Japanese “nameko” mushrooms (*Pholiota microspora*) produced via sawdust-based cultivation exhibit severe genetic bottleneck associated with a single founder

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**ABSTRACT**

*Pholiota microspora* (“nameko” in Japanese) is one of the most common edible mushrooms, especially in Japan, where sawdust-based cultivation is the most dominant method accounting for 99% of the production. The current strains for sawdust cultivation in Japan are considered to have been derived from a single wild strain collected from Fukushima, Japan, implying that commercial nameko mushrooms are derived from a severe genetic bottleneck. We tested this single founder hypothesis by developing 14 microsatellite markers for *P. microspora* to evaluate the genetic diversity of 50 cultivars and 73 wild strains isolated from across Japan. Microsatellite analysis demonstrated that sawdust-cultivated strains from Japan were significantly less genetically diverse than the wild strains, and the former displayed a significant bottleneck signature. Analyzing the genetic relationships among all genotypes also revealed that the sawdust-cultivated samples clustered into one monophyletic subgroup. Moreover, the sawdust-cultivated samples in Japan were more closely related than full-sibs. These results were consistent with the single founder hypothesis that suggests that all commercial nameko mushrooms produced in Japan are descendants of a single ancestor. Therefore, we conclude that cultivated *P. microspora* originated from a single domestication event that substantially reduced the diversity of commercial nameko mushrooms in Japan.

**Keywords**: domestication event, genetic diversity, log-cultivated strains, microsatellite markers, sawdust-cultivated strains, wild strains

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1. Introduction

*Pholiota microspora* (Berk.) Sacc. (synonym *P. nameko*) (“nameko” in Japanese) is one of the most popular edible mushrooms, especially in Japan. This species is a type of white-rot fungus that grows on dead or rotten trees in cool-temperate deciduous forests of East Asia, ranging from the Himalayas and China to Japan (Berkeley, 1850; Neda, 2008; Adgujarum, Watanabe, & Parajuli, 2014; GBIF.org, 2022). According to records, the nameko mushroom was first artificially log-cultivated in 1921 at the Tohoku region of northeastern Japan (Yoshimi, 1979; Pegler, 2003). According to records, the nameko mushroom was first artificially log-cultivated in 1921 at the Tohoku region of northeastern Japan (Yoshimi, 1979; Pegler, 2003), where this mushroom was traditionally consumed. Modern sawdust-based cultivation of nameko mushroom was established in 1930 by Kikosaburo Morimoto (US patent No. 1,833,089), the pioneer of sawdust-based cultivation of edible mushrooms (Hamada & Hagimoto, 1962; Yoshimi, 1979). Then, commercial production of selected strains in Japan expanded with the development of wooden tray cultivation during the 1960s up to the 1970s. Since the 1980s, the year-round production of mushrooms was possible using sawdust cultivation with air-conditioning, making it the dominant method accounting for 99.7% of its production in Japan (Forestry Agency, 2019). Subsequently, the cultivation of nameko mushroom was introduced to the southern part of the Liaoning Province, China, in the middle of the 1970s (Meng et al., 2019). Since then, China has become its largest producer. Finally, in 2012, China and Japan produced over 700,000 and 20,000 tons of nameko mushroom, respectively (Zhang, Chen, Huang, Gao, & Qu, 2015), causing the current spread of its cultivation to other parts of Asia, North America, and Europe.

Therefore, it has been reported that the cultivation of edible...
mushrooms has a relatively short history compared to crop plants and farm animals. For example, the cultivation of the shiitake mushroom (*Lentinula edodes*), one of the oldest cultivated mushrooms, likely occurred about 800 years ago in China (Chang & Miles, 1987). However, the nameko mushroom has been cultivated for approximately one hundred years. Although the first domesticated strains of nameko mushroom were isolated from wild fruit bodies sampled in 1929 in Yamagata Prefecture, Japan (Morimoto, 1930), these strains were proposed to have disappeared during the chaotic times after World War II. There are few records on the domestication process of *P. microspora*. However, it is likely that the current spawn strains used for sawdust cultivation are derived from a single wild strain, F27 (Kanno, Matsuksi, Shigihara, Kimura, & Suyama, 2016), that was collected from Kawairi, Ichinoki, Yama-to-machi, Fukushima Prefecture, Japan on Oct 16, 1962 (Nakamoto, Ito, Shoji, & Otake, 1967). Therefore, the vast majority of commercial nameko mushrooms are presumably derived from this severe bottleneck event, resulting in a very low level of genetic variation. However, a comparison of the genetic diversity of cultivars and wild strains has not been conducted.

