Original Research



Comparison of physiological and cultivation characteristics of wild strains of Nameko (*Pholiota microspora*) from Bhutan and Japanese commercial spawn

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Abstract

Wild fruiting bodies of Pholiota microspora (Berk.) Sacc., commonly known as nameko, were collected from five locations in western Bhutan at altitudes ranging from 1,769 to 3,100 m between 2018–2022. Morphological and microscopic observations of the specimens submitted to the national herbarium revealed no clear basis for subspecies differentiation. To support the development of domestic cultivation and potential overseas marketing of nameko in Bhutan, eight Bhutanese wild strains isolated from these specimens were evaluated and statistically compared with two Japanese commercial strains. The aim was to assess their industrial applicability and gather key traits for breeding strategies. The Bhutanese wild strains exhibited notable diversity in cap and stem coloration as well as in mycelial growth characteristics. Unlike the Japanese strains, none of the Bhutanese strains exhibited the formation of abnormal dedikaryotized flat sectors under incubation at 25 °C and 30 °C, a phenomenon known to negatively impact cultivation stability. All Bhutanese strains displayed bipolar mating systems and were capable of mating with Japanese strains, suggesting strong potential as breeding material to address the genetic bottleneck caused by the reliance on a single ancestral strain in Japan's air-conditioned nameko cultivation. All tested wild strains successfully formed fruiting bodies on sawdust substrate and tended to exhibit early fruiting. Two strains that showed yields comparable to Japanese strains and significantly earlier harvest times were selected as candidates for practical cultivation in Bhutan. These findings indicate that selective breeding from wild fruiting bodies represents a highly effective and accessible strategy for developing commercially viable nameko strains suited to Bhutanese agriculture and beyond.

Keywords: Cultivation, Domestication, Mating, Selection breeding, Spawn

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Introduction

Pholiota microspora (Berk.) Sacc. has historically been misidentified in Japan, often being confused with other fungi exhibiting a glutinous pileus, such as Collybia velutipes (Curtis) P. Kumm., the former scientific name for what is now recognized as Flammulina velutipes (Curtis) Singer (Kawamura, 1930, 1931). Ito (1929) initially designated the species as Collybia nameko T. Ito, which was subsequently revised to Pholiota nameko (T. Ito) S. Ito & S. Imai by Imai (1933). Neda (2008) later concluded that Pholiota nameko was conspecific with a specimen collected from Nepal, and accordingly consolidated the nomenclature under *Pholiota* microspora, the name originally assigned to the species by Berkeley (1850). However, a more recent specimen collected in Nepal was described as Pholiota microspora var. hymalensis in 2014 (Adhikari et al., 2014), based on four distinguishing characteristics: variation in pileus coloration, the presence of scales on the pileus and stipe, the presence of pleurocystidia, and differences in spore morphology. Given the geographic proximity of Bhutan to Nepal, it is plausible that wild Bhutanese strains identified as *Pholiota microspora* may represent a distinct subspecies. Nonetheless, in the present study, the name *Pholiota microspora* is provisionally applied to the Bhutanese strains, as DNA sequence data necessary to definitively resolve their taxonomic status at the subspecies level are not yet available.

Pholiota microspora is a white-rot fungus that colonizes dead or decaying wood in cooltemperate deciduous forests across East Asia, with a natural distribution extending from the Himalayas and China to Japan (Adhikari et al., 2014; Berkeley, 1850; Neda, 2008). In Bhutan, the National Mushroom Centre identified and recorded a total of 25 specimens between 2010 and 2023—three as *Pholiota nameko* and 22 as *Pholiota microspora*. These specimens have been catalogued on the Bhutan Biodiversity Specimen Portal (2024) and are preserved in the Bhutan National Herbarium (National Biodiversity Centre, Ministry of Agriculture and Livestock, 2024). The recorded collection months for these specimens are as follows: one in March, four in May, six in June, seven in July, one in August, two in September, one in October, and one in November. This temporal distribution indicates that the primary natural fruiting season for *P. microspora* in Bhutan occurs between May and July. This phenological pattern contrasts with that observed in Japan, where fruiting typically occurs from October to November (Hirao et al., 2022).

Pholiota microspora, commonly known as *nameko* in Japan, is one of the country's commercially important mushroom species, with an annual production of 24,063 tons reported in 2021 (Ministry of Agriculture, Forestry and Fisheries, 2021). Characterized by its distinctive glutinous cap, nameko is highly favoured in Japanese cuisine and is considered an essential ingredient in various traditional dishes. The cultivation of this species has expanded beyond Japan and is now practiced in several Asian countries, as well as in North America and Europe. China has emerged as the largest producer, with annual production ranging from 540,000 to 1,771,000 tons between 2010 and 2019 (Singh et al., 2021). The sawdust-based cultivation method for *Pholiota microspora (nameko)* was first established by Hikosaburō Morimoto in 1930 (Morimoto, 1930). A pivotal advancement occurred with the isolation of the F27 strain from a wild fruiting body collected in *Fukushima Prefecture* in 1962 (Nakamoto et al., 1967). This strain rapidly disseminated among *nameko* growers throughout Japan via commercial spawn suppliers. The favourable cultivation traits of the F27 strain facilitated the development



of air-conditioned cultivation systems, significantly enhancing productivity. Contemporary sawdust-cultivated *nameko* strains in Japan are believed to have originated from the F27 lineage (Kanno et al., 2016). This has been corroborated by nuclear simple sequence repeat (SSR) analysis, which demonstrated that all commercially cultivated *nameko* strains using sawdust substrates are genetically derived from F27 (Hirao et al., 2022). Furthermore, Hirao et al. (2022) revealed that while cultivated strains are derived from a very narrow genetic base, a wide range of genetic diversity persists among wild populations.

