Tianjin Daxue Xuebao (Ziran Kexue yu Gongcheng Jishu Ban)/
Journal of Tianjin University Science and Technology
ISSN (Online):0493-2137
E-Publication: Online Open Access
Vol: 57 Issue: 08:2024
DOI: 10.5281/zenodo.13283275

MOLECULAR PHYLOGENY AND PHENOLOGY REVEAL A NEW FUNGISTIC RECORD OF *PSEUDOOMPHALINA KHANSPURENSIS* (AGARICALES, TRICHOLOMATACEAE) FROM PAKISTAN

ALEENA IQBAL
Department of Botany, Lahore College for Women University, Lahore, Pakistan.
Email: aleenaiqbal012@gmail.com

SAMINA SARWAR *
Department of Botany, Lahore College for Women University, Lahore, Pakistan.
*Corresponding Authors E-Mail: Samina_boletus@yahoo.com

IRMGARD KRISAI-GREILHUBER
Department of Botany and Biodiversity Research, University of Vienna, Austria.

TALHA SAJAD
Department of Botany, Lahore College for Women University, Lahore, Pakistan.

MUHAMMAD FIAZ
Department of Botany, Hazara University, Mansehra, Pakistan.

MUHAMMAD HANIF
Department of Botany, Government College University, Lahore, Pakistan.

Abstract

The District Mansehra, situated in the Hazara division of Pakistan's Province Khyber Pakhtunkhwa is characterized by moist temperate conditions and is one of the regions with a high macrofungal diversity. The genus *Pseudoomphalina* is rare in Pakistan with *P. khanspurensis* as the only species known. We present a second record of *P. khanspurensis* described in detail and illustrated based on basidiomata collected during a mycological field trip across various forests in District Mansehra, Pakistan. The study uses an integrative approach with morphology, scanning electron microscopy, and maximum likelihood phylogeny of nrITS and LSU sequence data. Molecular genetic analyses revealed the sample as *P. khanspurensis*. We consider some minor phenotypic differences, when compared to the previously reported *P. khanspurensis* specimens, to lie within the species range, further completing the knowledge about its morphological variation.

Keywords: ITS Regions, LSU Region, SEM, Morphology, Taxonomy.

INTRODUCTION

Pseudoomphalina is a genus of the family Tricholomataceae comprising species that are widespread in northern temperate regions. Various morphological and molecular genetic studies have addressed the systematic position of different members of the genus *Pseudoomphalina* (Singer, 1936; Singer, 1948; Ballero & Contu, 1993; Kuyper, 1995; Bon, 1997; Contu, 2003; Consiglio *et al*., 2006; Knudsen, 2008; Lavorato *et al*., 2015; Malysheva *et al*., 2011; Singer, 1986; Voitk *et al*., 2020).

Tianjin Daxue Xuebao (Ziran Kexue yu Gongcheng Jishu Ban)/
Journal of Tianjin University Science and Technology
ISSN (Online):0493-2137
E-Publication: Online Open Access
Vol: 57 Issue: 08:2024
DOI: 10.5281/zenodo.13283275

The genus is characterized by small yellowish, brownish to pinkish or dark purplish basidiomata, convex, later umbilicate smooth or finely scaly pileus, thin flexuous stipe, decurrent lamellae, pileipellis as a cutis, cystidia mostly absent, rarely present, clamp connections, and amyloid ovoid to ellipsoid spores; terricolous and saprotrophic (Malysheva *et al*., 2011; Agerer 2018; Voitk *et al*., 2020). Currently, around 16 *Pseudoomphalina* species are known in North America, Asia and Europe (Malysheva *et al*., 2011; Voitk *et al*., 2020; Rani *et al*., 2023).

In the course of an ongoing investigation of the funga of Pakistan, a collection of *Pseudoomphalina* Singer was found in the Pakistani District Mansehra in the Khyber Pakhtunkhwa Province. The region, comprising Mansehra, Balakot, and Oghi tehsils and covering an area of 5959 km^2 (Shah& Khan, 2006), includes high mountains, lakes, valleys, plains, and diverse trees within a northern mountain system.

