New report of the edible mushroom *Pleurotus cystidiosus* from Pakistan

SHAH HUSSAIN[1]
NAJAM-UL-SAHAR AFSHAN[2]
HABIB AHMAD[1]
ABDUL NASIR KHALID[3]
Email: pakrust@gmail.com

[1]Department of Botany
Hazara University
Mansehra, Pakistan

[2]Centre for Undergraduate Studies
University of the Punjab
New Campus, Lahore, Pakistan

[3]Department of Botany
University of the Punjab
New Campus, Lahore, Pakistan

Accepted 15. April 2015

Key words: *Pleurotus*. – nrITS. – Mycobiota of Malakand.

Abstract: A well-known edible mushroom, *Pleurotus cystidiosus,* is described and illustrated macro- and microscopically. We also sequenced the internal transcribed spacer regions (ITS1−5.8S−ITS2) of the rDNA of this mushroom, which was collected in subtropical pine forests of Malakand, Pakistan, during the rainy season of July-August 2013. This taxon is a new species for Pakistan and an addition to its edible agaric flora.

Zusammenfassung: Ein bekannter Speisepilz, *Pleurotus cystidiosus*, wird beschrieben und makro- und mikroskopisch illustriert. Wir sequenzierten auch die intern transkribierten Spacer-Regionen (ITS1-5.8S-ITS2) der rDNS dieses Pilzes, der in den subtropischen Kiefernwäldern von Malakand, Pakistan, während der Regenzeit Juli-August 2013 gesammelt worden war. Dieses Taxon konnte für Pakistan als neu nachgewiesen werden und ist eine Ergänzung der Speisepilze in Pakistan.

Edible mushrooms are a precious source of nutrients and bioactive compounds in addition to a growing demand for humans by their flavours and beneficial effects on human health (GUILLAMÓN & al. 2010). These mushrooms are widely consumed in many countries as a food due to their attractive taste, aroma and nutritional values (DE PINHO & al. 2008). *Pleurotus* (FR.) P. KUMM. (commonly known as Oyster mushroom) is a genus of gilled mushrooms, containing 20 established biological species throughout the world (KIRK & al. 2008) and belongs to family *Pleurotaceae* of order *Agaricales* (VILGALYS & al. 1996). These mushrooms are found in tropical, subtropical and temperate climates throughout the world, and about 755 species, subspecies, varieties and forms of this genus are found in the literature (www.IndexFungorum.org). These are edible and one of the most cultivated mushrooms worldwide. The genus has high protein content of 30–40% on dry weight basis (SHARMA & MADAN 1993). Species of

this genus are not easily recognizable in the field macroscopically. For the accurate identification of these, microscopic tools should be supported with molecular study (SIDDIQUEE & al. 2012).

From Pakistan, only a few workers collected and described edible mushrooms (AHMAD 1941, 1952, 1978; KHAN & al. 1980; KHAN 1982; BATRA 1983; SULTANA & al. 2011), but most of their work is related to taxonomic identification of these fungi. Among described edible mushrooms, only eight species of *Pleurotus* have been reported from Pakistan (AHMAD & al. 1997, SULTANA & al. 2011).

In the present study, we have collected this important and world known edible mushroom, i.e. *Pleurotus cystidiosus* and described it morpho-anatomically and phylogenetically. This species is a new record for Pakistan and also an addition to the edible agaric flora of our country. This work is an initial step towards the identification and distribution of this edible mushroom and will be a motivation for those scientists who want to collect this mushroom for cultivation and commercialization.

Table 1. ITS-nrDNA sequences included in phylogenetic analysis for *Pleurotus cystidiosus* and related species.