In recent years, the Ministry of Agriculture and Livestock of Bhutan has placed increasing emphasis on the potential value of indigenous wild fungal strains. In response, the National Mushroom Centre has actively initiated the collection of not only herbarium specimens but also living wild strains, with the aim of developing viable cultivation methods. As part of these efforts, on-site cultivation trials using Bhutanese wild strains of *Pholiota microspora (nameko)* commenced in 2024, with the goal of promoting their adoption by local farmers. For the sustainable development of a national *nameko* industry, it is essential to clarify the taxonomic identity of Bhutanese wild strains and to establish a practically cultivable Bhutanese variety through targeted breeding efforts.

To support the development of *nameko* cultivation and its potential for overseas marketing in Bhutan, a comparative study was designed to evaluate domestic wild strains alongside Japanese commercial strains. This study presents an assessment of Bhutanese wild *Pholiota microspora* strains as breeding material for practical cultivation in Bhutan, focusing on morphological, basic physiological, mating compatibility, and cultivation characteristics. The objectives of each test were as follows: morphological analyses aimed to inform both taxonomic classification and commercial product value; basic physiological tests were conducted to ensure cultivation stability; mating compatibility tests were performed to support the future development of practical strains; and cultivation trials were designed to evaluate the potential for selection breeding from wild populations. Each trait was quantitatively measured to generate foundational data for establishing breeding strategies and objectives. This report seeks to advance the industrial application of Bhutanese wild strains. Results from internal transcribed spacer (ITS) region sequencing for determining taxonomic position will be presented in a separate report.

Material and methods

Strains collection

Wild fruiting bodies of *Pholiota microspora* were collected from five locations in western Bhutan, at altitudinal ranges between 1,769 m and 3,100 m, during the period from 2018 to 2022 (Table 1). Three of these specimens—NMC/02568, NMC/02604, and NMC/02605 were submitted to and are preserved in Bhutan National Herbarium. From these collections, eight Bhutanese wild strains were successfully isolated. These strains, designated as test numbers 3 through 10, were evaluated for mycelial growth, cultivation performance, and morphological characteristics in comparison with two Japanese commercial strains, used as controls (test numbers 1 and 2). The control strains included N405, a late-ripening variety, and N217, an early-ripening variety, both developed for natural cultivation and supplied by Hokken Co., Ltd. For the mating compatibility test, basidiospores derived from the wild fruiting bodies of NMC/02568 (test A), NMC/02604 (test B), and NMC/02605 (test C) were utilized. Statistical comparisons between Bhutanese wild strains and Japanese commercial strains were conducted using a two-sample t-test assuming equal variances, implemented in Microsoft Excel.



Mycelium growth characteristics

Mycelial growth characteristics were assessed for eight Bhutanese wild strains (test numbers 3-10) and two Japanese commercial strains (test numbers 1 and 2). Each strain was initially inoculated onto 20 mL of Potato Dextrose Agar (PDA; Pallav Chemicals and Solvents Pvt. Ltd., India) in 9 cm diameter Petri dishes and incubated at 25 °C for two weeks. From each culture, a 5 mm diameter plug was excised using a cork borer and transferred to fresh PDA plates. The inoculated plates were incubated at five different temperatures ($10 \,^\circ$ C, $15 \,^\circ$ C, $20 \,^\circ$ C, $25 \,^\circ$ C, and $30 \,^\circ$ C), with five replicates per temperature treatment. To measure mycelial growth, the radial expansion of the colony was marked in four directions at two time points: when the colony had grown approximately 5 mm and 25 mm from the point of inoculation. Growth rates were calculated in millimeters per day (mm/day) based on the distance and time elapsed between the two marks. To evaluate the uniformity of radial growth, the coefficient of variation (CV) of growth rates across the four directions on each plate was calculated. This provided an index of eccentric mycelial growth, which may be relevant to strain selection for stable cultivation.