Hence, a proper understanding of the evolutionary history of domesticated species is required to be able to use their genetic resources and facilitate further breeding. Such understanding requires knowledge of the genetic diversity and population structure of natural populations of domesticated organisms. Obatake, Matsumoto, Mimura and Fukumasa-Nakai (2002) investigated the genetic relationship among 36 wild isolates of *P. microspora* sampled from across Japan using RAPD and PCR-RFLP markers. They discovered no geographic basis in the population structure, and estimates of genetic diversity were uncertain. Thus, the level of genetic diversity in wild *P. microspora* has remained uncertain because of the low reproducibility of RAPD profiles (Jones, Edwards, & Castagnone, 1997) and the weak polymorphism of RFLP markers. Besides, although STS markers based on RAPD and ISSR analysis were developed to discriminate *P. microspora* strains (Sasaki et al., 2007), they were uninformative in assessing the genetic diversity. Therefore, we developed microsatellite markers for *P. microspora* to evaluate the genetic diversity of wild stains isolated from across Japan, including cultivars, and we further assessed the putative origin of these cultivars.

Microsatellite markers are highly polymorphic and reproducible, easy-to-score, and are assumed to be neutral and co-dominant. Thus, they have been invaluable in many fields of biology, including population genetics, forensic science, and food science. Another approach, genome-wide SNP genotyping, offers high-density information and has been more available in recent years. However, microsatellite analysis is better suited for individual-unit operations, requires a very small amount of sample DNA, and can analyze damaged DNA samples, such as those from processed food products. Thus, microsatellite markers have been developed for some economically important mushrooms (*e.g.*, *Agaricus bisporus* and *L. edodes*) to facilitate genetic and breeding studies (*e.g.*, Fourlongne-Oriol, Spartaro, & Savoie, 2009; Xiao, Liu, Dai, Fu, & Bian, 2010), even though microsatellites occur less frequently in the genomes of fungal species, and they often exhibit lower levels of polymorphism than those in angiosperms and mammals (Dutech et al., 2007). In the last decade, rapid developments in genome sequencing have accelerated the discovery of microsatellites in various organisms, but especially in fungal species, due to their relatively small-sized genomes. In fact, the draft genome sequences of *P. microspora*, as well as 89 other mushrooms in China, were published simultaneously (Li et al., 2018). This genome sequence assembly of a nameko mushroom in China can be used to develop microsatellite markers in this species, as well as provide insight into the domestication history of *P. microspora*.

In this study, we aimed to (1) develop reliable microsatellite markers for *P. microspora*; (2) evaluate the genetic diversities of cultivars mainly from Japan and China, including wild strains isolated from across Japan; and (3) evaluate the consistency of the domestication history of nameko mushroom with the single founder hypothesis stating that commercial nameko mushrooms descended from a single wild strain collected from Japan. Finally, we considered the importance of assessing the unexplored genetic resource of this recently domesticated organism and its role in enhancing breeding and cultivation.

### 2. Materials and methods

#### 2.1. The fungal material

First, 124 *P. microspora* samples comprising 51 cultivars mainly from Japan and China, in addition to 73 wild strains collected from across Japan, were analyzed using microsatellite genotyping (see detailed in Table 1). Of the 51 cultivars, 18 strains were isolated from spawn for sawdust cultivation in Japan, including not only most of the current strains in circulation but also past-historical strains, such as the putative founder strain F27. However, of the other 33 cultivars, seven strains were from spawn for log-bed cultivation in Japan, one strain was from spawn for sawdust cultivation in China, one strain was from spawn for log-bed cultivation in China, and the remaining 22 and two strains were commercial mushrooms produced in Japan and China, respectively. Alternatively, 73 wild strains were collected between 1993 and 2020 from 13 prefectures, covering much of the distribution range of *P. microspora* in Japan. Of the 124 samples, 82 samples were extracted for genomic DNA using a DNeasy Plant Mini Kit (QIagen, Hilden, Germany) following the manufacturer’s protocol. Then, DNA was extracted from the remaining 32 samples for subsequent experiments, as reported in previous studies (Sasaki et al., 2007; Sasaki et al., 2013; Suyama & Matsuksi, 2015; Kanno et al., 2016).

