adusta, Fomes fomentarius, Rigidoporus ulmarius* and *Tremetes versicolor* were reported first time from Pakistan. Another ten species *viz., Lepiota sistrata, Krombholziella scabra, Suillus leutus, Inocybe agardhii, Cyathus olla, Phallus impedicus, Schizophylum commune, Coprinus domesticus, Morchella esculenta* and *M. conica* were reported first time from the study area. The fourteen fungal species were attended by eleven Randomly Amplified Polymorphic (RAPD) DNA primers. Bivariate data was used to estimate genetic diversity in different fourteen fungal accessions using unpaired group of arithmetic mean (UPGMA) procedure. High level genetic diversity ranging from 40% to 80% was observed in the fungal species. Samples of accessions were also grouped in to 7 clusters using dendrogram analysis.

Key words: Macro fungi, Genetic distance, Basidiomycetes, Ascomycetes, PCR, Phylogeny, RAPD.

Introduction

The Kingdom of fungi commonly known as mushrooms, bracket or shelf fungi, bread mold, yeast, puff ball, morels, smut and rust fungi. Macro fungi include mushrooms; toadstools, puffballs, bracket fungi, polypore's, and other fungi. In Asian countries, for long time a variety of macro fungi have been taken as mineral supplement and vitamins. Recent development in pharmacological studies focused on edible macro fungi due to their valuable activities with biological modification (Waseer *et al*., 2002). These have chemo preventive, chemotherapeutic, hypoglycemic effect from Gilgit-Baltistan. Estimation of genetic diversity is essential for the understanding and improvement of macro fungi in the region. Currently DNA based markers have been prepared which are infinite in number and which are not forced by environment and taken as improved marker system for detection of species. Molecular protocols have a substantial effect on systematic study of bacteria, fungi and Protista and other similar microbs (Bruns *et al*., 1991; Hillis *et al*., 1991; Bowman *et al*., 1992). Molecular procedures provide sample sensitivity to allow the finding of unevenness between organisms at the stage of a base change and so can be used to establish relations between very closely linked organisms (Schlick *et al*., 1994; Lanfranco *et al*., 1995). Various fungal species from mountain areas of Pakistan are also explored to western countries meant for medicinal uses especially as immune stimulants, antioxidants and anti-tumor agents (Pankaj *et al*., 2002). Variety of various procedures have been published for the segregation and expansion of DNA from lichens and fungi (Bruns *et al*., 1990; Lee & Taylor, 1990; Armeleo & Clerc, 1991, 1995; Grube *et al*., 1995; Crespo *et al*., 1997; Cubero *et al*., 1999; Grube, 2005). With the help of PCR extension with given primers the key barrier of DNA extraction of a sole species of fungi has overcome (Crespo *et al*., 1997). In the past morphological studies have been done by different mycologist from Gilgit-Baltistan, Pakistan, but the genetic diversity being the most important component for knowing diversification among different species of macro fungi has not been done from the District Astore.

Material and Methods

Different specimens of macro fungi were taken from multiple locations of District Astore, Gilgit-Baltistan (Fig. 2) the attributes of natural environment and the other pertinentparticulars were also documented. The samples were brought to Bio- Sciences Dept, KIU, Gilgit, and identification was done to species level using reference to taxonomic keys of Demoulin & Mirriott (1981), Surcek (1988), Buczacki (1989), Shibata (1992), Swann & Taylor (1993), Murakami (1993), Ahmad *et al*., (1997), Leelavathy & Ganesh (2000), Sultana *et al*., (2011) and Razaq *et al*., 2012. Synonymy of the species was checked at www.speciesfungorum.org. The specimens were dried at room temperature for future references.

