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AGARICUS BRUNNEOSPORUS, A NEW SPECIES FROM PUNJAB, PAKISTAN

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Abstract

Agaricus species are ecologically and economically significant, yet their diversity in underexplored regions like Pakistan remains poorly documented. This study investigated *Agaricus* species from Bahawalnagar district, Punjab, Pakistan. Specimens were collected from different locations in Bahawalnagar, followed by morphological examination based on macroscopic and microscopic features. For molecular identification, DNA was extracted, and the ITS region of the nrDNA was sequenced. Phylogenetic analysis was conducted using Maximum Likelihood methods to determine the taxonomic placement of the species under investigation. The newly discovered species is morphologically similar to *A. dunensis*, with a brownish-orange central disk and light brown squamules becoming sparser at the margins. Basidiospores were subglobose to broadly ellipsoid, measuring 3.9–4.8 × 3.1–4.1 µm, and the species displayed clavate to narrowly clavate cheilocystidia. Phylogenetic analysis of the ITS region confirmed the distinctiveness of this species within *Agaricus* sect. *Minores*. The findings highlight the diversity of macrofungi in this region, with this species representing a previously undocumented taxon. This discovery enhances knowledge of Pakistan's fungal diversity and emphasizes the importance of exploring understudied regions.

Introduction

Agaricus L. (*Agaricales*, *Agaricaceae*) is a saprobic genus with a wide ecological distribution, ranging from arctic tundra to tropical rainforests. Species within this genus are found in diverse habitats, including alpine meadows, grassy dunes, salt-tolerant seaside grasslands, coniferous and deciduous forests, and various soil types. However, they generally avoid highly acidic and waterlogged soils (Bas, 1991). The genus is characterized by species that have white, yellow, or brown pileus, free gills lamellae with a regular trama when young, which later becomes irregular. The spore print appears in dark brown, featuring spores of a dark to purple-brown coloration, devoid of any discernible germ pore basidiospores smooth with a compound wall not visibly pseudo amyloid. The genus commonly displays a partial veil that develops into a ring on the stipe (Mitchel and Bresinsky, 1999; Karunarathna *et al*., 2016; Kuo, 2018; Saini *et al*., 2018; Ismail *et al*., 2023).

Agaricus is represented by 500 species divided into seven subgenera and twenty-eight sections (Heinemann, 1974; Stoichev and Lacheva, 2002; Lacheva and Stoichev, 2004; Zhao *et al*., 2016; Chen *et al*., 2017; Callac and Chen, 2018; He *et al*., 2018; Parra *et al*., 2018; Bashir *et al*., 2018, 2021, 2023, 2024; Jaichaliaw *et al*., 2021; Wang and Bau 2024). From Pakistan, more than 40 species in this genus are known. Among these, 16 species have been reported based on

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morphological characteristics; however, two species are given in Ahmad *et al*. (1997); *A. callipelus* Berk. & Br. and A. *lateritiocolor* Heinm. lack records in Index Fungorum. The remaining species have been reported on molecular basis (Thongklang *et al*., 2014; Chen *et al*., 2016; Bashir *et al*., 2018, 2021, 2023, 2024; Hussain and Sher, 2019; Niazi *et al*., 2022; Nawaz *et al*., 2024; Ullah *et al*., 2024).

Agaricus subgen. *Minores* comprises three sections: *A*. sect. *Leucocarpi* Linda J. Chen & Callac, *A*. sect. *Minores* (Fr.) Henn., and *A*. sect. *Pantropicales* L.A. Parra, Angelini, B. Ortiz, Linda J. Chen & Callac (Zhao *et al*., 2016; Chen *et al*., 2017; Parra *et al*., 2018). The characteristic feature of the *A*. sect. *Minores* is that the lower surface of the annulus is neither floccose nor squamose. The universal veil is poorly developed or absent (Zhao *et al*., 2016). *Agaricus* sect. *Minores* stands out as the most diverse section within the genus comprising 80 known species (He *et al*., 2017, 2018). It is anticipated that this section encompasses around 200 species (Chen *et al*., 2017). Among these, approximately 40 species in this section are documented in Asia and 21 are reported from Europe (Chen *et al*., 2017; Hussain and Sher, 2019). Data of the *A*. sect. *Minores* in Pakistan is extremely limited, with only two known species; *A. glabriusculus* S. Hussain and *A. latiumbonatus* S. Hussain (Hussain and Sher, 2019).