The district experiences seasonal periods of rainfall, snowfall, and drought in a moist temperate environment (Mustafa, 2003). The district's forests are rich in trees including Blue Pine, Chirr, Cherry, Deodar, Kao, Poplar and Walnut sustaining a wide variety of macrofungi, particularly during the monsoon season (SMEDA, 2009).

MATERIAL AND METHODS

Site description and sampling

During fungal field surveys in the District Mansehra in the Hazara civil division of Khyber Pakhtunkhwa (KPK) region, we collected *Pseudoomphalina* basidiomata from the forests of the district in monsoon season. Field notes were prepared from fresh basidiomata and photographs were taken in their natural habitat. Basidiomata were dried by a fan heater and then placed in envelops for further processing in the laboratory.

Macro- and micromorphological studies

The sample was studied macro- and microscopically following Zang (2006). Macroscopic characters were recorded from fresh fruiting bodies:

1) Pileus shape, diameter, texture, ornamentation, surface color and bruising reaction of the context, margin color and shape.
2) Stipe width, length, color, shape, texture, ornamentation, stipe attachment to the pileus, presence/absence of annulus on stipe, color and bruising reaction of the context.
3) Hymenium: color of lamellae, bruising reactions of the hymenial surface.

Microscopic examination was conducted in lactic acid, KOH, or Congo red, examining the size, shape, cytoplasmic contents, and color reactions of basidia, hymenial cystidia, stipitipellis, pileipellis, basidiospores and terminal cells following Osemwegie *et al*. (2006). Spore dimensions are given as range of lengths and widths and average length/width ratio of an individual spore. A total of 30 spores from two basidiomata were measured.

Tianjin Daxue Xuebao (Ziran Kexue yu Gongcheng Jishu Ban)/
Journal of Tianjin University Science and Technology
ISSN (Online):0493-2137
E-Publication: Online Open Access
Vol: 57 Issue: 08:2024
DOI: 10.5281/zenodo.13283275