DNA isolation: Meager chunk of DNA extraction process (minipreparation) of fourteen species of macro fungi were carried out at the Department of Biological Sciences, KIU by method described by Weining & Langridge (1991) and Kobayashi *et al*., (1998). The following steps were adapted for DNA isolation about 0.5 gm of fruiting body was crushed with a mortar pistil to make extra fine powder. 500 µl DNA extraction buffer was supplemented in each eppendorf tube and dissolved well. 500 µl of chloroform, isoamyl alcohol (in ratio of 24:1) was added and mixed vigorously till complete mixture was prepared. The tubes were kept in centrifuge chamber at 5000 rpm for 5 minutes. Aqueous (top) stage was shifted to a new

tube and also added 50 µl 3M sodium aetate (PH=4.8). Tubes were filled with isopropanol and mixed smoothly till the DNA is precipitated and then centrifuge again at 5000 rpm for 5 minutes to make the DNA pellet. Supernatant was put at side and DNA pill was dried in air for 10 minutes. Pill was then mixed in 50 µl TE buffer. Polymerase Chain Reactions (PCR) were carried out using four 10n base pair RAPD primers (obtained from Gene Link, Inc. 1052 NY, USA) for estimation of genetic diversity (calculated as genetic distance).

Agarose gel electrophoresis: The value and number of the DNA was checked on 1% agarose/TBE gel. Agarose powder (1.0 g) was mixed in 100 ml tris-borate (TBE) buffer. The blend was boiled in microwave oven, after heating 5 µl Ethidium Bromide was mixed in the gel. Gel was casted in a gel tray with comb. After settling, gel was placed in gel tank having TBE buffer. Five µl DNA from each samples were taken, mixed with 2 µl loading dye (Bromophenol Blue) and poured in the wells, after that the gel was run at 70 volts for one and half hour. Gel was seen under UV light using "UVitech" Gel Documentation System.

Polymerase chain reaction (PCR): RAPD primers (obtained from Gene Link, Inc, USA) were used to intensify genomic DNA isolated from 14 samples of macro fungi. Part of a characteristic PCR is the genomic DNA used as template 1µl, DNTPs (mixture 3.5 µl of DATP, DCTP, DGTP and DTTP), RAPD Primer 1µl, Taq Polymerase buffer 2.5µl, Taq DNA Polymerase 1.0 µl and double distilled de ionized autoclaved water 16µl. Reactions were performed in 25 µl volume. Thermo cycling conditions *viz;* de-naturation at 94°C, annealing at 34°C and extension at 72°C was carried out in thermo cycler. Sequence information of the decamer RAPD primers 1µl are used during present study and dendrogram constructed for macro-fungi accessions using 11 RAPD primers and DNA isolated from 14th samples shows in Table 1.

Thermo cycler situation applied during PCR

Step: Temp: Time:
1. 94ºC 4 min, 2.94ºC 1 min, 3.34ºC 1 min, 4. 72ºC 2 min, 5. Repeat Steps 2-4, 40 times, 6. 72ºC 10 min, 7. 70ºC Hold.

Primer name	% GC	Sequences	Molecular weight
GL Decamer I-07	60%	CAGCGACAAG	3117.04
GL Decamer I-09	60%	TGGAGAGCAG	3053.01
GL Decamer I-10	60%	ACAACGCGAG	3068.02
GL Decamer I-11	60%	ACATGCCGTG	2987.98
GL Decamer I-15	60%	TCATCCGAGG	2947.96
GL Decamer I-17	60%	GGTGGTGATG	3019
GL Decamer I-20	60%	AAAGTGCGGG	3019
GL Decamer J-04	70%	CCGAACACGG	3108.04
GL Decamer J-05	70%	CTCCATGGGG	2954.97
GL Decamer J-06	60%	TCGTTCCGCA	2954.97
GL Decamer J-14	70%	CACCCGGATG	2994.99

Result

During the present work, five species *viz., Bjerkandra adusta, Fomes fomentarius, Rigidoporus ulmarius, Tremetes versicolor* and *Lepiota sistrata* were reported new from Pakistan, using morphological, distributional and molecular characteristics while nine species *viz,. Krombholziella scabra, Suillus leutus, Inocybe agardhii, Cyathus olla, Phallus impedicus, Schizophylum commune, Coprinus domesticus, Morchella esculenta* and *M. conica* are first time reported from the District Astore.