A field survey of Haroonabad, Bahawalnagar district, Punjab Pakistan was conducted during the rainy season of 2023 to collect the fungal specimens for a research project. During the survey, various basidiomata were collected. Among these, two specimens from two collections were found similar and closely resembled the species in *Agaricus*. The specimens were observed morphologically and phylogenetically, which represent a new species in *A*. sect. *Minores*.

Materials and Methods

Sample collection, *isolation*, *and specimen examination*

The specimens were collected from Haroonabad, located in the Bahawalnagar district of Punjab, Pakistan. Based on the Köppen climate classification, this area experiences a hot desert climate (BWh), characterized by extremely high temperatures and low precipitation. The temperature in the region varies between 11°C and 50°C, with an average annual rainfall of approximately 99 mm (Asif *et al.,* 2023). The vegetation in this area predominantly includes species such as *Azadirachta indica* A. Juss., *Albizia lebbeck* (L.) Benth., *Dalbergia sissoo* Roxb., *Eucalyptus camaldulensis* Dehnh. and *Vachellia nilotica* (L.) P.J.H. Hurter & Mabb. (Ahmed *et al*., 2014).

The specimens were photographed at the collection site, morphological features were noted, and the collections were dried using a fan heater. Key characteristics, such as the pileus' size, shape, and color, lamellae attachment and color, and the presence and type of annulus on the stipe, were noted. For color specification, Munsell color chart mobile application was used (https://munsell.com/color-blog/tag/color-app/). An EU 2230436 microscope with a 100X objective lens was used to observe anatomical features. Various chemicals, including 5% KOH for rehydration and Congo red for staining hyaline tissues, were used as mounting media. Measurements were recorded using the Calibrated Motic Images Plus 2.0 software. Microscopic characteristics were based on a minimum of 20 measurements for each structure. The symbol '(e) f–g (h)' was used to indicate the basidiospore size, where 'f–g' represented 90% of measured values, and 'e' and 'h' indicated extreme values. 'Q' denoted the length-to-width ratio of a single basidiospore from the side view, while 'avQ' represented the average 'Q' value for all specimens. Other microstructural measurements included the range between extreme length and width measurements. The specimens were deposited in the Herbarium at the Department of Botany, University of Education, Lahore, Pakistan (UEH).

DNA extraction, *PCR amplification*, *and sequencing*

The DNA extraction was done by Bruns (1995) CTAB method. PCR and sequencing used the ITS1F and ITS4 primers (White *et al*., 1990). Sequences were analyzed in BioEdit version 7.2.5 (Hall, 1999). Phylogenetic reconstruction involved selecting close matches from GenBank, excluding sequences with inadequate query coverage. The phylogenetic tree included published sequences of species' closest relatives with corresponding GenBank accessions. To root the tree, sequences from *A*. sect. *Agaricus* were chosen as the out group (Bashir *et al*., 2021, 2024; Hussain and Sher, 2019). Multiple sequence alignment was performed with the online MUSCLE tool at EMBL-EBI (https://www.ebi.ac.uk/jdispatcher/msa/muscle). Phylogenetic analysis was performed to investigate the evolutionary relationships of the sequences. The best-fit nucleotide substitution model was selected based on model selection criteria, using the MEGA version 6 software (Tamura *et al*., 2013). Kimura 2-parameter model (Kimura, 1980) was found to be the most suitable model and applied to account for the nucleotide substitution patterns. Phylogeny was inferred using Maximum Likelihood (ML) for evolutionary distance estimation. The support for the branches was evaluated by performing 1000 bootstrap replicates to assess the robustness and statistical significance of the tree topology.