Table 1. List of <i>I notioid microspord</i> strains included in this paper	Table 1.	List of Pholiota	microspora	strains	included	in this paper
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	DNA	Date of	Place	e of collection				Physiological	Mating
Herbarium No. ^{**1}	Sample No. ^{**2}	collection	Place name	Latitude and Longitude	Altitude (m asl)	Collector	Isolator	and cultivational test no.	test no.
-	J1	-	Japanese commercial strain (N405)	-	-	-	-	1	J1
-	J2	-	Japanese commercial strain (N217)	-	-	-	-	2	J2
-	N1	2018/5/18	Pelela	N27.529784 E90.244740	3,100	D	RG	3	-
-	N2	2022/6/25	Hontsho, Tashigang Goenpa, Thimphu	N27.472972 E89.727439	2,900	D	D	4	-
NMC/02568	N6	2022/8/19	Thimphu (Royal Botanical Garden)	N27.4254 E89.6496	2,420	SP, DL	(Specimen)	-	А
NMC/02604	N8	2022/9/19	Thimphu (Royal Botanical Park)	N27.507 E89.751	2,800	AK, SP, DL	(Specimen)	-	В
-	N9	"	"	"	"	"	AK	5	-
-	N10	"	"	"	"	"	"	6	-
-	N11	"	"	"	"	"	"	7	-
NMC/02605	N12	"	Phunaka, Lumitsawa	N27.31.17 E89.46.45	2,220	"	(Specimen)	-	С
-	N13	"	"	"	"	"	AK	8	-
-	N14	"	"	"	"	"	"	9	-
-	N15	"	"	"	"	"	"	10	-

Notes: ^{**1}; Specimen number at the Bhutan National Herbarium. ^{**2}; DNA sample number at the NMC (unpublished). D: Dorji, SP: Sabitra Pradhan, RG: Rita Gurung, DL: Deki Lhamo, AK: Atsushi Kumata

The proportion of the colony area occupied by flat sectors—regions with reduced aerial hyphal development—was quantified using photographic image analysis with the open-source software ImageJ (Wayne Rasband, NIH). Photographs were taken 30 days post-inoculation under standardized lighting conditions using a digital camera (Sony RX100 III) set to auto mode. Images were captured in RGB color format at 10-megapixel resolution and converted to 8-bit grayscale for analysis. Thresholding was applied to isolate and measure the flat sector area. The occupied ratio was calculated by dividing the measured area of the flat sector by the total area of mycelial spread. The presence of clamp connections within the flat sectors was examined microscopically to confirm the dikaryotic nuclear phase, particularly in colonies incubated at 25 °C or 30 °C, where flat sectors were most prominently observed.



Zone line formation was assessed among all pairwise combinations of the ten test strains using the same Potato Dextrose Agar medium employed in the mycelial growth tests. The method followed the guidelines established in the *Hericium erinaceum* (Bull.: Fr.) Pers. Variety Registration Examination Criteria (Ministry of Agriculture, Forestry and Fisheries, Japan, n.d.). Dual inoculations were performed with a 25 mm spacing between 5 mm diameter plugs taken from each strain. The inoculated plates were incubated at 25 °C for approximately 30 days. Zone line formation at the interface of paired colonies was classified into three categories: (1) coloured zone line, (2) uncoloured zone line, and (3) absence of a zone line.

Mating characteristics

Ten monokaryotic strains were isolated from germinated basidiospores derived from spore prints of three wild fruiting body specimens (test numbers A, B, and C), using the dilution plate method. Self-mating tests were performed to determine mating types and to select appropriate tester strains for mating type identification. Additionally, di-mon mating tests were conducted by pairing each monokaryotic tester strain with dikaryotic mycelia of the two Japanese commercial strains.

All mating tests were conducted on the same PDA medium used for mycelial growth studies. Inoculation spacing on the plates mirrored that of the dual culture method, with each plate incubated at 25 °C for 30 days. For the self-mating and mon-mon pairing tests, the formation of clamp connections was examined microscopically at the interaction zones between colonies on both inoculated sides. In the di-mon mating tests, clamp connections were specifically assessed in the area of the monokaryon colony outside the interaction zone to determine successful nuclear migration and dikaryon formation.

Cultivation characteristics and morphological feature of fruiting body

For the cultivation trials, a substrate composed of 83 % broad-leaved sawdust (*Alnus nepalensis*) and 17 % rice bran (by dry weight) was prepared, with the final moisture content adjusted to 64 %. A total of 580 g of the moist substrate was packed into 850 mL polypropylene bottles (65 mm diameter), which were then sterilized at 121 °C for 60 minutes. After overnight cooling, the bottles were inoculated with spawn from eight Bhutanese wild strains (test numbers 3–10) and two Japanese commercial strains (test numbers 1 and 2), with seven replicates per strain. The inoculated bottles were incubated for 70 days at 15–20 °C in complete darkness, followed by transfer to a fruiting chamber maintained at 13–18 °C with relative humidity exceeding 90 %. Lighting in the fruiting room consisted of indirect sunlight supplemented with at least 200 lux from a fluorescent lamp during the daytime, which was switched off at night. Ventilation was provided by opening a small window for six hours each day.

Morphological measurements and color evaluations were conducted during the first flush of fruiting. For each bottle, ten representative fruiting bodies were selected, and the diameters and lengths of the caps and stems were measured in two perpendicular directions using a digital vernier caliper. Color characteristics of the cap, gills, and stem were quantified for three randomly selected fruiting bodies per bottle. High-resolution RGB images (10 megapixels), taken with a digital camera (Sony RX100 III) under standardized fluorescent lighting against a black background, were analysed using ImageJ software. The images were converted to HSV (hue, saturation, brightness) format, and specific regions of interest were outlined—caps and gills with ovals, and stems with rectangles—ensuring maximal coverage of the targeted structures. Color component distributions (0–255 scale for each HSV channel) were extracted, and mean values were calculated to represent the color characteristics of each morphological



part per strain.