Fig. 1A. *Bjerkandra adusta* (A-C), *Fomes fomentarius* (D-E), *Rigidoporus ulmarius* (F-G) and *Tremetes versicolor* (H-J).

1. *Bjerkandra adusta* (Willd. Ex Fr.) Karst. Soc. Faun.

Synonyms:
Polyporus adusta Willed.ex. Fr. *Bjerkandera adusta* (Wild) P. Karast. 1879.
Bjerkandera adustaf.adusta (Wild) P.Karst. 1879.
Bjerkandera adustaf.carpinea (Sowerby) Donk 1799
Bjerkandera adustaf.resupinata(Bourdot and Galzin) 1967.
Description of species: Fruit body velvety, bracket like. Flesh white fibrous and leathery partly bracket spores on lower side and black, edge initially paler, wavy Flesh whitish and fibrous and leathery. The fertile surface covered with tubes with pores is 4-5mm. **Spores**: Spores ellipsoid, smooth, 3.5-3x4-5.5µm. **Spore print**: White. **Season of Fruiting:** July –August. **Edibility:** Inedible, used for joint pain. **Habitat:** Usually in fused masses
Previous report from Pakistan: None

2. *Fomes fomentarius* **(**L.ex Fr) Kickx. *Crypt. Fland. Res*. 2:237 (1867).

Synonyms:
Polyporus fomentarrius L., Fr., Syst.Myc.1: 374.1821.
Fomes abiets P.karast.`882.
Fomes abrabramisianus (Murill) Murrill 1915
Fomes alni (Sorokin) Sacc.1895.
Description of species: Fruiting body13- 16cm long, club shaped, brown surface margin. Margin curved and light colored. Tubes arranged parallel. **Fig. 1A. Pores and tubes:** rounded and minute with thick wall which first white then brownish. **Spores:** 13-14x 4-5µm in diameter. **Spore print**: Pale. **Occurrence:** Harcho Nalla
Edibility: Inedible. **Habitat:** Solitary on dead wood
Previous report from Pakistan: None

3. *Rigidoporus ulmarius* Fr. Imazeki, Bull. *Meguro,* 57: 119 (1952).

Table 1. DNA isolated from 14th samples, Sequence of RAPD primers used.

Primer name	%GC	Sequences	Molecular weight
GL Decamer I-07	60%	CAGCGACAAG	3117.04
GL Decamer I-09	60%	TGGAGAGCAG	3053.01
GL Decamer I-10	60%	ACAACGCGAG	3068.02
GL Decamer I-11	60%	ACATGCCGTG	2987.98
GL Decamer I-15	60%	TCATCCGAGG	2947.96
GL Decamer I-17	60%	GGTGGTGATG	3019
GL Decamer I-20	60%	AAAGTGCGGG	3019
GL Decamer J-04	70%	CCGAACACGG	3108.04
GL Decamer J-05	70%	CTCCATGGGG	2954.97
GL Decamer J-06	60%	TCGTTCCGCA	2954.97
GL Decamer J-14	70%	CACCCGGATG	2994.99

Synonymy:
Rigidoporus Murril, Bull. Torrey bot.Club 32 (9): 478(1905).
Rigidoporus adnatus Corner 1987.
Rigidoporus dimiticus (Corner) T. Hatt. 2001.
Rigidoporus durus (Jungh) Imazeki 1952
Rigidoporus populinus (Schumach) Pouzar 1966
Description of species: Fruiting body flesh first whitish, hard fibrous, then extra hard woody. Fruit body irregular warty, concentric ridges margin thick, rounded. Variable shape with 8-11cm, perennial **Fig. 1A. Pores:** pore surface at first pink and then brownish. Pores 5-8mm, rounded, pointed.
Spore print: white. **Spores:** sphere-shaped, soft, 5-6x5µm in size, non-colored. **Season of fruiting:** July-August
Occurrence: Rama. **Habitat:** solitary or overlapping in small group on wood. **Edibility:** Inedible
Previous report from Pakistan: None

4. *Trametes versicolor* (L. ex Fr.) Pil. Vandendries and Brodie 1933, Bull. Acad.R. Belg.Sci., Ser., 5, 19: 4.