Phylogenetic analyses

The ITS sequence of 677 base pairs from our Pakistani collection UEH-F0020 was BLAST searched at NCBI. It showed 100 % similarity with a sequence from Pakistan and 99.12–99.38% similarity with *Agaricus* sequences from Pakistan and India. These sequences along with closely related sequences of *Agaricus* spp. from *A*. sect. *Minores* were retrieved from GenBank. The final aligned dataset contained a total of 50 nucleotide sequences, including two sequences: *A*. *campestris* L. (KM657927) and *A*. *langei* (F.H. Møller) F.H. Møller (JF797181) as out group (Table 1). The final dataset contained a total of 706 positions in the final dataset. Out of which, 493 sites were conserved, 201 variable, 125 parsimony informative and 74 singletons. The sequences from our collections appeared in a clade of *A*. sect. *Minores* separating from other *Agaricus* spp. in the same section. The sequences generated from the current collections clustered in a clade that includes recently published taxon, *A*. *dunenesis* H. Bashir & M. Asif sequences from India and Pakistan along with *A*. *parvibicolor* Linda J. Chen, R.L. Zhao & K.D. Hyde (NR_151751) from Thailand with 86% boot strap value. The clade representing *A*. *dunensis* is split into two lineages. The sequences generated during this investigation are separated from the clade that includes the type sequence of *A*. *dunensis* (ON137217) and two additional sequences labeled as *A*. *dunensis* (ON158599 & ON158600), suggesting that these sequences represent a separate taxon distinct from *A*. *dunensis* (Fig. 1).

Results and Discussion

Taxonomy

Agaricus brunneosporus Raza, Bilal & Jabeen sp. nov. (**Figs 2, 3**)

MycoBank: MB 852517

Etymology: The specific epithet "*brunneosporus*" (Latin) refers to the brown basidiospores.

Diagnosis: Agaricus brunneosporus differs from *A*. *dunensis* by its brownish orange central disk and light brown squamules which become sparse towards margins, subglobose to broadly ellipsoid smaller (3.9–4.8 × 3.1–4.1 μm) basidiospores and clavate to narrowly clavate cheilocystidia.

Holotype: PAKISTAN. Punjab, Bahawalpur division, Bahawalnagar district, Haroonabad, 160 m a. s. l., on soil, 26 July 2023, Muhammad Bilal Sharif P325 (UEH-F0020; GenBank for ITS: PP262619).

Table 1. Taxa used for constructing the phylogenetic tree, along with their voucher numbers, geographical localities and GenBank accession numbers.