No.	Taxon	Origin	GenBank Accession No
1.	*Agaricus bisporus*	USA	EF460355
2.	*Pleurotus australis*	USA	AY315760
3.	*Pleurotus australis*	USA	AY315761
4.	*Pleurotus australis*	USA	AY450342
5.	*Pleurotus cystidiosus*	China	AY540321
6.	*Pleurotus cystidiosus*	China	AY540320
7.	*Pleurotus cystidiosus*	Pakistan	KJ648452
8.	*Pleurotus cystidiosus*	Pakistan	KR149589
9.	*Pleurotus cystidiosus*	China	JF758878
10.	*Pleurotus cystidiosus*	Republic of Korea	AY368655
11.	*Pleurotus cystidiosus*	Republic of Korea	AY265819
12.	*Pleurotus cystidiosus*	USA	AY315800
13.	*Pleurotus cystidiosus*	India	DQ978222
14.	*Pleurotus cystidiosus*	USA	AY315810
15.	*Pleurotus cystidiosus*	USA	AY315795
16.	*Pleurotus cystidiosus*	China	EU424280
17.	*Pleurotus cystidiosus*	USA	AY315796
18.	*Pleurotus fuscosquamulosus*	USA	AY315789
19.	*Pleurotus fuscosquamulosus*	USA	AY315788
20.	*Pleurotus smithii*	USA	AY315784
21.	*Pleurotus smithii*	Mexico	GU722288
22.	*Pleurotus smithii*	Republic of Korea	AY265851
23.	*Pleurotus tuberregium*	USA	AF109972
24.	*Pleurotus tuberregium*	USA	AY450344

Materials and methods

Fungal isolates

The specimens were collected from three localities, Qaldara, Pirano and Hazar Nao of Malakand district, KP Pakistan. The samples were photographed, tagged, dried in sunlight and kept in sealed plastic bags for further studies. The study area lies between 34° 33'-34° 56' N, 71° 55'-71°52' E, and features a subtropical climate with heavy rainfall (600−1100 mm, annually) at monsoon (KARIM 2008). Vegetation is mostly subtropical and falls under the forest type of Coniferous Subtropical Pine Forest of Pakistan (SHEIKH 1993). The dominant forest trees in the area are the *Pinus roxburghii* mixed with *Quercus incana* and *Olea ferruginea*. *Acacia modesta* and *Dodonaea viscosa* are found at the lower elevation (BARKATULLAH & IBRAR 2011).