The scale-occupied ratio on the stem surface was also analysed from RGB images, which were converted to 8-bit grayscale. Thresholding was used to isolate and quantify the area occupied by stem scales, expressed as a percentage of the total visible stem surface. Spore prints were obtained on white paper from three fruiting bodies per strain and analysed using the same HSV method as for the cap. Microscopic observations were conducted using a ZEISS AxioLab 5 microscope equipped with an AxioCam 208 color camera. The shapes of basidiospores and the presence of pleurocystidia were documented. The long and short axes of 20 basidiospores per strain were measured using ZEISS ZEN software for research applications.

Results

Morphological description (Fig. 1)

Pileus 15–80 mm in diameter, fleshy, initially hemispherical to weakly conical, becoming convex to nearly plane at maturity. Surface shiny and smooth, dark brown to dark yellow at the center, becoming paler toward the margin and further fading with age; surface glabrous and glutinous. Margin thin, initially incurved, later flattening and occasionally becoming wavy; not striate; appendiculate with remnants of a glutinous, dark-coloured veil. Context light in color, with the flesh ranging from white to slightly creamy. *Lamellae* crowded to very crowded, broad, light cream in immature stages, becoming rust-coloured at maturity. *Stipe* 20–90 mm in length, 5–20 mm thick, generally cylindrical but slightly thickened toward the base; viscid, nearly solid, coloured similarly to or slightly paler than the pileus margin; glutinous below the annulus and nearly white, with surface scales matching the pileus in color. *Annulus* is initially cobwebby and ephemeral, eventually disappearing and leaving behind a dark-coloured veil remnant on the stipe. *Basidiospores* measuring $3.0-3.4 \times 4.4-4.9 \mu m$, predominantly ellipsoid-oval to slightly ovoid-ellipsoid, smooth, lacking a germ pore; spore print dark rust brown. *Cystidia* were not observed. Taste, odour, and texture were all reported to be favourable.

Specimens examined: NMC/02568, NMC/02604, and NMC/02605.



Fig. 1 - *Pholiota microspora* fruiting bodies collected in the wild and submitted to Bhutan National Herbarium. Specimens shown correspond to herbarium accessions NMC/02568, NMC/02604, and NMC/02605 (see Table 1). Arrows indicate scale bars of 25 mm.



Characteristic value for the size of fruiting body and stem

The status of fruiting body formation for each strain under bottle cultivation is presented in Fig. 2, while quantitative characteristics of cap and stem dimensions are shown in Fig. 3. The cap diameter of strains 4, 6, 7, and 10 was significantly larger than that of the Japanese commercial strain no. 2 ($\alpha = 0.05$). Similarly, cap thickness in the Bhutanese wild strains—except for strains 3 and 9—was also significantly greater than that of strain no. 2 ($\alpha = 0.05$). In contrast, stem diameter for all Bhutanese wild strains, except for strain 8, was significantly smaller than that of one or both Japanese commercial strains ($\alpha = 0.01$ or 0.05). Stem length in most Bhutanese wild strains was not significantly different from that of the Japanese strains, except in strains 7, 9, and 10, which exhibited significant differences.



Fig. 2 - Fruiting body formation of each *Pholiota microspora* strain under bottle cultivation. Strain numbers 1–10 correspond to those listed in Table 1. The bottle neck diameter is 65 mm. Each scale division in the black background photographs represents 10 mm.

Colour characteristics

Color characteristics of the cap, stem, and gills were analysed in terms of hue, saturation, and value (HSV), with results presented in Fig. 4. Among the Bhutanese wild strains, only strain 5 showed no significant differences from both Japanese strains in all three parameters (hue, saturation, and value) for the cap. The remaining Bhutanese strains generally exhibited lower saturation and higher brightness (value) compared to the Japanese strains. For stem hue, strains 4, 7, and 10 did not differ significantly from either Japanese strain. In terms of stem saturation, all Bhutanese wild strains showed no significant differences from the Japanese strains. However, except for strains 3 and 5, the Bhutanese strains tended to exhibit higher brightness (value), with significant differences observed relative to one or both Japanese strains. Regarding the gills, only strain 9 exhibited a significant difference in hue and value compared to Japanese strain no. 2. For gill saturation, strains 4, 7, 9, and 10 showed no significant differences from either Japanese strain in the strain strain of stem saturation.





Fig. 3 - Characteristic values for cap and stem size in each *Pholiota microspora* strain. Strain numbers correspond to those listed in Table 1. Error bars indicate standard deviations. $\Rightarrow (\alpha = 0.05)$ and $\Rightarrow \Rightarrow (\alpha = 0.01)$ denote significant differences from strain no. 1 (t-test). $\Rightarrow (\alpha = 0.05)$ and $\Rightarrow \pm (\alpha = 0.01)$ denote significant differences from strain no. 2 (t-test).