Species	Voucher	Country	ITS	References
Agaricus armandomyces	ZRL2015992	China	KX684860	He *et al*. (2017)
A. bonussquamulosus	ZRL2010106	China	KX657047	He *et al*. (2017)
A. brunneolus	LAPAG938	Spain	KU975082	Chen *et al*. (2017)
A. brunneosporous as *A. dunensis*	LAH36806	Pakistan	ON158599	Bashir *et al*. (2024)
A. brunneosporus	UEH-F0020	Pakistan	PP262619	This study
A. brunneosporus	UEH-F0021	Pakistan	PP262620	This study
A. brunneosporus	UEH-F40027	Pakistan	PV796086	This study
A. brunneosporus as *A. dunensis*	BWN-67 (LAH36805)	Pakistan	ON158600	Bashir *et al*. (2024)
A. campestris	LAPAG370	China	KM657927	Ling *et al*. (2021)
A. coccyginus	275412	China	KU245981	Unpublished
A. comtulus	2692	Canada	KM248904	Unpublished
A. diminutivus	WC912	USA	AY484681	Geml *et al*. (2004)
A. dulcidulus	PRM-909627	Czech Republic	KF447894	Parra (2013)
A. dunensis	LAH36806	Pakistan	ON158599	Bashir *et al*. (2024)
A. dunensis	LAH35748	Pakistan	ON137218	Bashir *et al*. (2024)
A. dunensis	LAH 35747	Pakistan	ON137217	Bashir *et al*. (2024)
A. dunensis	CUHAM737	India	OM654930	Bashir *et al*. (2024)
A. dunensis	LAH36808	Pakistan	ON158597	Bashir *et al*. (2024)
A. dunensis	LAH21719	Pakistan	ON158598	Bashir *et al*. (2024)
A. dunensis	LAH36807	Pakistan	ON158596	Bashir *et al*. (2024)
A. dunensis	LAH35749	Pakistan	ON137219	Bashir *et al*. (2024)
A. edmondoi	LAPAG80	Spain	KF447902	Parra (2013)
A. elongatestipes	ZRL2013271	China	KX657002	He *et al*. (2017)
A. friesianus	LAPAG592	France	KT951316	Parra (2013)
A. glabriusculus	SH7	Pakistan	MK751852	Hussain and Sher (2019)
A. heinemannianus	LAPAG302	Spain	KF447906	Parra (2013)
A. indicus	TBGT16128	India	OR661746	Arya and Pradeep (2024)
A. jacobi	AH-44505	Spain	NR_158300	Parra (2013)
A. jingningensis	ZRL20151562	China	KX684877	He *et al*. (2017)
A. kerriganii	AH-44509	Spain	KF447893	Parra (2013)
A. lamelliperditus	MDBF61/96	Australia	JX984559	Lebel (2013)
A. langei	LAPAG141	Spain	JF797181	Zhao *et al*. (2011)
A. latiumbonatus	SH166	Pakistan	MK751861	Hussain and Sher (2019)
A. luteomaculatus	CA331	France	KF447901	Parra (2013)
A. marisae	LAPAG138	Spain	KU975083	Chen *et al*. (2017)
A. matrum	AH-44506	Spain	KF447896	Parra (2013)
A. megalosporus	MFLU:100774	Belgium	NR_119951	Unpublished
A. midnapurensis	CUH AM718	India	OL467539	Tarafder *et al*. (2022)
A. neimengguensis	HMAS:254648	China	NR_189789	He *et al*. (2017)
A. pallens	LAPAG441	Spain	KF447898	Parra (2013)
A. palodensis	TBGT17483	India	OR661748	Arya and Pradeep (2024)
A. parvibicolor	MFLU:12-0953	Thailand	NR_151751	Liu *et al*. (2015)
A. parvibrunneus	HMAS0278356	China	MG137001	He *et al*. (2018)
A. pseudopallens	ZRL20151552	China	KX684874	He *et al*. (2017)

A. purpurellus	TRgmb01309	Italy	KF447903	Parra (2013)
A. purpurellus	-	Canada	MN620489	Unpublished
A. purpureosquameus	MFLU:17-1306	Thailand	NR_157484	Unpublished
A. purpureosquamulosus	CUH AM716	India	OL467541	Tarafder *et al*. (2022)
A. robustulus	CA847 (MFLU16-0973)	Thailand	KU975086	Chen *et al*. (2017)
A. yanzhiensis	HMAS0281083	China	MG137003	He *et al*. (2018)