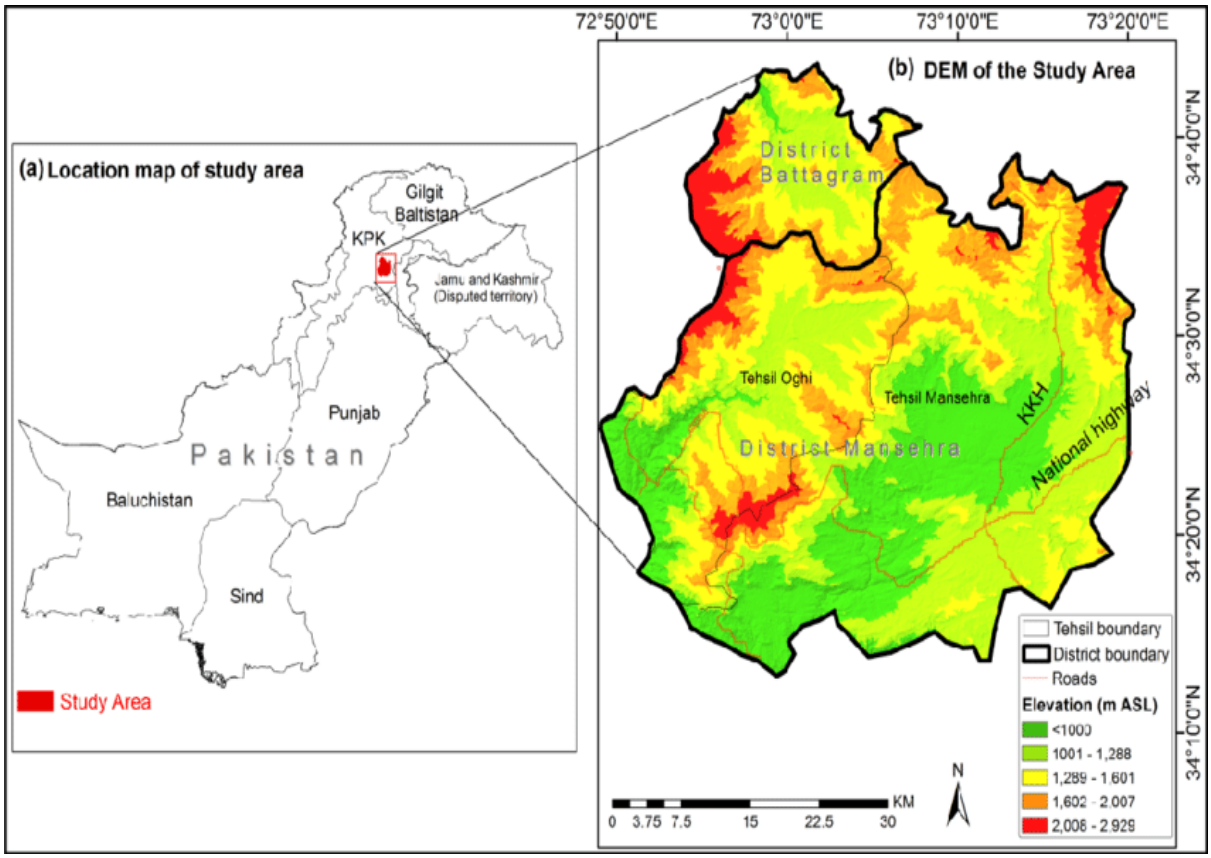

Figure 1: Map of District Mansehra (Ullah *et al*. 2019)

Molecular phylogenetic studies

DNA was isolated by using a Thermo Scientific GeneJET Plant Genomic DNA Purification Kit. Bands were visualized using the Bio-Rad Gel Doc™ 2000 gel documentation system. Polymerase Chain Reaction (PCR) was carried out using the fungus specific as well as universal primers (ITS1F, ITS4 and ITS4B) to amplify the nuclear ribosomal internal transcribed spacer (ITS) region, and LR0R and LR5 for the larger subunit (LSU) region (White *et al*., 1990). PCR products were confirmed with the nanodrop technique and cleaned using an enzymatic PCR cleanup (Werle *et al*., 1994) as described in Voglmayr & Jaklitsch (2008). DNA was cycle-sequenced using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit v. 3.1 (Applied Biosystems, Warrington, UK).

Sequencing was performed on an automated DNA sequencer (ABI 3730xl Genetic Analyzer, Applied Biosystems). BioEdit sequence alignment editor version 7.2.5 (Hall, 1999) was used for analyzing the obtained sequences. Consensus sequence was BLAST searched at NCBI (http://www.ncbi.nlm.nih.gov/). To reconstruct phylogeny, sequences from GenBank with closest match and additional *Pseudoomphalina* species were selected (Table 1). All sequences were aligned by using online MUSCLE tool at EMBL-EBI (http://www.ebi.ac.uk/) and manually modified where required in BioEdit (Hall, 1999).

Tianjin Daxue Xuebao (Ziran Kexue yu Gongcheng Jishu Ban)/
Journal of Tianjin University Science and Technology
ISSN (Online):0493-2137
E-Publication: Online Open Access
Vol: 57 Issue: 08:2024
DOI: 10.5281/zenodo.13283275

Table 1: GenBank accession numbers and geographical origins of taxa used for the phylogenetic analysis

Taxa	Voucher/isolate	Origin	GenBank accession number		Reference
			ITS	nrLSU	
Pseudoomphalina angelesiana	MushroomObserver.org/319024	USA	MH459165	-	Unpublished
Pseudoomphalina angelesiana	JLF9366 iNaturalist # 81790365	USA	-	OQ891234	Unpublished
Pseudoomphalina umbrinopurpurascens	MCVE 28275	China	NR_158907	-	Lavorato *et al*., 2015
Pseudoomphalina umbrinopurpurascens	C. Lavorato 091101-11	China	-	KP987563	Lavorato *et al*., 2015
Pseudoomphalina compressipes	DAOM11115	Canada	MK982246	-	Voitk *et al*., 2020
Pseudoomphalina compressipes	CMMF002076	Canada	-	MK400232	Voitk *et al*., 2020
Pseudoomphalina_kalchbrenneri	TU114963	Switzerland	MK982241	-	Voitk *et al*., 2020
Pseudoomphalina_kalchbrenneri	TU109855	Estonia	-	MK400229	Voitk *et al*., 2020
Pseudoomphalina intermedia	MICH10152	USA	MN326458	-	Voitk *et al*., 2020
Pseudoomphalina intermedia	MICH55730	USA	-	MN326460	Voitk *et al*., 2020
Pseudoomphalina anticostica	MIN956134	USA	MK982247	-	Voitk *et al*., 2020
Pseudoomphalina khanspurensis	LCWUBOT.SS.08082212	**Pakistan**	**OR528637**	**OR528639**	-
Pseudoomphalina khanspurensis	LAH37779	Pakistan	OQ550146	-	Rani *et al*., 2023
Pseudoomphalina khanspurensis	LAH37777	Pakistan	OQ550145	-	Rani *et al*., 2023
Pseudoomphalina khanspurensis	LAH37778	Pakistan	-	OQ550151	Rani *et al*., 2023
Pseudoomphalina khanspurensis	LAH37777	Pakistan	-	OQ550149	Rani *et al*., 2023
Pseudoomphalina cokeri	TENN 012963	USA	NR_121485	-	Unpublished
Clitocybe nebularis	ZMU129_ITS	China	MW724242	-	Unpublished
Clitocybe nebularis	GLM 45883	Germany	-	AY207222	Walther *et al*., 2005
Clitocybe cokeri	TENN:012963	USA	-	HQ179663	Unpublished
Pseudoomphalina felloides	DAOM11115	USA	-	AF261442	Moncalvo *et al*., 2002
Clitocybe vibecina	GLM 45888	Germany	-	AY207160	Walther *et al*., 2005