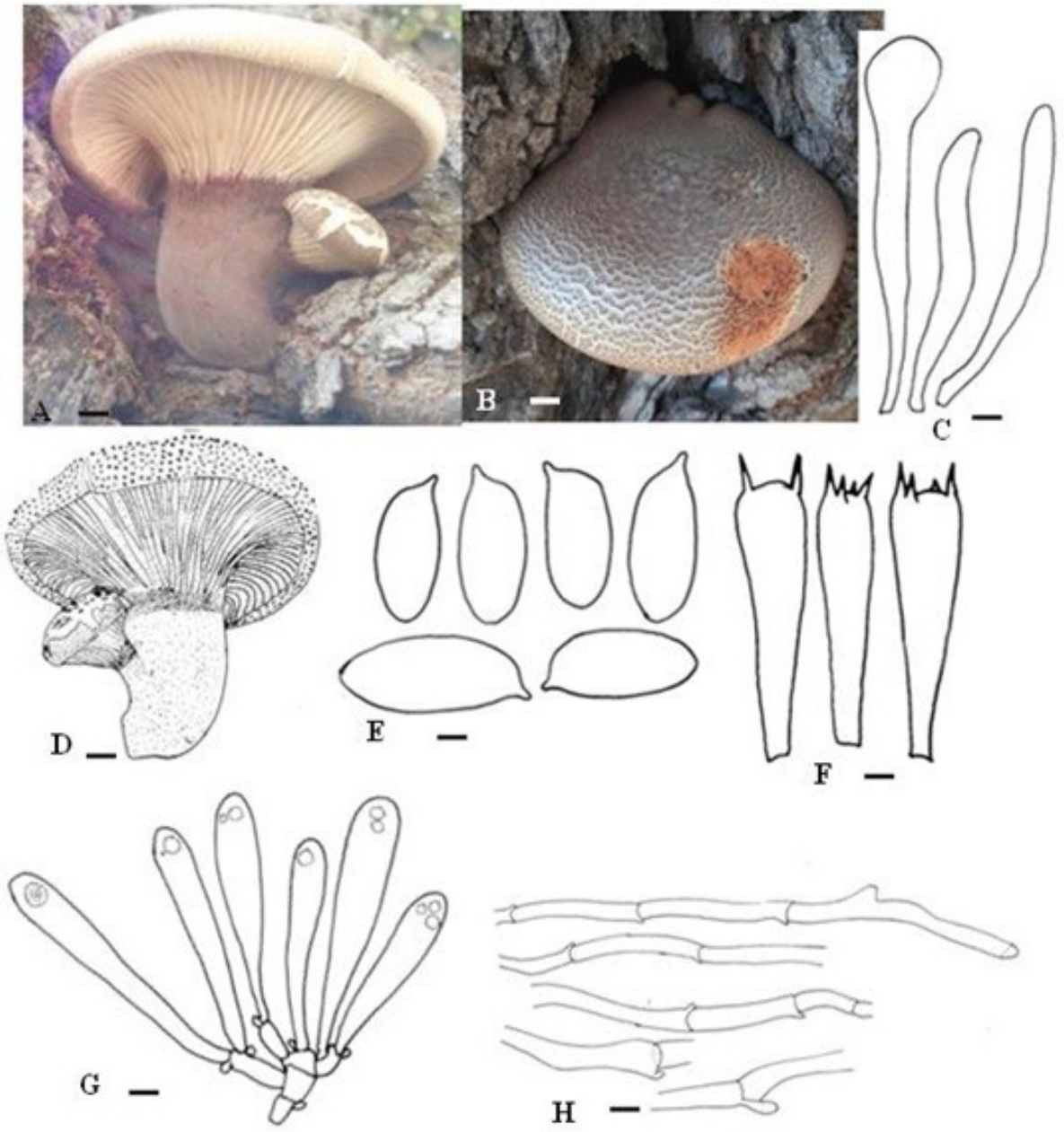

Fig. 1. *Pleurotus cystidiosus*. *A, B* Basidiomata on *Morus alba*, *C* pleurocystidia, *D* drawing of fruit body, *E* basidiospores, *F* basidia, *G* cheilocystidia, *H* stipe hyphae. Scale bars: A–C= 1.2 cm, D = 6.3 mm, E = 9 µm, F = 10 µm, G = 8 µm, H = 11 µm.

Microscopic features

Sections of gills were smashed in 5% KOH for microscopic features under a biological microscope (MX4300H, Meiji Techo Co., Ltd., Japan). Features like shape and size of basidiospores, basidia, cystidia and generative hyphae as well as the colour reaction in MELZER'S reagent (LARGENT & al. 1977) of the spores were studied. Line drawings were made with a camera lucida. For colour designations, MUNSELL (1975) was followed. Measurements of morpho-anatomical features (basidiospores, basidia, cystidia, pileus hyphae and stipe hyphae) are presented from at least 20 measurements made with an ocular micrometre under a 100× oil-immersion objective, with following parameters (n/m/p); n: number of spores measured, from m: number of specimen out of p collections. Average width and length of basidiospores were represented by avl and avw, respectively, along with the quotient length by width of spores by Q and the average quotient by avQ (LIANG & al. 2011).

DNA extraction and amplifications

For DNA extraction, a small piece (30−50 mg) of gills was kept in 2% CTAB buffer, following the BRUNS (1995) protocol with little modifications. The ITS regions of nrDNA was amplified using the universal primer pair ITS−1F and ITS−4 (WHITE & al. 1990). The polymerase chain reaction (PCR) was performed in 20 µl reaction volume following the protocol given by GARDES & BRUNS (1993). The amplified products were visualized in Agarose Gel Electrophoresis through Gel Documentation System UVtec (Avebury House, Cambridge CB4 1QB UK), using default settings.

ITS sequencing

The PCR products were directly sequenced in both directions using the same primer set. Sequencing was carried out under BigDye™ terminator cycling conditions. The reaction products were purified using ethanol precipitation and run through Automatic Sequencer 3730XL (Macrogen, Korea). Sequences were manually edited and assembled using BioEdit (www.mbio.ncsu.edu/bioedit/bioedit.html). Sequences of *Pleurotus cystidiosus* along with the other related sequences from GenBank were used in the phylogenetic analysis (Tab. 1). All the sequences were aligned through Multiple Sequence Comparison by Log- Expectation (MUSCLE) online (http://www.ebi.ac.uk/Tools/msa/muscle). For phylogenetic tree construction Molecular Evolutionary Genetics Analysis (MEGA6) softer (TAMURA & al. 2013) was used. Maximum likelihood (ML) analysis was performed by MEGA6 using Jukes-Cantor model of nrITS sequences and nearest-neighbor-interchange (NNI) as ML heuristic search method. Phylogeny was tested by bootstrap value of 1000 replicates.