Fig. 4 - Color characteristic values (hue, saturation, and value) of the cap, stem, and gills for each *Pholiota microspora* strain. Strain numbers correspond to those listed in Table 1. For the meaning of star symbols and error bars, refer to Fig. 3.



Scales on Stem Surface

Scales were observed on the stem surfaces of both the Bhutanese wild strains and the two Japanese commercial strains (Fig. 5). Strains 3 and 9 exhibited significantly higher scale-occupied ratios on the stem surface compared to both Japanese strains. In contrast, strain 10 showed a significantly lower scale-occupied ratio than Japanese strain no. 2.



Fig. 5 - Scale-occupied ratio on the surface of the stem for each *Pholiota microspora* strain. Strain numbers correspond to those listed in Table 1. For the meaning of star symbols and error bars, refer to Fig. 3.

Colour of the spore print

The results of HSV color analysis of spore prints are presented in Fig. 6. No significant differences in hue were observed between the two Japanese strains and Bhutanese strains 4, 5, and 9. For saturation, no significant differences were found for strains 7, 8, 9, and 10, while for value, strains 4, 9, and 10 showed no significant differences. Notably, only strain 9 exhibited no significant differences from both Japanese strains across all three HSV parameters.



Fig. 6 - Results of HSV color analysis of spore prints for each *Pholiota microspora* strain. Strain numbers correspond to those listed in Table 1. For the meaning of star symbols and error bars, refer to Fig. 3.

Microscopic features

Basidiospore dimensions were measured as $4.8 \pm 0.5 \times 3.4 \pm 0.4 \mu m$ for Japanese strain 1, and $4.2 \pm 0.4 \times 2.7 \pm 0.3 \mu m$ for Japanese strain 2 (Table 2). The Bhutanese wild strains exhibited spore size ranges of $4.4-4.9 \times 3.0-3.4 \mu m$, generally overlapping with the ranges observed in the Japanese strains. Photomicrographs of basidiospores for each strain are shown in Fig. 7. The basidiospores of both Japanese strains and Bhutanese strains 3-8 were primarily ellipsoid-oval in shape. In contrast, basidiospores of strains 9 and 10 were slightly ovoid-ellipsoid. Pleurocystidia were not observed in any of the Japanese commercial strains (1 and 2) or the Bhutanese wild strains (3–10).

Mycelium growth characteristics

At an incubation temperature of 30 °C, all Bhutanese wild strains exhibited significantly lower mycelial growth rates ($\alpha = 0.01$ or 0.05) compared to the two Japanese commercial strains (Fig. 8). The mean coefficient of variation (CV) of radial growth across each plate was highest at



30 °C and lowest at 15 °C across all strains, suggesting eccentric hyphal growth under higher temperature conditions and nearly perfect circular growth at cooler temperatures. Significant differences ($\alpha = 0.05$) in CV at 15 °C were observed between Bhutanese strains 3, 6, 8, and 9 and Japanese strain no. 1, indicating that these Bhutanese strains demonstrated more uniform (circular) growth under low-temperature conditions. The flat sector occupied ratio was highest at 30 °C for the Japanese strains and at 25 °C for the Bhutanese wild strains. For the Japanese strains, dedikaryotization was confirmed in the hyphae of flat sectors in all but one of the ten replicate plates at 30 °C. In contrast, the flat sector occupied ratios at 25 °C, 20 °C, and 15 °C were significantly higher than those observed in the Japanese strains.

			U		1		
Strain	No. of	Long dian	neter (µm)	Short dian	neter (µm)	Flatten	ing 🔆
no.	basidiospores	Avg.	STD	Ave.	STD	Avg.	STD
1	20	4.8	0.5	3.4	0.4	0.28	0.12
2	20	4.2	0.4	2.7	0.3	0.35	0.07
3	20	4.4	0.4	3.1	0.4	0.30	0.10
4	20	4.4	0.4	3.0	0.4	0.33	0.09
5	20	4.7	0.4	3.3	0.3	0.31	0.09
6	20	4.7	0.4	3.2	0.3	0.32	0.10
7	20	4.5	0.2	3.2	0.3	0.29	0.06
8	20	4.8	0.3	3.4	0.3	0.28	0.06
9	20	4.9	0.4	3.2	0.3	0.35	0.07
10	20	48	0.4	34	03	0.28	0.09

Table 2. The size and the flattening of basidiospores in each strain

Note: % Flattening = (Long diameter - Short diameter) / Long diameter



Fig. 7 - Photomicrographs of basidiospores from each *Pholiota microspora* strain. Strain numbers 1–10 correspond to those listed in Table 1.





Fig. 8 - Comparison of physiological characteristics between Bhutanese wild strains (bar with dots) and Japanese commercial strains (white bars) of *Pholiota microspora*. Strain numbers correspond to those listed in Table 1. For the meaning of star symbols and error bars, refer to Fig. 3. "5/5", "4/5", and "0/5" indicate the number of replicate plates (out of five) in which dedikary sector among five plate media.