#### 2.2. Microsatellite identification, primer design, and genotyping

Microsatellites were identified based on a draft genome assembly of *P. microspora* (GenBank/EMBL/DDBJ accession number QFFD00000000: Li et al., 2018) using QDD (ver. 3.1.2) software (Meglécz et al., 2014). Primers were designed using the Primer3 algorithm (Rozen & Skaletsky, 1999) with the following parameters: amplicon sizes of 90–350 bp, GC contents of 30%–60% with an optimum at 50%, primer melting temperatures (Tm) of 56°C–62°C with an optimum at 60°C, and primer lengths of 18–28 bp.

PCR amplification was performed in a total volume of 6 μL, containing 1 μL of template DNA, 3 μL of Multiplex PCR Master Mix (QIAGEN), and 0.2 μL of each primer. Each forward primer was labeled with either FAM, VIC, PET, or NED fluorescent dye. Microsatellites were appended with the “PIG-tail” sequence (GTTCCTCTT) at the 5’ end to promote non-templated A addition and facilitate subsequent genotyping (Brownstein, Carpent, & Smith, 1996) (see Table 2 for details). The amplification parameters were initial denaturation at 95°C for 15 min followed by 45 cycles of 95°C for 10 s, 58°C for 90 s, and 72°C for 1 min; and a final elongation step of 60°C for 30 min. Allele-specific fragment sizes of PCR amplicons were determined using GeneMapper ver. 4.0 (Applied Biotech Inc.).
Table 1. Collections of Pholiota microspora used in this study.