Synonymy:
Boletus versicolor., sp. Pl. 2: 1176 (1753).
Poria versicolor (L.) Scon., Flcarniol., Edn 2 (Wien) 2: 468, 592 (1772).
Trametes versicolorf. (Velen) Pilat 1939.And Popoff 1998.
Trametes verscicolor f. tucumanesis (Rajchenb) J. E Wrigh *Trametes versicolorf*. (L.) Lloyd 1921.
Description of species: upper surface 3-7cm wide 1-2cm thick, and the total length consisting 12-8cm, leathery bracket-like; surface velvet-like with concentric bands of brown-red-yellow-gray-blue colors **Fig. 1A. Pores:** surface first white and then cream. Pore 3-5mm pointed. **Spores:** Spores spherical 1.5-3x 5-6µm in size, non-colored.
Spore print: white. **Edibility:** Inedible. **Season of fruiting:** June-July
Habitat: densely overlapping groups, fruit body attached with tree trunk of broad-leave trees.
Occurrence: Mushkin forest.
Previous report from Pakistan: Non

5. *Lepiota sistrata* *Fr.syst. Myc. I, p. 24, Saccardo, syl. Fung, V.5.1988.p.50*

Synonyms:
Agaricus seminudus Lash, Linnaea 3:157(1828).
Lepiota seminude (Lash) p. kumn., Fuhr. (Zerbst): 136 (1871).
Description of species: Cap is2-5 cm; convex, growing to oval shaped or broadly convex, enclosed with a powderedgrainy dusting; developing reddish to pink areas; the margin not arranged. **Stipe:** equal; when fresh and young enclosed with finestuff like the cap; becoming nearly smooth; white when young, becoming reddish to pink from the base up; white and copious. Slender. **Spore print**: White. **Spores**: Spore ellipsoid, 3-5x 2-3µm in size. Cylindric to long-ellipsoid. **Season of fruiting**: July and August.**Occurrence**: Lashtang forest. **Edibility**: Inedible. **Habitat.** Usually foundin grasses. **Previous report from Pakistan:** None

6. *Krombholziella scabra* *Bull. Ex. Fr. R. Maire, Fungi, Cot,* Series Altera 1937: 46 (1937). *Krombholziella*

Synonyms:
Krombholziella oxydabilis singer, Revue Mycol., Paris 3: 189(1938).
Krombholzielaa chalybae (Singer) sutara, Ceska Mykol.36:81(1982).
Description of species: Cap is brown wide, 5-12cm. **Stipe:** white covered with scales and soon tough like wood. Flesh unchanged when cut. It has pleasant taste and smell. **Spores**: Spores club shaped smooth, 14-17x 5-6µm in size, and yellowish. **Season of fruiting:** June-July. **Edibilit**y: edible, tasty. **Habitat: s**olitaryor in small groups. **Occurrence**: Rama, District Astore. **Previous report from Pakistan**: Razaq *et al*., (2007).

7. *Suillus leuteus* L. Ex. Fr. S.F. Gray.

Synonyms:
Boletus luteus L., sp pl.2: 1177(1753).
Suillus luteus (L) Roussel, F. Calvados: 34 (1796)
Suillus grevillei (Klotzch) Singer
Description of species: Cap dome shapebut soon convex Cap 3.5cm wide. Peeling cuticals when fresh it glossy and slimy. Flesh yellowish, pleasant smell. **Stipe:** Stipe cylindrical sheath with leathery veil, yellow brown, greasy and lustrous when wet, fibrillose when dry. Flesh yellowish. **Spores:** Spores mass brown and ellipsoid, pale yellow, 8-10x 3-3.5µm in size. **Season of fruiting:** July-August. **Habitat**: On soil, along sides of canal. **Edibility:** Edible and containing pleasant test. **Occurrence:** Mushkin forest. **Previous report from Pakistan**: Razaq *et al*., (2007).