Results and discussion

Characters

Pleurotus cystidiosus O. K. MILL. Mycologia 61: 889 (1969) (Fig. 1 A−C)

– *Stilbum macrocarpum* ELLIS & EVERH. = *Antromycopsis macrocarpa* (ELLIS & EVERH.) STALPERS, SEifert & SAMSON = *Antromycopsis broussonetiae* PAT. & TRAB.

P i l e u s : 10−13 × 6.3−8 cm, convex in young stage then depressed, parabolic modification of pileus disc, surface scaly, dry velvety in touch, smooth and slightly lobed margin, with greyish yellow pink context (2.5YR 7/2 − 10R 7/2), scales moderate reddish brown (7.5R 3/4 − 2.5YR 4/4).

L a m e l l a e : decurrent, 4−8 cm in length, closed, with marginate edge, moderate reddish brown (7.5R 3/4 − 2.5YR 4/4), face brilliant yellow (2.5Y 9/10 − 5Y 9/), thin and remaining unchanged on drying.

S t i p e : eccentric, 3.7−5 × 2.4−3.5 cm, equal and flattened, smooth, slightly curved at the base, greyish brown (7.5YR 3/2 − 7.5YR 4/2), dry velvety, solid, veil absent, base bulbous.

B a s i d i o s p o r e s : [30/2/2], 7.4−14 × 3−6 µm, [avl × avw = 11 × 5 µm, Q= 1.35–1.85, avQ= 2.2], cylindrical to ellipsoidal, thin walled, hyaline, smooth and in-amyloid (Fig. 1 E).

B a s i d i a : 23.7−35 × 3.8−6 µm, cylindrical to clavate, thin walled, hyaline, 2− or 4− spored, with sharp and thin sterigmata of 1.9−2.18 µm (Fig. 1 F).

C h e i l o c y s t i d i a : 24−40 × 3.03−5.66 µm, frequent, pyriform to cylindrical, thin walled (Fig. 1 G).

P l e u r o c y s t i d i a : 26−42 × 6−9 µm, thin walled, hyaline, sub-fusiform to sub-globose (Fig. 1 C).

S t i p e h y p h a e : 6−10 µm in diam., septate, thin-walled, hyaline with frequent clamp connections (Fig. 1 H).