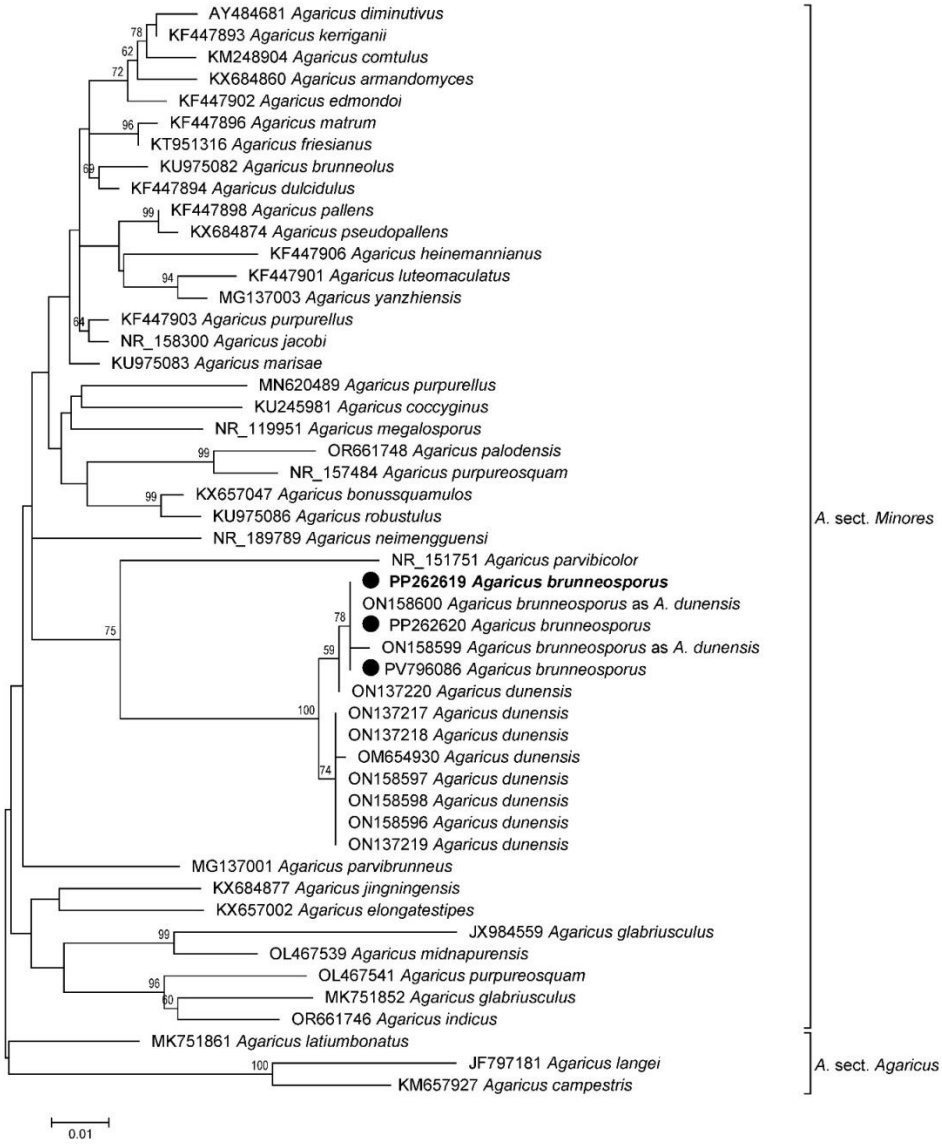

Fig. 1. Molecular phylogenetic analysis of *Agaricus brunneosporus* and related species based on ITS sequences. GenBank accession numbers are provided for each taxon. Sequences generated during this study are marked with bullets. Bold face represents sequence from type species.

Fig. 2. *Agaricus brunneosporus*. a, b. UEH-F20020 (Holotype), c, d. UEH-F0021. Scale bars: 1 cm.

Description

Pileus 3–7 cm diam., parabolic to hemispherical; surface squamulose, dense at center, sparse towards margins; margins entire to eroded; central disk brownish orange (7.5YR 7/8), light brown (10YR 8/6), white fibrillose context. Lamellae free, alternate with lamellulae; brownish pink (5RP 7/4); lamellulae frequent, variable in length. Stipe 4.2–5.0 × 0.6–0.8 cm, central, cylindrical, bulbous base; surface smooth to slightly fibrillose, brown (7.5YR 7/3) from base to annulus, white above annulus. Annulus superior, white. Context white, firm, no color change on exposure to air.

Basidiospores [40/2/2] (3.5)3.9–4.8(4.9) × (3)3.1–4.1(4.2) µm, Q = 1–1.3, avQ = 1.15, subglobose to broadly ellipsoid, smooth, thick walled, apiculus prominent, monoguttulate, brown in 5 % KOH. Basidia (11)13–14(15.5) × (5.1)5.5–5.6(5.7) µm, clavate, basal cell present, 2–4 sterigmata. Cheilocystidia (10)11.5–12(12.7) × (4.1)4.7–5.1(5.3) µm, clavate to narrowly clavate, septa present at the base. Pileipellis hyphae (4.5)5–5.8(6.7) µm wide, septate, cylindrical,

branched. Stipitipellis hyphae (4.1)5.1–6(6.9) µm wide, septate, branched. Clamp connections absent in all tissues. All tissues hyaline in 5 % KOH.