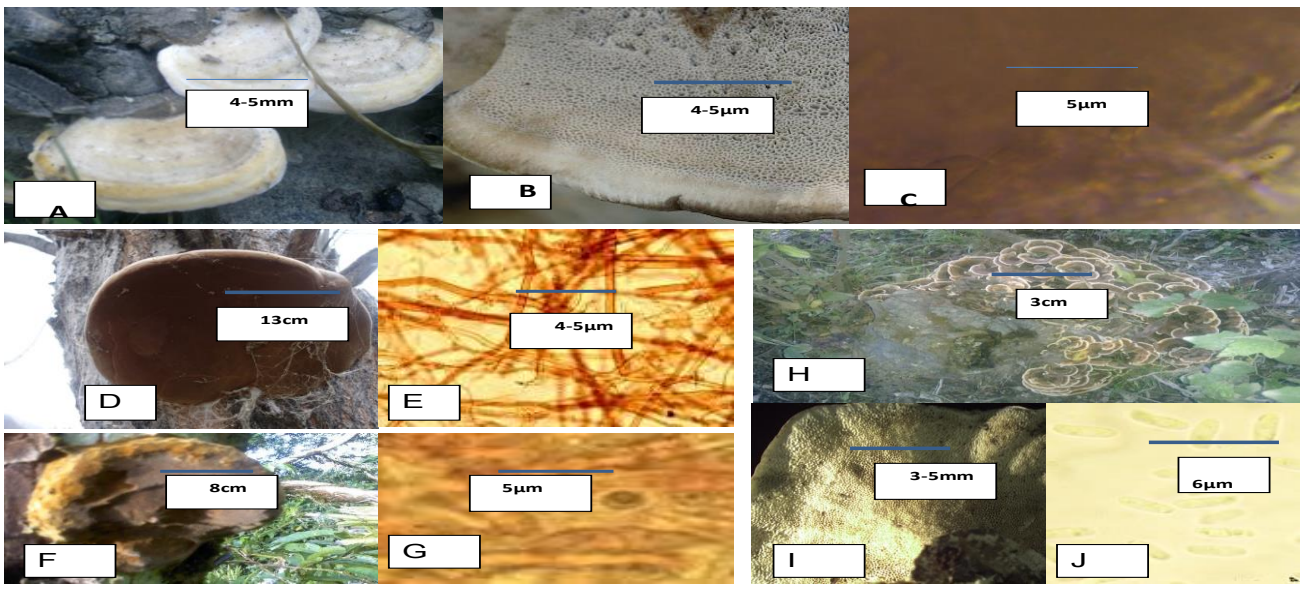

Fig. 1A. *Bjerkandra adusta* (A-C), *Fomes fomentarius* (D-E), *Rigidoporus ulmarius* (F-G) and *Tremetes versicolor* (H-J).