Note: Sequences generated for this study are shown in bold.

Tianjin Daxue Xuebao (Ziran Kexue yu Gongcheng Jishu Ban)/
Journal of Tianjin University Science and Technology
ISSN (Online):0493-2137
E-Publication: Online Open Access
Vol: 57 Issue: 08:2024
DOI: 10.5281/zenodo.13283275

MEGA11 software was used to reconstruct the phylogenetic tree (Tamura *et al*., 2021). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Jukes-Cantor model, and then selecting the topology with superior log likelihood value.

RESULTS

Molecular phylogenetic characterization

The BLAST results of ITS-LSU region revealed a concordance of more than 99 % with *Pseudoomphalina khanspurensis* A.K. Rani, A. Izhar, Usman & Khalid (ITS sequences with OQ550146, OQ550146 and LSU sequences with OQ550151, OQ550149), a species described from Pakistan recently. The final ITS dataset comprised 12 sequences and 737 characters. Similarly, the final aligned data set of nrLSU sequences included 11 sequences and 1134 characters. Maximum Likelihood method and Jukes-Cantor model (Jukes & Cantor, 1969) was used for inferring the evolutionary history. The tree with the highest log likelihood -2698.53 for ITS phylogeny and -3960.34 for LSU phylogeny is shown (Figs. 2, 3). Bootstrap consensus tree was determined from 1000 replicates, and the corresponding bootstrap values (> 50%) was mentioned in the tree.