| ID | MLG* | Type | Organ | Date | Collector | Location / Source | DNA extract | Collection | Taxonomy |
|----|-------|------|------|------|----------|-----------------|-------------|------------|----------|
| 103 | G09 | Wild strain | Hypha | 1993/10/17 | KB | Otari-mura, Nagano, Japan | | | |
| 104 | G10 | Wild strain | Hypha | 1998/11/11 | AK | Mt. Kinpoku-san, Sado, Niigata, Japan | | | |
| 105 | G03 | Wild strain | Hypha | 1982/10/21 | MW | Yaheishiro, Nishiaizu, Fukushima, Japan | | | |
| 106 | G09 | Wild strain | Hypha | 1993/10/17 | AK | Otari-mura, Nagano, Japan | | | |
| 107 | G39 | Cultivated strain | fruit body | 2021/12/16 | AH | Oyamacho-nokyo Coop., Oita, Japan | | | |
| 108 | G32 | Wild strain | Hypha | 1993/10/28 | KM | Nishikawa-machi, Yamagata, Japan | | | |
| 109 | G47 | Wild strain | Hypha | 1997/10/30 | AK | Ishizuchi-yama, Kochi, Japan | | | |
| 110 | G32 | Wild strain | Hypha | 1996/10/24 | AK | Ammonnotaki, Nisimeya-mura, Aomori, Japan | | | |
| 111 | G21 | Wild strain | Hypha | 1995/10/01 | SG | Etanbetsu, Asahikawa, Hokkaido, Japan | | | |
| 112 | G10 | Wild strain | Hypha | 2000/10/18 | KB | Akiyamago, Sakae-mura, Nagano, Japan | | | |
| 113 | G54 | Wild strain | Hypha | 1997/10/30 | KB | Kamui-yama, Okushiri, Hokkaido, Japan | | | |
| 114 | G10 | Wild strain | Hypha | 1997/10/30 | AK | Ishizuchi-yama, Kochi, Japan | | | |
| 115 | G32 | Wild strain | Hypha | 1993/10/28 | KM | Nishikawa-machi, Yamagata, Japan | | | |
| 116 | G21 | Wild strain | Hypha | 2000/10/18 | KB | Akiyamago, Sakae-mura, Nagano, Japan | | | |
| 117 | G12 | Wild strain | Hypha | 1993/10/28 | KM | Nishikawa-machi, Yamagata, Japan | | | |
| 118 | G32 | Wild strain | Hypha | 1993/10/28 | KM | Nishikawa-machi, Yamagata, Japan | | | |
| 119 | G21 | Wild strain | Hypha | 1995/10/01 | SG | Etanbetsu, Asahikawa, Hokkaido, Japan | | | |
| 120 | G09 | Wild strain | Hypha | 1993/10/17 | KB | Otari-mura, Nagano, Japan | | | |
| 121 | G45 | Wild strain | Hypha | 1993/10/28 | AK | Otari-mura, Nagano, Japan | | | |
| 122 | G10 | Wild strain | Hypha | 1998/11/11 | AK | Mt. Kinpoku-san, Sado, Niigata, Japan | | | |
| 123 | G21 | Wild strain | Hypha | 1995/10/01 | SG | Etanbetsu, Asahikawa, Hokkaido, Japan | | | |
| 124 | G09 | Wild strain | Hypha | 2000/10/18 | KB | Akiyamago, Sakae-mura, Nagano, Japan | | | |
| 125 | G09 | Wild strain | Hypha | 1993/10/17 | KB | Otari-mura, Nagano, Japan | | | |
| 126 | G21 | Wild strain | Hypha | 1995/10/01 | SG | Etanbetsu, Asahikawa, Hokkaido, Japan | | | |
| 127 | G10 | Wild strain | Hypha | 1998/11/11 | AK | Mt. Kinpoku-san, Sado, Niigata, Japan | | | |
| 128 | G09 | Wild strain | Hypha | 2000/10/18 | KB | Akiyamago, Sakae-mura, Nagano, Japan | | | |
| 129 | G09 | Wild strain | Hypha | 1993/10/17 | KB | Otari-mura, Nagano, Japan | | | |
| 130 | G12 | Wild strain | Hypha | 1993/10/28 | KM | Nishikawa-machi, Yamagata, Japan | | | |
| 131 | G32 | Wild strain | Hypha | 1993/10/28 | KM | Nishikawa-machi, Yamagata, Japan | | | |
| 132 | G10 | Wild strain | Hypha | 1998/11/11 | AK | Mt. Kinpoku-san, Sado, Niigata, Japan | | | |
| 133 | G21 | Wild strain | Hypha | 1995/10/01 | SG | Etanbetsu, Asahikawa, Hokkaido, Japan | | | |
| 134 | G09 | Wild strain | Hypha | 2000/10/18 | KB | Akiyamago, Sakae-mura, Nagano, Japan | | | |
| 135 | G09 | Wild strain | Hypha | 1993/10/17 | KB | Otari-mura, Nagano, Japan | | | |
| 136 | G32 | Wild strain | Hypha | 1993/10/28 | KM | Nishikawa-machi, Yamagata, Japan | | | |
| 137 | G21 | Wild strain | Hypha | 1995/10/01 | SG | Etanbetsu, Asahikawa, Hokkaido, Japan | | | |
| 138 | G09 | Wild strain | Hypha | 2000/10/18 | KB | Akiyamago, Sakae-mura, Nagano, Japan | | | |
| 139 | G09 | Wild strain | Hypha | 1993/10/17 | KB | Otari-mura, Nagano, Japan | | | |
| 140 | G32 | Wild strain | Hypha | 1993/10/28 | KM | Nishikawa-machi, Yamagata, Japan | | | |

- **MLG**: Multi-locus genotype based on 14 microsatellite markers
- *Date*: Sampling date for wild strain or purchase date for commercial product
- *Collector*: A. S. Hirao; AK: Atsushi Kuma; FFPC, Fukushima Prefectural Forest Research Center, KB: Kitahiroshibubashi, KS: Kominokoge Co., KM: Kagawashiitake Co., Ltd., SAKAI: Sakegawa, SG: Shisso Gakari, SK: Shingo Kami, SN: Shingo Oya, TK: Takasaki Kishimoto, TT: Toshitaka Takagi, MW: Masaaki Watanabe