Colony morphology of each strain is shown in Fig. 9. Table 3 presents the proportion of PDA plates showing mycelial regrowth after transferring cultures of Bhutanese wild strains from a 30 °C incubator to 20 °C, along with the corresponding nuclear phase. Overall, 75 % of the PDA plates inoculated with Bhutanese wild strains exhibited renewed mycelial growth following the temperature shift. Microscopic examination confirmed that all regrowing colonies retained the dikaryotic phase, indicating that dikaryotic mycelia remained viable despite prior exposure to inhibitory high-temperature conditions.





Fig. 9 - Representative colony morphology of each *Pholiota microspora* strain at different incubation temperatures. Strain numbers 1–10 correspond to those listed in Table 1.

Stock	Ratio of plates with	Nucleus phase of grown hyphae		
	mycelial regrowth (%)	Dikaryon (%)	Monokaryon (%)	
3	75	100	0	
4	100	100	0	
5	75	100	0	
6	80	100	0	
7	100	100	0	
8	100	100	0	
9	100	100	0	
10	75	100	0	

Table 3. Proportion of PDA plate cultures showing mycelial regrowth following transfer from 30 °C to 20 °C, along with nuclear phases of Bhutanese wild strains

The formation of a confrontation line

The formation of confrontation (zone) lines in dual-culture assays between Japanese commercial strains and Bhutanese wild strains on PDA medium is shown in Fig. 10. Zone line formation was categorized as coloured, uncoloured, or absent, as summarized in Table 4. For Japanese strain no. 1, no clear zone line was formed against three of the eight Bhutanese wild strains. In the case of Japanese strain no. 2, one Bhutanese wild strain failed to form a clear zone line. Among the 36 possible pairwise combinations of Bhutanese wild strains, 12 combinations showed no formation of a clear zone line.

Mating characteristics

The three Bhutanese wild *Pholiota microspora* specimens exhibited a confirmed bipolar mating system, as demonstrated through self-mating tests of monokaryons derived from basidiospores (Table 5). Based on these results, provisional tester strains representing mating types A1 through A6 were established. However, a notable proportion of monokaryotic combinations exhibited loss of mating ability: 56 % in strain A, 65 % in strain B, and 64 % in strain C. The results of outcross mating tests are presented in Table 6. Although certain



combinations showed mating incompatibility, no single mating type was found to be shared among the provisional types A1–A6, indicating that these mating types could be treated as distinct. Therefore, the provisional mating types A1–A6 were considered to represent ultimate mating type distinctions within this sample set. Di-mon mating tests confirmed that all tester strains (A1–A6) were capable of mating with the Japanese strains, except for two combinations involving the J2 Japanese strain and specific monokaryons.

Strain	1	2	3	4	5	6	7	8	9	10
no.										
1		±	±	-	±	-	+	±	Ŧ	-
2			±	+	+	+	+	+	-	+
3				±	±	±	-	±	±	-
4					±	-	H	-	-	-
5						-	Ŧ	I	±	±
6							-	Ŧ	±	±
7								Ŧ	±	Ŧ
8									-	-
9										±
10										

Table 4. Results of zone line formation observations

Notes: + means colored zone line, ± means uncolored zone line, - means no zone line, Strain number refers to Table 1.

Cultivation characteristics

Cultivation performance of each *Pholiota microspora* strain in bottle cultivation is presented in Fig. 11. The total number of fruiting bodies formed by Bhutanese wild strains 3, 6, and 10 was 33, 42, and 54, respectively, while Japanese strains produced 40 and 38 fruiting bodies. No significant differences were observed between these strains. However, strain 8 produced a significantly higher number of fruiting bodies than both Japanese strains ($\alpha = 0.01$ or 0.05) (Fig. 11a). Yield per bottle for Bhutanese strains 8 and 10 was 118 g and 126 g, respectively, compared to 108 g and 114 g for the Japanese strains. These differences were not statistically significant (Fig. 11b). In terms of earliness, all Bhutanese wild strains—except for strains 3 and 9—fruited earlier than one or both Japanese strains. Strains 8 and 10 produced fruiting bodies on days 8 and 12, respectively, whereas the first flush for Japanese strains occurred on days 27 and 29. These differences were statistically significant ($\alpha = 0.01$) (Fig. 11c). The average unit weight of fruiting bodies for Bhutanese strains 4, 5, 7, and 10 ranged from 2.5 to 3.1 g per fruiting body. The corresponding values for the Japanese strains were 2.9 g and 3.1 g, with no significant differences detected (Fig. 11d).

Discussion

Morphological feature of fruiting body

A strain isolated from a wild fruiting body collected in Nepal was described as *Pholiota microspora* var. *himalensis* (Adhikari et al., 2014). A comparative summary of morphological characteristics between the Nepalese specimen and the Bhutanese strains examined in this study is presented in Table 7. Adhikari et al. (2014) cited five distinguishing features in support of subspecies designation when compared to the Japanese commercial strain: cap color and size, presence of scales on the surface of the cap and stipe, and basidiospore shape. The most decisive trait was the cap color, described as bright yellow to orange-brown in *P. microspora*



var. *himalensis*, contrasting with the umber-brown coloration characteristic of *P. microspora* var. *microspora*. In the present study, the cap colours of Bhutanese strains 3, 4, 6, 8, 9, and 10 were more similar to those reported for *P. microspora* var. *himalensis*, whereas strain 5 showed no significant differences from the Japanese strains in hue, saturation, or brightness. Interestingly, strains 5 and 6 were both isolated from fruiting bodies collected at the same location (NMC/02604; see Table 1), suggesting that variation in cap coloration may be strain-specific rather than site-dependent. Neda (2008), in the taxonomic revision that integrated *P. microspora* included pileus coloration with livid shades near the margin. This feature, however, is not observed in Japanese commercial *nameko* strains. Neda (2008) concluded that such minor color differences are insufficient for species-level separation and that these forms should be unified under *P. microspora* var. *microspora*. Similarly, differences in cap size observed among the Bhutanese wild strains relative to Japanese strains are likely attributable to inherent genetic variation among strains rather than taxonomic divergence.