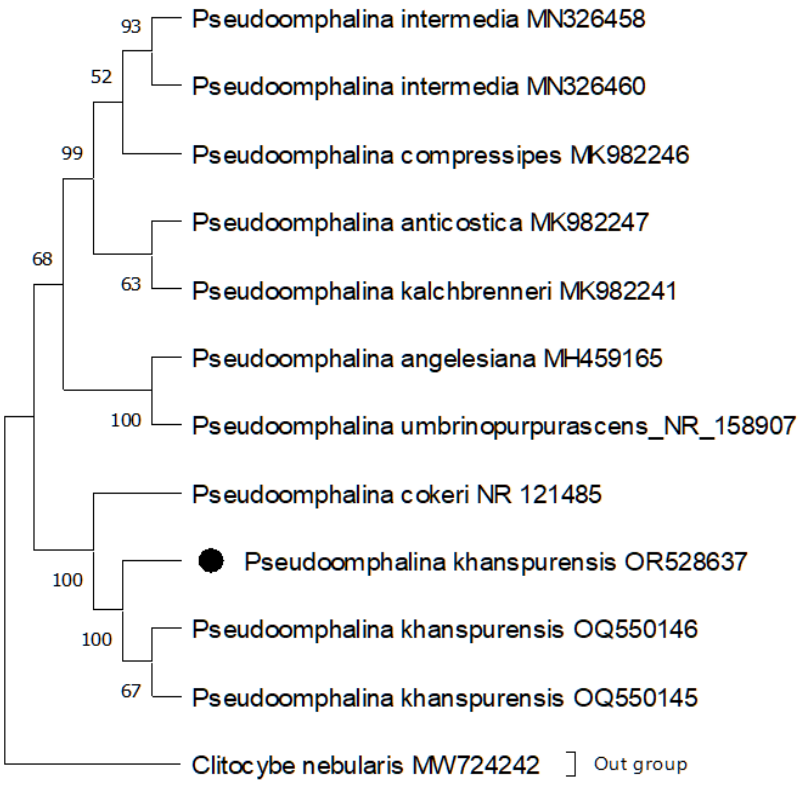

Figure 2: Phylogenetic tree of *Pseudoomphalina khanspurensis* represented by (•) based on Maximum Likelihood (ML) analysis of nrITS sequences

Tianjin Daxue Xuebao (Ziran Kexue yu Gongcheng Jishu Ban)/
Journal of Tianjin University Science and Technology
ISSN (Online):0493-2137
E-Publication: Online Open Access
Vol: 57 Issue: 08:2024
DOI: 10.5281/zenodo.13283275

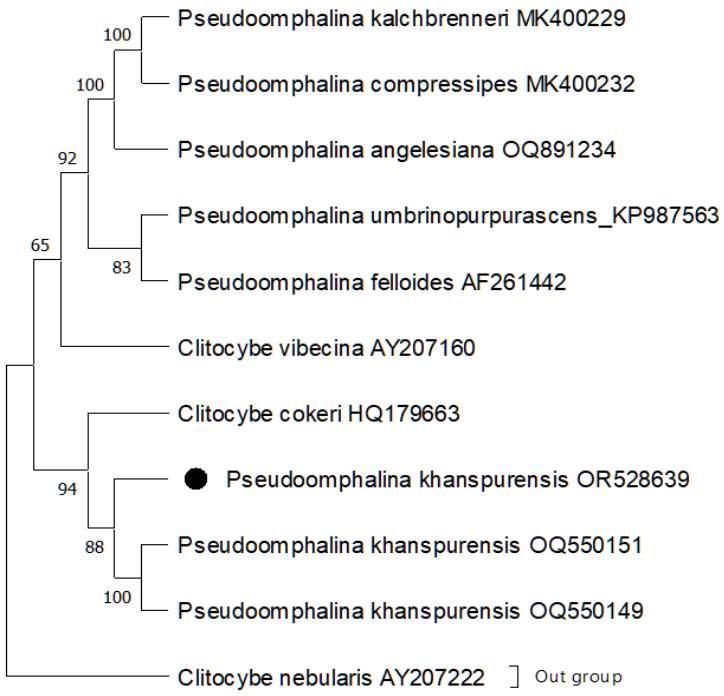

Figure 3: Phylogenetic tree of *Pseudoomphalina khanspurensis* represented by (•) based on Maximum Likelihood (ML) analysis of nrLSU sequences

Description of the Pakistani specimen

Pseudoomphalina khanspurensis A. K. Rani, A. Izhar, Usman, & Khalid Figures 4-5

Figure 4: Basidiomata of *Pseudoomphalina khanspurensis*. (A) Pileus, (B) Hymenium (C) Stipe. Bar = 1 cm

Tianjin Daxue Xuebao (Ziran Kexue yu Gongcheng Jishu Ban)/
Journal of Tianjin University Science and Technology
ISSN (Online):0493-2137
E-Publication: Online Open Access
Vol: 57 Issue: 08:2024
DOI: 10.5281/zenodo.13283275