*Note: The table continues with additional entries not fully transcribed in the image.*
was established from a hybrid between the wild strain and To-
Fukushima-N2 strain was not a descendant of a single founder, it
assigned to “the other cultivated strains” because although the
strains, Fukushima-N2 for sawdust cultivation was exceptionally
wild strains, sawdust-cultivated strains from Japan, and the other
among genotypes within each of the following group of strains:
the observed and expected heterozygosity ($H_e$), and allele richness ($P_{AR}$ loci ($A$)), number of alleles ($A$), including
A2008) packages in R to calculate the percentages of polymorphic
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The draft genome of $P$. microspora yielded a total of 1714 micro-
satellite regions, with a relative density of 20.5 microsatellites per
Mb. The most abundant motif was dinucleotide (55.4%), followed
by tri- (35.7%), hexa- (3.4%), tetra- (3.3%), and pentanucleotide
(2.1%) motifs. We carried out a preliminary PCR amplification test
on 24 microsatellites consisting of 14 di- and 10 trinucleotide micro-
satellites with a minimum of nine repetitions per unit. Twenty-
one out of the 24 candidate markers were amplified, and we sele-
ted 14 polymorphic microsatellites with easy-to-score profiles
for subsequent analyses (Table 2).

One sample with missing alleles (ID: K23 from China) was ex-
cluded from the following analysis. Using the 14 microsatellites
across 123 samples yielded 93 alleles, ranging from 4 to 16 alleles
per locus with an average of 6.6. Furthermore, we used a 5% genet-
ic distance threshold to identify 74 multi-locus genotypes (MLGs)

### Table 2. Characteristics of 14 microsatellite markers developed for $Pholiota$ microspora.

| Locus | Primer sequences (5'-3') | Repeat motif | $T_a$ (°C) | Allele size range (bp) | Fluorescent label | Primer ratio$^*$ | Multiplex combination | $A$ |
|-------|--------------------------|--------------|-----------|-----------------------|-----------------|-----------------|---------------------|-----|
| Phm01 | F-AAAGCTTATTTTCACGCCAAG | (ATC)$_A$   | 59        | 85–94                 | FAM             | 1:7             | A                   | 4   |
| Phm02 | F-TGTCCCTTTCTTTTGCTT    | (AG)$_B$    | 60        | 99–107                | VIC             | 1:7             | A                   | 5   |
| Phm03 | F-AGCTCAGGATCCTGGAAC    | (AGC)$_A$   | 61        | 106–115               | PET             | 1:3             | A                   | 4   |
| Phm04 | F-GGGTATTCTCGAATATTTAGCGC | (AG)$_B$   | 61        | 102–110               | NED             | 1:7             | A                   | 4   |
| Phm05 | F-TACCTCAGGCACACTTGAGCA | (GAC)$_B$   | 61        | 120–159               | FAM             | 1:3             | B                   | 11  |
| Phm06 | F-TTAACGGGTCTTCTGAGCGC  | (AC)$_B$    | 59        | 135–147               | PET             | 1:3             | B                   | 5   |
| Phm07 | F-ACATGCAGCATCTAGAAGT  | (AT)$_A$    | 60        | 146–154               | VIC             | 1:7             | B                   | 7   |
| Phm08 | F-TAACACCGATTTGCAAGTG   | (AC)$_A$    | 58        | 133–145               | NED             | 1:5             | B                   | 4   |
| Phm09 | F-GGTTCCTACCTGGTCTTTC   | (AC)$_B$    | 60        | 151–175               | PET             | 1:3             | A                   | 8   |
| Phm10 | F-GATGGCCAGGCATTAGACAGT | (AG)$_A$    | 60        | 142–183               | VIC             | 1:7             | A                   | 8   |
| Phm11 | F-TATATGCCGCTTCCTTTCGG  | (AC)$_B$    | 60        | 213–238               | FAM             | 1:3             | A                   | 16  |
| Phm12 | F-CGGAGTGAACATATACGAGCA | (AC)$_B$    | 61        | 282–292               | PET             | 1:3             | B                   | 7   |
| Phm13 | F-ACAGGACAATCGACAGGAGGG  | (AT)$_A$   | 60        | 286–313               | VIC             | 1:7             | B                   | 5   |
| Phm14 | F-GTCTGTAAGCCAGGCAACCAG  | (AG)$_B$    | 59        | 309–321               | FAM             | 1:3             | A                   | 7   |