Fig. 10 - Confrontation line formation in dual cultures between Japanese commercial strains and Bhutanese wild strains of *Pholiota microspora* on PDA medium. Strain numbers 1–10 correspond to those listed in Table 1.



Table 5. Results of self-mating experiments in three Bhutanese wild specimens



Notes: 1~10 means the strain number of monokaryon from the basidiospore of each specimen, \bigcirc means success to mate. \times means unsuccess to mate

Blue-shaded strain number means each selected tester strain of mating factor,

Yellow-shaded combinations normally can be mated; however, it was observed inability combinations in 56 % (14/25) of strain A, 65% (17/25) of strain B, and 64 % (17/25) of strain C

Mon-mon mating of each tester strain									
No.	A_1	A_2	A ₃	A ₄	A ₅	A ₆			
A ₁			0	×	0	0			
A ₂			0	0	×	×			
A ₃		0	\backslash		0	0			
A ₄	0	0	\backslash		0	0			
A ₅	×	×	0	0		\backslash			
A ₆	×	×	0	0					

	Table 6.	The results	of out	-mating
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Di-mon mating between 2 Japanese commercial									
strain and each tester strain									
No.	A ₁	A ₂	A ₃	A4	A ₅	A ₆			
J1	0	0	0	0	0	0			
J2	×	0	0	×	0	0			

Notes: For A1~A6, refer to Table 5, For ○ and ×, refer to table 5, For J1 and J2, refer to Table1.



Fig. 11 - Cultivation characteristics of each *Pholiota microspora* strain under bottle cultivation.

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*The first flush date indicates the number of days from the completion of incubation to the first harvest. Strain numbers correspond to those listed in Table 1. For the meaning of star symbols and error bars, refer to Fig. 3.

		Description for N	Venal specimen ^{%1}	Results	of this naner	
Part of organ	Characteristic	P. microspora	P. microspore ver. humalensis	Japanese commercial stocks (Nos.1, 2)	Bhutanese wild stocks (No. 3–10)	
Сар	Color	Umber-brown	Bright yellow to orange brown	Umber-brown Hue:17, 21 Saturation:168, 177 Value:143, 144	For No. 5, no significant difference to Japanese commercial stocks	
	Size	Lage	Small	Nos. 4, 6, 7, and 10 were larger than No. 2 $(\alpha = 0.05)$		
	Scales	×	\bigcirc	0	0	
Stipe	Scales	×	\bigcirc	\bigcirc	\bigcirc	
Spore	Shape	Ellipsoid-oval	Ovoid, Ovoid-ellipsoid	Ellipsoid-oval	Ellipsoid-oval (Nos. 3-8) Ovoid-ellipsoid (Nos .9, 10)	
	Color of spore print	No description	Dull brown	For No.9, no signi Japanese cor	ficant difference from mmercial stocks	
Pleurocystidium	Existence	No description ^{*2}	0	×	×	

Table 7. Comparison of morphologi	cal characteristics	between a	a Nepalese	specimen	description	and
the results of this study						

Notes: ^{**1}; Adhikari et. al. (2014)

**2; Pleurocystidium was not observed on the type specimen of *Agaricus microsporus* (type, K(M):130654) (Neda, 2008).

The Japanese strains used in this study, which were developed specifically for natural (outdoor) cultivation, exhibited scales on both the cap and stem surfaces (Fig. 2). Although approximately 99.7 % of Japanese commercial nameko mushrooms-descended from the F27 strain (Nakamoto et al., 1967) and adapted for air-conditioned indoor cultivation-typically lack surface scales (Hirao et al., 2022), the presence of scales is not uncommon in strains specialized for natural cultivation or in wild fruiting bodies collected in Japan. Therefore, the presence of scales on the cap and stem is not considered a valid criterion for subspecies differentiation. Similarly, no Bhutanese wild strain (strains 3–10) exhibited the ovoid spore shape reported as a distinguishing feature of the subspecies *P. microspora* var. *himalensis*. Given this, there is no morphological basis in the current study to separate Bhutanese wild strains as a distinct subspecies. Consequently, products derived from Bhutanese strains 3-10 may be marketed under the commercial name "nameko" without concern for taxonomic misidentification. Nonetheless, the Bhutanese wild strains exhibited some unique and distinctive morphological features. For instance, strain 3 displayed a prominently protruding central region of the capmorphologically distinct from both the Japanese strains and the other Bhutanese strains. Such traits have the potential to contribute to the visible diversity of nameko products in commercial markets, enhancing consumer appeal.