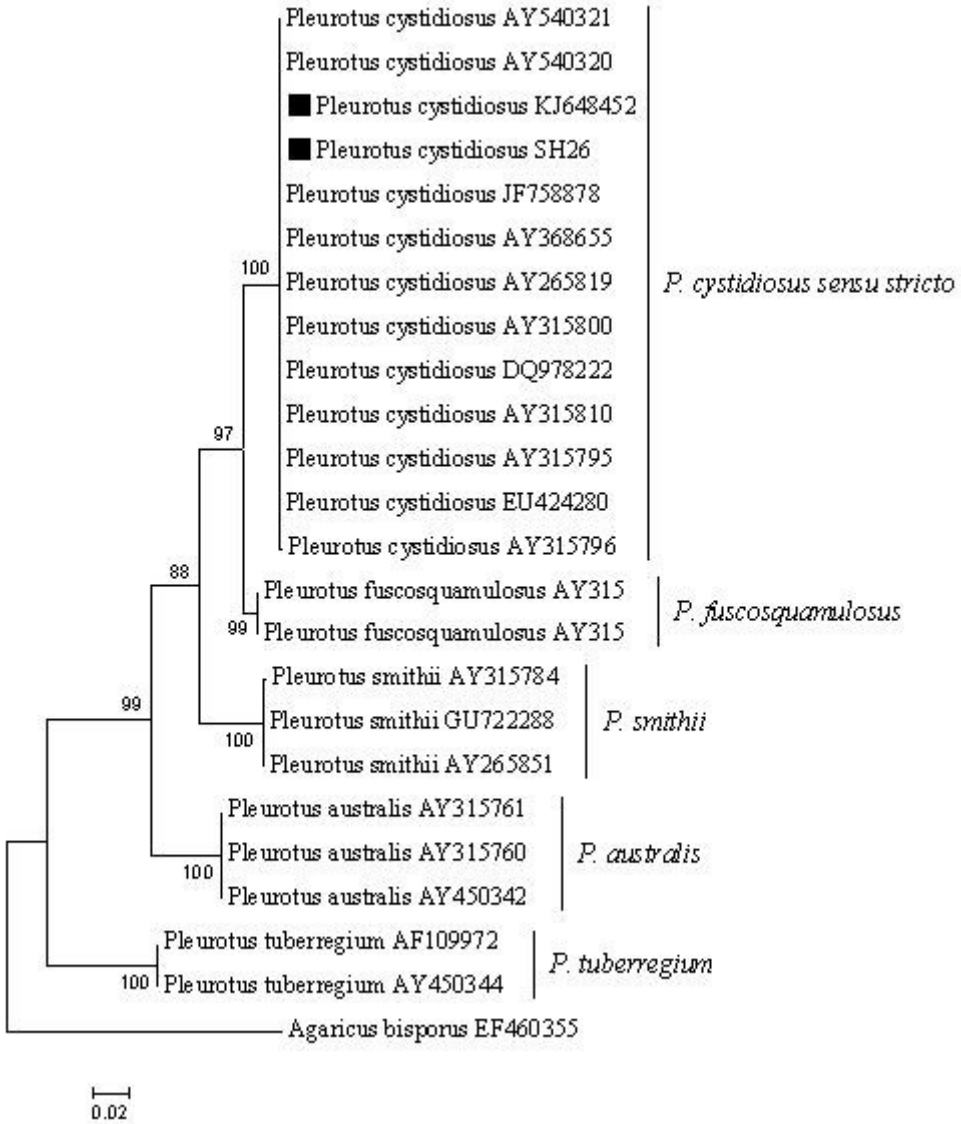

Fig. 2. Phylogenetic relationship of Pakistani (■) *Pleurotus cystidiosus* with other members of *Pleurotus* inferred from ITS sequencing using Maximum Likelihood from 24 nucleotide sequences along with *Agaricus bisporus* (EF460355) as an outgroup. Bootstrap values based on 1000 replicates are shown above the branches. All positions containing gaps and missing data were eliminated. There were a total of 516 positions in the final dataset.

Material examined: Pakistan, KP, Hazar Nao Hills Agra, 23 km west of Swat-Malakand Highway, 34°31'59.3'' N, 71°45'13.5'' E, 1245 m a.s.l., *Morus alba* L., 05. 8. 2013, Shah Hussain (HUH−SH22,26; LAH−SH22,26).

Molecular characterization and phylogenetic analysis of *Pleurotus cystidiosus*

The target region comprising of internal transcribed spacers gave fragments of 636−700 base pair length on amplification in polymerase chain reaction using ITS primers (ITS1 F and ITS4). Initial blast analysis of the amplified product revealed that our sample matches best with *Pleurotus cystidiosus* (GenBank no. JF758878). Sequences from GenBank database were retrieved for further alignment and phylogenetic analysis. In the alignment it was noted that the ITS region of the current sequence is shorter than the GenBank sequences. Therefore extra parts of the other sequences were removed during the analysis. Phylogenetic analysis based on sequence alignment showed its clustering with P. *cystidiosus*. 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, five clades were formed of all *Pleurotus* taxa. All *P. cystidiosus* s. str. species including the Pakistani collections were monophyletic, morphologically homogenous and clustered in clade I (Fig. 2), and those having distinct cystidial characters were separated in another clade (Fig. 2: clade II). In clades III, IV and V the strains are easily distinguished by their morphology (SEGEDIN & al. 1995, ISIKHUEMHEN & al. 2000). *Agaricus bisporus* (GenBank no. EF460355) was considered as an outgroup for phylogenetic tree construction (Fig. 2).