Fig. 3. Microscopic characters of *Agaricus brunneosporus* UEH-F0020 (holotype). a. Basidiospores; b. Basidia; c. Cheilocystidia; d. Stipe hyphae; e. Pileipellis hyphae. Scale bars: a–c = 5 µm; d & e = 10 µm.

Habitat and distribution: Found solitary in a hot desert climate with dominant vegetation of *Azadirachta indica*, *Albizia lebbeck*, *D. sissoo*, *E. camaldulensis*, and *V. nilotica*.

Additional material examined: PAKISTAN. Punjab, Bahawalpur division, Bahawalnagar district, Haroonabad, 160 m a. s. l., on the soil, 26 July 2023, Muhammad Bilal Sharif P220 (UEH-F0021; GenBank for ITS: PP262620); 9 August 2024, Muhammad Bilal Sharif P654 (UEH-F40027; GenBank for ITS: PV796086).

Agaricus brunneosporus is characterized by its hemispherical pileus covered with brownish orange squamules on white fibrillose surface, having pink and later brownish lamellae and slightly fibrillose stipe bearing a white superior annulus. The basidiospores are brown, subglobose to broadly ellipsoid (3.9–4.8 × 3.1–4.1 µm) without a germ pore.

Agaricus dunensis, a recently reported taxon from Punjab, Pakistan differs from *A. brunneosporous* by its pileus thoroughly covered by orange-yellow squamules, comparatively small and thicker stipe, subglobose to ellipsoid larger (6.3–6.9 × 5–5.3 µm) basidiospores and polymorphous cheilocystidia (Bashir *et al*., 2024). *Agaricus parvibicolor*, a closely related taxon, differs from *A. brunneosporus* by its hemispherical to convex pileus surface having violet-brown

fibrils and a crenulate annulus on the stipe. Its basidiospores are ellipsoid to oblong and larger (4.7–5.5 × 3–3.5 µm) (He *et al*., 2018). *Agaricus glabriusculus* S. Hussain, a Pakistani taxon, differs from *A*. *brunneosporus* by its fibrillose pileus surface having pinkish fibrils, pendant annulus and relatively larger (6–6.5 × 4–4.5 µm), cylindrical to broadly ellipsoid basidiospores (Liu *et al*., 2015).

Agaricus latiumbonatus S. Hussain another Pakistani species differs from *A*. *brunneosporus* by a white pileus having grayish-red to dark-red squamules, arranged in rings and by a broadly umbonate disc, a stipe bearing pendant ascending annulus and by ellipsoid to broadly ellipsoid or amygdaliform, and relatively small (4.8–5.5 × 3.2–3.8 µm) basidiospores (Hussain and Sher 2019). *Agaricus megalosporus* J. Chen, R.L. Zhao, Karun. & K.D. Hyde from Thailand is distinct from *A*. *brunneosporus* by its hemispherical, purplish-brown to brown pileus with an eroded surface, and by a membranous pendant annulus. Its basidiospores are relatively larger (5.5–6.5 × 7–7.5 µm) and ellipsoid to oblong, rarely cylindrical, smooth, reddish-brown, and thick-walled (Chen *et al*., 2012).

Agaricus parvibrunneus M.Q. He, K.D. Hyde & R.L. Zhao another Asian species from China is different from *A*. *brunneosporus* by its brown fibrillose pileus surface, pinkish brown lamellae and a white pendent annulus. It further has smooth, thick-walled, and larger (5.0–5.8 × 3.7–4.1 µm) basidiospores (He *et al*., 2018). *Agaricus velutinosus* T. Bau & S.E. Wang from Northeast China differs from *A*. *brunneosporus* by fibrillose minutely floccose pileus and stipe surfaces and by initially white lamellae, and a stipe with superior white annulus. The basidiospores are relatively smaller (2.9–3.0 × 3.7–3.8 µm) ellipsoid to elongate-ellipsoid, thick-walled, and guttulate (Wang and Bau, 2024).

Besides these morpho-anatomical differences, molecular phylogenetic analysis based on sequences from ITS region of nrDNA showed that this species formed its own lineage separated from already known taxa in *A*. sect. *Minores* with a strong bootstrap support.

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