Mycelium growth characteristics

Unlike the two Japanese commercial strains, all eight Bhutanese wild strains retained the



dikaryotic phase at an incubation temperature of 30 °C. In Japan's air-conditioned nameko cultivation systems, undesirable phenomena such as delayed fruiting and reduced yield frequently occur under elevated incubation temperatures (Kumata et al., 1995a; Kumata et al., 1998). Previous studies have suggested that these cultivation issues are associated with abnormal dedikaryotization in flat-sector hyphae, which tends to be triggered by high incubation temperatures (Kumata et al., 1995b). The current study revealed that Bhutanese wild strains did not undergo dedikaryotization under the same thermal conditions, indicating a potential advantage for breeding more stable and heat-tolerant nameko strains. These mycelial growth characteristics could thus contribute significantly to improving the resilience and reliability of commercial nameko cultivation. However, it is also notable that the Bhutanese wild strains exhibited slower mycelial growth at 30 °C and nearly perfect circular growth at 15 °C, indicating an adaptation to lower temperature ranges. For context, the monthly average maximum temperature in August 2021 at Thimphu—located at an altitude of approximately 2,300 m—was 26.2 °C (National Statistics Bureau, 2021). The Bhutanese wild strains examined in this study were collected from forests at altitudes ranging from 2.220 to 3,100 m, where temperatures rarely, if ever, reach 30 °C. In contrast, Japanese nameko strains may originate from habitats with higher ambient temperatures. This environmental distinction likely plays a key role in shaping the thermal response and growth patterns observed in Bhutanese strains, and should be considered in future strain development and breeding programs.

Mating characteristics

The Bhutanese wild strains were confirmed to be compatible with Japanese commercial strains through di-mon mating tests, indicating the potential for cross-breeding. This raises the prospect of transferring desirable traits—such as the thermal stability of the dikaryotic phase observed in Bhutanese strains—into new hybrid lines for improved cultivation performance. However, a relatively high proportion of self-mating combinations (56–68 %) exhibited loss of mating ability when incubated at 25 °C. *Pholiota microspora* is known to possess a bipolar mating system, and previous studies have reported that approximately 10–20 % of progeny may lose mating ability due to the presence of an incompatible A β factor arising during meiosis. Additionally, recombination within the A mating-type locus has been observed to result in progeny with incompatibility factors different from the parent strain, although such events occur at a much lower frequency (approximately 0.3 %) (Ratanatraigooldacha et al., 2002).

The higher incidence of mating incompatibility observed in this study (56–68 %) suggests that other factors may be influencing the mating system, beyond the rates expected from meiotic segregation and recombination alone. One possible explanation is the influence of incubation temperature during mating. Given the sensitivity of Bhutanese wild strains to elevated temperatures, as demonstrated in the mycelial growth tests, it is plausible that the mating process in these strains is similarly temperature-sensitive. Further investigations are needed to determine whether environmental conditions, particularly temperature during mating, contribute to the elevated rate of mating incompatibility observed in the Bhutanese wild strains.

Cultivation characteristics

Strains 8 and 10 were identified as promising candidates for domestic commercial cultivation in Bhutan, as they demonstrated comparable yields and significantly earlier fruiting times than the Japanese commercial strains. These findings suggest that selective breeding from wild fruiting bodies is both an effective and practical approach for developing cultivable strains suited to Bhutanese conditions. In addition to their potential for local production, the early fruiting characteristics of strains 8 and 10 also position them as valuable genetic resources for breeding new strains adapted to air-conditioned cultivation systems in Japan. Most Bhutanese



wild strains exhibited relatively soft cap flesh, which may influence market preference and shelf life. Notably, strain 5 displayed firmer cap tissue similar to that of the Japanese commercial strains, indicating its potential utility as a breeding parent. It is therefore considered a promising candidate for improving the physical and commercial quality of strains 8 and 10 through mating-based breeding strategies.

Conclusion

This study demonstrates that while Bhutanese wild strains 3–10 of *Pholiota microspora* are not morphologically distinct enough to warrant subspecies classification based on the current data, they exhibit unique and valuable characteristics. Final taxonomic determination will depend on forthcoming DNA analyses. These strains show notable variation in cap and stem coloration, mycelial growth behaviour, and compatibility in mating tests—both among themselves and with Japanese commercial strains. Given their confirmed mating compatibility and phenotypic diversity, Bhutanese wild strains represent promising breeding material. They offer a valuable opportunity to broaden the genetic base of Japanese *nameko* strains, which currently suffer from a severe genetic bottleneck due to their derivation from a single ancestral strain used in air-conditioned cultivation systems. Furthermore, two Bhutanese strains (no. 8 and no. 10) were identified as practical candidates for domestic cultivation in Bhutan, owing to their comparable yields and significantly earlier fruiting periods relative to the Japanese strains. This highlights the effectiveness and simplicity of selective breeding from wild fruiting bodies as a method for developing locally adapted, commercially viable strains in Bhutan.

Conflict of Interest

There is no conflict of interest.

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