Discussion

Pleurotus cystidiosus is an edible mushroom (LAU & al. 2013) and a good representative of the genus found mostly on the rotted stem of *Morus* species a.o. It was collected from the subtropical pine forest of Hazar Nao, Malakand, Pakistan, at 1500 m altitude. It was previously reported from India, China, Korea (ATRI & al. 2012) and America as the geographical boundaries of this species are not strict (ZERVAKIS & al. 2004). To confirm our molecular results a detailed morphological description is also provided. All the morphological characters were compared with the descriptions available in literature (GONZALEZ & LABARÈRE 2000, LECHNER & al. 2004, ATRI & al. 2012). The presence of moderate reddish brown scales (7.5R 3/4 − 2.5YR 4/4) on pileus and monomitic hyphae are the key features of the species (LECHNER & al. 2004). Molecular characterization of Pakistani collections showed its similarity of sequences within *P. cystidiosus* s. str. with 100% BS value (Fig. 2 Clade I). The phylogenetic analysis and morpho-anatomical characterization and comparison proved the Pakistani collections as *Pleurotus cystidiosus*. This is an addition to the edible mycoflora of Pakistan.

The authors express their gratitude to the staff members of Fungal Biotech Lab, Department of Botany, University of the Punjab, Lahore Pakistan for the continuous help and valuable suggestion. We greatly thank Ms. MALKA SABA and Ms. TAYYIBA QASIM, Ph.D students in the above mentioned lab for their cooperation during molecular study. Financial support for this study was provided by Research Project, University of the Punjab Lahore, Pakistan.

References

AHMAD, S., 1941: Gasteromycetes of Western Himalayas. I. – J.Indian Bot. Soc. **20**: 173−182.

AHMAD, S., 1952: Gasteromycetes of Western Pakistan. – Department of Botany, University of the Punjab, Lahore, Pakistan..

AHMAD, S., 1978: *Ascomycetes* of Pakistan. – Biological Society of Pakistan, Monograph **7**: 1−146.

AHMAD, S., IQBAL, S. H., KHALID, A. N. 1997: Fungi of Pakistan. – Lahore, Pakistan: Sultan Ahmad Mycological Society of Pakistan.

ATRI, N. S., SHARMA, S. K., KAUR, N., RAHI, P., GULATI, A., 2012: Morpho-cultural, molecular and nutraceutical studies on Coremiopleurotus from India. – World Applied Sci. J. **17**: 759−763.

BARKATULLAH, H. K., IBRAR, M., 2011: Plants profile of Malakand Pass Hills, District Malakand, Pakistan. – African J. Biotechnol. **10**: 16521−16535.

BATRA, L. R., 1983: Edible Discomycetes and Gasteromycetes of Afghanistan, Pakistan and North-Eastern India. – Biologia **29**: 293−306.

BRUNS, T. D., 1995: Thoughts on the processes that maintain local species diversity of ectomycorrhizal fungi. – Plant Soil **170**: 63−73.

DE PINHO, P. G., RIBEIRO, B., GONÇALVES, R. F., BAPTISTA, P., VALENTÃO, P., SEABRA, R. M., 2008: Aroma compounds in eleven edible mushroom species: relationship between volatile profile and sensorial characteristics. – J. Agriculture Food Chemistry **56**: 1704–1712.

GARDES, M., BRUNS, T. D., 1993: ITS primers with enhanced specificity of *Basidiomycetes*: application to the identification of mycorrhizae and rusts. – Molec. Ecol. 2: 113−118.

GONZALEZ, P., LABARÈRE, J., 2000: Phylogenetic relationships of *Pleurotus* species according to the sequence and secondary structure of the mitochondrial small-subunit rRNA V4, V6 and V9 domains. – Microbiologia **146**: 209–221.

GUILLAMÓNA, E., LAFUENTEA, A. G., LOZANOA, M., ARRIGOA, M. D., ROSTAGNOA, M. A., VILLARES, A., MARTÍNEZ, J. A., 2010: Edible mushrooms: role in the prevention of cardiovascular diseases. – Fitoterapia **81**: 715–723

ISIKHUEMHEN, O. S., MONCALVO, J. M., NERUD, F., VILGALYS, R., 2000: Mating compatibility and phylogeography in *Pleurotus tuberregium*. – Mycol. Res. **104**: 732–737.