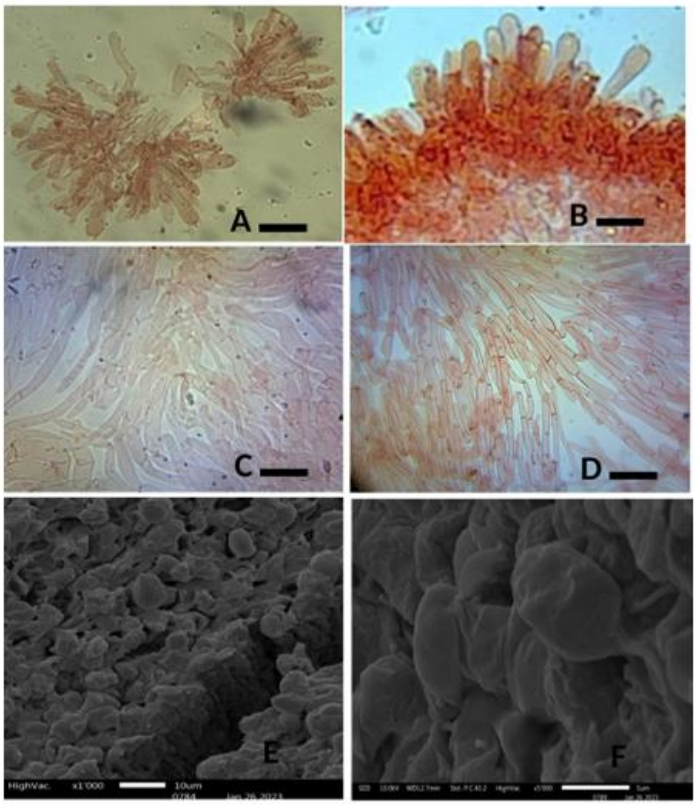

Figure 5: Microscopic features of *Pseudoomphalina khanspurensis*. (A-B) Basidia & Cystidia Bar = 10 µm, (C) Pileipellis Bar = 15 µm, (D) Stiptipellis Bar = 15 µm, (E-F) SEM photographs of Basidiospores

Macroscopic features

Pileus 3–4 cm in diameter, smooth, convex to broadly convex, center depressed, umbonate, overall brownish pink, margin incurved when young, highly wavy to irregular at maturity. Lamellae sub-decurrent to decurrent, regular, concolorous with pileus surface, 2-3 lamellulae, dicho- to polytomously branched towards margin. Stipe 5–6 × 0.6–1.0 cm, smooth, hollow, central, equal, cylindrical to slightly tapering upward, sub-bulbous, brownish pink with whitish to off-white background. Annulus and volva absent. Basidiospores 6–9.5 × 2.5–5 µm, avl × avw= 5.53 × 4.72 µm, oblong to globose, hilar appendix well-developed with suprahilar depression, thin-walled, guttulate, weakly amyloid, hyaline in 5% KOH. Basidia 25-50 × 4.5–6 µm, long, 2-4 spored thick-walled, clavate, clamp connections present, cytoplasmic content dense, hyaline in KOH. Hymenophoral trama irregular, hyphae thin-walled, hyaline in 5% KOH. Cheilocystidia 26-55× 4-5 µm, cylindrical-clavate, sometimes cylindrical and fasciculate, thin-walled, hyaline. Pileipellis a filamentous cut is made up of narrow thin-walled, cylindrical-clavate or cylindrical hyphae with rounded ends, hyaline, clamp connections present, intricate to irregular. Stipitipellis hyphae thin-walled, septate, parallel, cylindrical to clavate, branched, hyaline, clamp connections present.

Tianjin Daxue Xuebao (Ziran Kexue yu Gongcheng Jishu Ban)/
Journal of Tianjin University Science and Technology
ISSN (Online):0493-2137
E-Publication: Online Open Access
Vol: 57 Issue: 08:2024
DOI: 10.5281/zenodo.13283275

Material examined: Pakistan, Khyber Pakhtunkhwa province, District Mansehra at 1122m a.s.l, on soil, in mixed conifers tree stand, 08-08-2022, leg. S. Sawar, LCWUBOT. SS.08082212 (Herbarium, Dept. of Botany, LCWU, Lahore). (Genbank no. OQ550148 for nrITS and OQ550151 for nrLSU).

DISCUSSION

Pseudoomphalina khanspurensis was described from Pakistan by Rani *et al*. (2023) being the first record of this genus for the country. One of our specimens found during the mycological survey of the district Mansehra was revealed to belong to this species, when analyzing it macro- and microscopically, as well as by sequencing ITS and LSU regions (Figs. 2-3). In both phylogenetic trees, our sequences form a clade with *P. khanspurensis* with high bootstrap value. There are some phenotypic differences from *P. khanspurensis* as described by Rani *et al*. (2023).