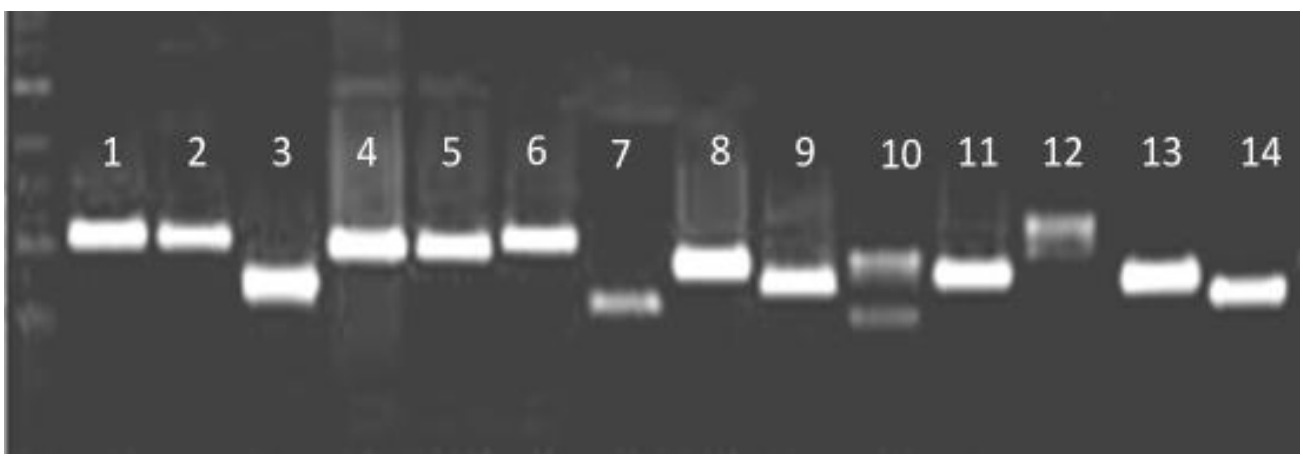

Fig. 1B. DNA isolated from 14 samples of macro fungi *viz., 1= Lepiota sistrata, 2= Krombhoziella scabra, 3= Suillus luteus, 4= Inocybe agardhii, 5= Cythus olla, 6= Phallus impedicus, 7= Bjerkandera adusta, 8= fomes fomentarious, 9= Rigidoporus ulmarius, 10= Trametes versicolor, 11= Schizophylum commune, 12= Coprinus domesticus, 13= Morchella esculents, 14= Morchella conica.*

8. *Inocybe agardii* (N. Land) *Orton as Inocybe* (*Inocybe*) in Trans. *Brit. Mycol. Soc.*, 43, p. 177, 196.

Synonyms:
Agaricus subgen. Clypeus Britzelm. Ber, naturhist. Augsburg 26: 137(188).
Agaricus trib.Inocybe Fr., *Syst.mycol*. (Lundae) 1:11, 254 (1821).
Description of species: Cap 4-9cm, slightly depressed at center, fibrous line when wet, reddish brown later. **Stipe:** 3-6cm, narrowing upward and rounded at base. **Gills:** olive brown. Smell obnoxious. Flesh pale yellowish buff. **Spores**: Spore print brown. Spores ellipsoid, smooth 9-12x 6-7µm in size. **Season of fruiting**: August to September. **Habitat:** solitary or in small trooping groups. On soil. **Occurrence:** Mushkin forest. **Edibility**: Inedible. **Previous report from Pakistan:** Razaq *et al*., (2007).

9. *Cyathus olla* (*Batsch*) *Pers.*199.

Synonyms:
Cyathus nitidus, Roth, *Neue Ann. Bot.*1*:* 1791.
Peziza olla Batsch, Elench. *fung*, Helle.127:1873.

Description of species: Fruit bodies are up to 1-1.5cm tall and 1cm wide. Outer surface, brownish, finely bushy, growingsoft and gray with passage of time. Interior, soft, gray to black. The peridioles are gray to darkish or blackish and 2-3.5mm wide. **Spores**: Spore ellipsoid, non-amyloid, 6-7x9µm in size. **Season of fruiting:** July-August. **Occurrence:** Rama forest. **Habitat**: In patches, in field of wheat. **Previous report from Pakistan:** It has been reported, Khagan vally, Muree, Kashimr, Ahmed (1972).

10. *Phallus impudicus*L inn.ex. *Pers., Syn.Meth. Fung.* 242. (1801).