KARIM, A., 2008: Constituency NA 35, Malakand. – Monthly Magazin "*Nazar*" **1**: 9–14.

KHAN, S. M., 1982: Research studies on wild and exotic mushrooms in Pakistan. – PL480 Project Report FG. Pa 252 (PK. ARS71) (N).

KHAN, D. A., KHAN, S. M., AKHTAR, M. I., 1980: Four edible fungi from Baluchistan Pakistan. – Pakistan J. Agriculture Research **1**(2): 142–145.

KIRK, P. M., CANNON, P. F., MINTER, D. W., STALPERS, J. A., 2008: Ainsworth and Bisby's Dictionary of Fungi. 10th edn. – Wallingford, UK: CABI.

LARGENT, D., JOHNSON, D., WATLING, R., 1977: How to identify mushrooms to genus III: microscopic features. – Arcata: Mad River Press.

LAU, C. C., ABDULLAH, N., SHUIB, A. S., 2013: Novel angiotensin I-converting enzyme inhibitory peptides derived from an edible mushroom, *Pleurotus cystidiosus* O. K. MILLER identified by LC-MS/MS. – BMC Complementary and Alternative Medicine **13**: 313.

LECHNER, B. E., WRIGHT, J. E., ALBERTO, E., 2004: The genus *Pleurotus* in Argentina. – Mycologia **96**: 845–858.

LIANG, J. F., YANG, Z. L., XU, D. P., 2011: A new species of *Lepiota* from China. – Mycologia **103**: 820–830.

MUNSELL, T. M., 1975: Soil color charts. – Baltimore.

SEGEDIN, B. P., BUCHANAN, P. K., WILKIE, J. P., 1995: Studies in the *Agaricales* of New Zealand: new species, new records and renamed species of *Pleurotus* (*Pleurotaceae*). – Australian Syst. Bot. **8**: 453–482.

SHARMA, S., MADAN, M., 1993: Microbial protein from leguminous and non-leguminous substrates. – Acta Biotechnol. **13**: 131−139.

SHEIKH, M. I., 1993: Trees of Pakistan. – Islamabad, Pakistan: Pictorial Printers.

SIDDIQUEE, S., YEE, W. Y., TASLIMA, K., FATIHAH, N. N., KUMAR, S. V., HASAN, M. M., 2012: Sequence analysis of the ribosomal DNA internal transcribed spacer regions in *Termitomyces heimii* species. – Annals Microbiol. **62**: 797–803.

SULTANA, K., RAUF, C. A., RIAZ, A., NAZ, F., IRSHAD, G., HAQUE, M. I., 2011: Checklist of agarics of Kaghan valley. – Pakistan J. Bot. **43**: 1777−1787.

TAMURA, K., STECHER, G., PETERSON, D., FILIPSKI, A., KUMAR, S., 2013: MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. – Mol. Biol. Evol. **30**: 2725−2729.

VILGALYS, R., MONTCALVO, J. C., LIOU, S. R., VOLOVSEK, M., 1996: Recent advances in molecular systematics of the genus *Pleurotus*. – In: VILGALYS, R., MONTCALVO, J. C., LIOU, S. R., VOLOVSEK, M., (Eds): Mushroom biology and mushroom production: 91−101. – Pennsylvania: Pennsylvania State Univ. Press.

WHITE, T. J., BRUNS, T. D., LEE, S., TAYLOR, J., 1990: Amplification and direct sequencing of fungal ribosomal DNA genes for phylogenetics. – In: INNIS, M. A., GELFAND, D. H., SNINSKY, J. J., WHITE, T. J., (Eds): PCR protocols. A guide to methods and applications: 315–322. – San Diego: Academic Press.

ZERVAKIS, G. I., MONCALVO, J. M., VILGALYS, R., 2004: Molecular phylogeny, biogeography and speciation of the mushroom species *Pleurotus cystidiosus* and allied taxa. – Microbiology **150**: 715–726.