They expand the morphological range of this species. Our basidiomata showed brownish-pink color, an irregular pileus margin when overmature, and more branched lamellae compared to the reddish-purple color of the pileus and dichotomously branched lamellae mentioned in the original publication by Rani *et al*. (2023). These differences are attributed to the higher age of the basidiomata. Interpretation of the cystidia as unequivocal is difficult in our sample, because they look very similar to immature basidia. Most *Pseudoomphalina* species do not have cystidia, but flagellate pseudocystidia are known to occur (Ludwig, 2012).

Pseudoomphalina khanspurensis is placed together with *P*. *cokeri* (Hesler) Vizzini, Contu & Z.W. Ge in one subclade. The latter is distinct by its stouter habit, a stipe covered with white fibrils and geographically, being a strictly North American species (Lavorato *et al*., 2015; Smith & Hesler, 1943).

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

Acknowledgements

Authors would like to express sincere gratitude to Austrian Academy of Science for funding to support this research under JESH postdoc fellowship by Joint Excellence in Science and Humanities (JESH) and to University of Vienna, Department of Botany and Biodiversity Research for providing the opportunity to conduct research by using all available facilities. We are highly indebted to Mag. Dr. Michael Barfuss University of Vienna, Department of Botany and Biodiversity Research, Division of Systematic and Evolutionary Botany, Plant Evolutionary Genomics and Systematics for his help in molecular work. We are also thankful to Mag. Dr. Silvia Ulrich, University of Vienna, Department of Botany and Biodiversity Research, Division of Structural and Functional Botany for her support in SEM analysis.

References

1) Agerer, R. 2018. Agaricomycotina. In: Begerow, D., McTaggart, A., Agerer,R.: Engler´s Syllabus of Plant Families. Part 1/3 Basidiomycota and Entorrhiomycota. Stuttgart: Borntraeger Science Publishers.

Tianjin Daxue Xuebao (Ziran Kexue yu Gongcheng Jishu Ban)/
Journal of Tianjin University Science and Technology
ISSN (Online):0493-2137
E-Publication: Online Open Access
Vol: 57 Issue: 08:2024
DOI: 10.5281/zenodo.13283275

2) Ballero, M. and M. Contu. 1993. Taxonomy and ecology of the genera *Pseudoclitocybe* Sing. And *Pseudoomphalina* Sing. (Basidiomycetes, Agaricales) in Sardinia. *Candollea*, 48: 601–606.

3) Bon, M. 1997. Les *Clitocybes, Omphales* et ressemblants. *Tricholomataceae* (2). *Clitocyboideae*. Flore Mycologique d'Europe. 4. Documents Mycologiques, Mémoire Hors Série No 4. Amiens, p. 181.

4) Consiglio, G., M. Contu and V. Caroti. 2006. Il genere *Pseudoomphalina* in Italia. I. Nuova descrizione di alcune specie note. *Bollettino AMER*. 68–69. Anno XXII, 2–3, 5–33.

5) Contu, M. 2003. An extraordinary new species of *Pseudoomphalina* (Basidiomycetes, Leucopaxilloideae) from Sardinia. *Micologia e Vegetazione Mediterranea*, 18(1): 61–68.

6) Hall, T. A. 1999. BioEdit: a user–friendlybiological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, 41: 95–98.

7) Jukes, T.H. and C.R. Cantor. 1969. Evolution of protein molecules. In Munro HN, editor, Mammalian Protein Metabolism, pp. 21-132, Academic Press, New York.

8) Knudsen, H. 2008. *Pseudoomphalina* (Singer) Singer. In H. Knudsen & J. Vesterholt (Eds.), Funga Nordica (pp. 238–239). Nordsvamp.

9) Kuyper, T.W. 1995. *Pseudoomphalina* (Singer) Singer. In C. Bas, T. W. Kuyper, M. E. Noordeloos, & E. C. Vellinga (Eds.), Flora Agaricina Neerlandica (pp. 93–94). Balkema.

10) Lavorato, C., A. Vizzini, Z.-W. Ge and M. Contu. 2015. Redescription of *Clitocybe umbrinopurpurascens* (Basidiomycota, Agaricales) and revision of *Neohygrophorus* and *Pseudoomphalina*. *Phytotaxa,* 219(1): 43–57. https://doi.org/10.11646/phytotaxa.219.1.3

11) Ludwig, E. 2012. Pilzkompendium Band 3 Beschreibungen. Berlin: Fungicon.