Synonym:
Ithyphallus impudicus (L.) Fr.1886
Dictyophora duplicate var. Obliterata Malencon 1957
Description of species: upper surface up to 20 cm tall; head up to 4 cm wide; stalk up to 3 cm broad. Fruit body at first white than to pinkish. The egg is bonded to clear to pinkish mycelia strands (rhizomorphs). The outer region (peridium) of the egg scludes' and a empty, spongy,

having a head covered with a greasy, light green fetid spores. **Spores**: spores yellowish brown, ellipsoid, smooth, 3.5-4x 1.5-2µm in size. **Habitat**: Saprobes; individual or in groups on wood debris and stump. **Occurrence:** Rama forest. **Edibility**: uneatable although Miller states the eggs are edible. **Previous report from Pakistan:** It has been reported, Khagan vally, Muree, Kashimr, Ahmed (1972), Razaq *et al*., (2007).

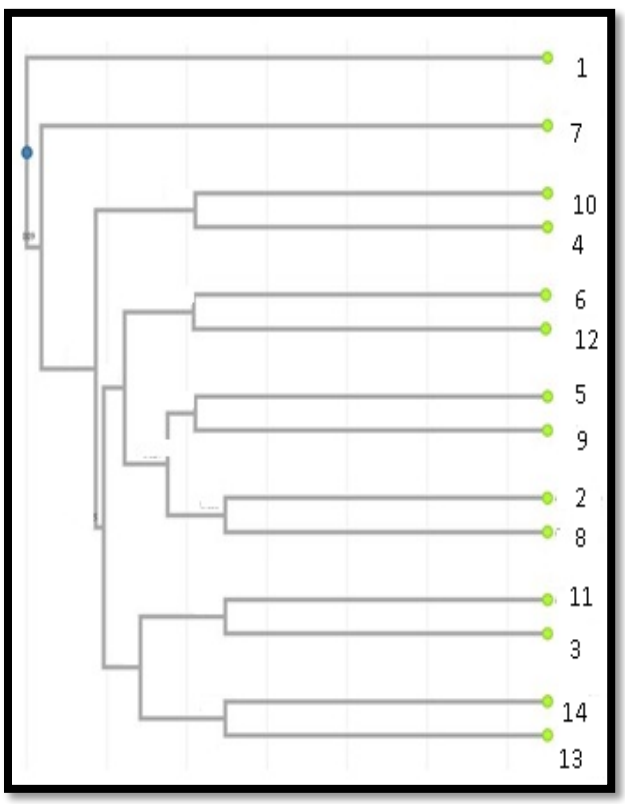

Fig. 1C. Dendrogram developed for macro-fungi accessions using 11 RAPD primers.

1= Lepiota sistrata, 2= Krombhoziella scabra, 3= Suillus luteus, 4= Inocybe agardhii, 5= Cythus olla, 6= Phallus impedicus, 7= Bjerkandera adusta, 8= fomes fomentarious, 9= Rigidoporus ulmarius, 10= Trametes versicolor, 11= Schizophylum commune, 12= Coprinus domesticus, 13= Morchella esculents, 14= Morchella conica

11. *Schizophylum commune* (*Fr. Syst. ycol.1:330*(1821).

Synonyms:
Schizophylum alneum (L) J. Schrot. 1889
Merulius communis (Fr.) Spirin and Zmitr. 2004
Description of species: Caps 1-4.5 cm wide, White to gray; densely hairy; fan to shell- shaped in lateral attachment, saucer shaped when centrally attached. Gills: folds are white to gray or pinkish- gray hairy; split lengthwise. Stipe: Absent or simply a narrow extension of the cap. **Spores**: spore 2.5-3x6-7µm in size, smooth elongated colorless. **Spore print**: White. **Habitat**: Saprobic; solitary, scattered or in overlapping cluster on decaying hardwoods; year-round Dimension: **Occurrence**: Rama. E**dibility:** Inedible. **Previous report from Pakistan:** *S. Commune* has previously reported on various wood, *Dalbergia sisso, Mangifera indica, Daphar* Plantation. (Ahmed 1972), (Delete some reference) Murakami (1993), Razaq *et al.*, (2007).