12) Malysheva, E.F., O.V. Morozova and M. Contu. 2011. New combinations in *Clitocybula*: A study of cystidiate *Pseudoomphalina* species (Basidiomycota, Agaricomycetes). *Sydowia*, 63(1): 85–104.

13) Moncalvo, J.M., R. Vilgalys, S.A. Redhead, J.E. Johnson, T.Y. James, C.M. Aime, V. Hofstetter, S.J.W. Verduin, E. Larsson, T.J. Baroni, R.T. Greg, S. Jacobsson, H. Clémençon and O.K. Miller. 2002. One hundred and seventeen clades of euagarics. Molec. *Phylogen. Evol*., 23: 357-400.

14) Mustafa, G. 2003. Mansehra, an introduction, gazetteer of the Hazara District. *Sang-e-Meel Publications, Lahore, Pakistan.*

15) Osemwegie, O.O., E.G. Eriyaremu and J. Abdulmalik. 2006. A survey of macrofungi in Edo/Delta region of Nigeria, their morphology and uses. *Global Journal of Pure and Applied Science*, 12(2): 149-157.

16) Rani, A.K., S. Hina, H. Iqbal, Z. Shabbir, A. Izhar, M. Usman and A.N. Khalid. 2023. Identification and taxonomic position of a new *Pseudoomphalina* species from Pakistan based on light, scanning electron microscopy, and molecular analysis. *Microscopy Research and Techniques*, 86(9):1144-1153.

17) Shah, G.M. and M.A. Khan. 2006. Check list of medicinal plants of Siran Valley Mansehra–Pakistan. *Ethnobotanical Leaflets*, 10: 63–71.

18) Singer, R. 1936. Notes sur quelques Basidiomycètes. II. *Revue de Mycologie*, 1: 279–2

19) Singer, R. 1948. Diagnoses fungorum novorum Agaricalium. *Sydowia*, 2(1–6): 26–42

20) Singer, R. 1986. The Agaricales in modern taxonomy (4th ed., p. 981). Koeltz Scientific Books.

21) SMEDA, NWFP. 2009. District profile Mansehra. Small and Medium Enterprises Development Authority, Ministry of Industries and Production. Government of Pakistan.

Tianjin Daxue Xuebao (Ziran Kexue yu Gongcheng Jishu Ban)/
Journal of Tianjin University Science and Technology
ISSN (Online):0493-2137
E-Publication: Online Open Access
Vol: 57 Issue: 08:2024
DOI: 10.5281/zenodo.13283275

22) Smith, A.H. and L.R. Hesler. 1943. New and interesting agarics from Tennessee and North Carolina. *Lloydia*, 6: 248–26.

23) Tamura K., G. Stecher and S. Kumar. 2021. MEGA 11: Molecular Evolutionary Genetics Analysis Version 11. *Molecular Biology and Evolution,* 38(7): 3022–3027. https://doi.org/10.1093/molbev/msab120.

24) Ullah, S., A.A. Tahir, T.A. Akbar, Q.K. Hassan, A. Dewan, A.J. Khan and M. Khan. 2019. Remote sensing-based quantification of the relationships between land use land cover changes and surface temperature over the lower Himalayan region. *Sustainability,* 11: 5492. https://doi.org/10.3390/su11195492

25) Voitk, A., I. Saar, R. Lebeuf and P. Kennedy. 2020. The *Pseudoomphalina kalchbrenneri* complex in North America. *Botany*, 98(2): 91–101. https://doi.org/10.1139/cjb-2019-0011

26) Werle, E., C. Schneider, M. Renner, M. Völker and W. Fiehn. 1994. Convenient single-step, one tube purification of PCR products for direct sequencing. *Nucleic Acids Research,* 22(20): 4354-5.

27) White, T.J., T.D. Bruns, S. Lee and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. PCR Protocol, a Guide to Methods and Applications. San Diego, CA, USA: Academic Press, pp. 315−322.

28) Walther, G., S. Garnica and M. Weiß. 2005. The systematic relevance of conidiogenesis modes in the gilled Agaricales. *Mycol. Res.,* 109: 525-544.

29) Zang, M. 2006. Boletaceae (I). Flora Fungorum Sinicorum, *Science Press, Beijing*, 22: 205.