12. *Coprinus domesticus* *(Pers)) Fr. Epicr. p. 251. sarccardo, syl, Fung.* Vol.5, 1887, p. 1102.

Synonymy: *Coprinus xanthothrix Romagn*., Revue Mycol., Paris 127(1941).
Description of species: Fruiting body up to3-4cm long, at first egg shaped then convex then flattened, margin split, bulbous at base. Flesh white at young. **Stipe**: 4-8cm long bulbous and slender at the base. **Gills**: first white and then black **Spores**: Spores ellipsoid, kidney shaped 7-8x 6-7µm in size **Spore print**: dark brown. **Season of fruiting**: June-July **Occurrence:** Dashkin, **Edibility: P**oisonous. **Habitat:** small group on soil or leaf litter. **Previous report from Pakistan**: Basidiomycota of Northern Areas Gilgit baltistan Razaq *et al*., 2007.

13. *Morchella esculenta* Pers., Mycolo.eur.1:204 (1821).

Synonyms:
Morchella Dill. ex Pers., *Neues Mag*. Bot. 1:116 (1794).
Morchella rotunda (Pers.) Boud. 1897
Description of species: Fruiting body 3 to 8cm tall, sometimes conical, but more often globular or an elongated, hollow and are covered in irregular array of pits, Flesh tough, hollow, and 1.5cm in diameter at base usually tapering towards the apex. **Habit and Habitat**: originate on the fieldamid aspen and many other hardwood species. **Spore Print:** Spore print creamy white, **Spores:** ellipsoid, smooth 12.5-15x 9.11µm. **Occurrence**: Lashtang forest. **Edibility:** good in taste and most required after edible mushrooms. **Previous report from Pakistan**: *M. esculenta* has been reported from on the ground, Madyan (Swat) Nathia Gali. Ahmed (1972), Khalid (1995), Razaq and Shahzad (2012a, 2012b).

14. *Morchella conica* Pers., Mycol.eur.1:204 (1821).

Synonyms: *Morchella sect. Adnatae., Bull. Soc. mycol.*, 13:134 (1897).
Description of species: Cap 3-5cm wide x 5-8cm tall. Stem 1-3cm wide x 4-6cm tall. Overall the size can range from 8 to 15 cm tall. The surface color varies from dark brown to dark grey, sometimes almost black. The hollow conical or egg-shaped cap deeply pitted, rather like an irregular honeycomb. **Habitat**: Often in groups and sometimes in very large numbers where it is found like gardens and woodchip mulch. **Spores print**: spore print pale cream. **Spores**: Spores ellipsoid smooth 18-25x 11-15µm. **Occurrence**: Rama. **Edibility**; Edible they are good in taste. **Previous report from Pakistan**: Razaq and Shahzad (2012a, 2012b). Fig. 1B shows the DNA of 14 samples of macro fungi.

Discussion

The genomic DNA was successfully isolated from different 14 samples of macro fungi, small scale DNA separation process was followed by (Weining and Langridge, 1991). The DNA was good as seen by

visualizing the 0.8% agarose gel. Approximately equal amount of DNA was isolated from each sample. No RNA contamination was observed in the samples hence DNA samples were not treated with RNA and were used directly for Polymerase Chain Reaction after making 1:4 dilutions in double distilled de-ionized autoclave water 16µl. Average genetic distances in all possible combinations were constructed using procedure outlined by (Nei and Li 1979), and the associations between accessions were presented graphically in the form of a dendrogram in (Fig. 1C). Genetic distance among the macro fungi accessions ranged from 40.2% to 71.8%. Total genomic DNA was successfully isolate from 4 samples of macro fungi using small scale DNA isolation procedure (Weining and Langridge, 1991). The procedure adopted in this study proved convenient for DNA isolation from diverse genera and the DNA thus yielded was appropriate for PCR amplification.

Fig. 2. Map of study area.

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