$T_a$, annealing temperature; $A$, number of alleles across the 123 samples analyzed.

* Ratio of fluorescent and unlabeled forward primers for multiplex PCR. See text for details.

**, modified with a “PIG-tail” (GTTTCTT) at the 5’ end (Brownstein et al., 1996). ***, modified with an adjusted “PIG-tail” (GTTTCT) at the 5’ end.

2.3. Genetic data analysis

We used “hierfstat” (Goudet, 2005) and “adegenet” (Jombart, 2008) packages in R to calculate the percentages of polymorphic loci ($P$), number of alleles ($A$), and allele richness ($AR$), including the observed and expected heterozygosity ($H_e$ and $H_o$, respectively) among genotypes within each of the following group of strains: wild strains, sawdust-cultivated strains from Japan, and the other cultivated strains from Japan and China. During the grouping of strains, Fukushima-N2 for sawdust cultivation was exceptionally assigned to “the other cultivated strains” because although the Fukushima-N2 strain was not a descendant of a single founder, it was established from a hybrid between the wild strain and To-
hoku-N127 strain (PVP Office of Japan, 2022). Thus, to test the ev-
idence accounting for recent bottlenecks in each group of
strains, we used the Wilcoxon signed-rank test of heterozygosity ex-
cess (Cornuet & Luikart, 1996) under the two-phase model with
95% single-step mutations, 5% multi-step mutations, and a variance
of 12%, as implemented in the BOTTLENECK program (v. 1.2.02)
(Piry, Luikart, & Cornuet, 1999).

Furthermore, we constructed a neighbor-joining dendrogram of
the genetic relationships among individual genotypes using the
Bruvo distance (Bruvo, Michiels, D’Souza, & Schulenburg, 2004) as
implemented in the R package, “adegenet” (Jombart, 2008). Then,
we visualized the differences in allelic composition among individ-
ual genotypes using principal component analysis (PCA), using the
“adegenet” package. We also evaluated the pairwise relatedness ($r_p$) (Queller & Goodnight, 1989) among genotypes within wild
strains, sawdust-cultivated strains from Japan, and other cultivated
strains, after which the $r_p$ estimate was calculated using the “relat-
ed” package (Pew, Muir, Wang, & Frasier, 2015) in R (version 3.6.1).
All R scripts for data analysis can be obtained from https://github.
com/akihirao/nameko_SSR.

3. Results

3.1. Microsatellite markers and identified genotypes

The draft genome of $P$. microspora yielded a total of 1714 micro-
satellite regions, with a relative density of 20.5 microsatellites per
Mb. The most abundant motif was dinucleotide (55.4%), followed
by tri- (35.7%), hexa- (3.4%), tetra- (3.3%), and pentanucleotide
(2.1%) motifs. We carried out a preliminary PCR amplification test
on 24 microsatellites consisting of 14 di- and 10 trinucleotide micro-
satellites with a minimum of nine repetitions per unit. Twenty-
one out of the 24 candidate markers were amplified, and we sele-
ted 14 polymorphic microsatellites with easy-to-score profiles
for subsequent analyses (Table 2).

One sample with missing alleles (ID: K23 from China) was ex-
cluded from the following analysis. Using the 14 microsatellites
across 123 samples yielded 93 alleles, ranging from 4 to 16 alleles
per locus with an average of 6.6. Furthermore, we used a 5% genet-
ic distance threshold to identify 74 multi-locus genotypes (MLGs)

A.S. Hirao et al. / Mycoscience VOL.63 (2022) 79-87

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--- 82 ---
from 123 samples (Table 1; Supplementary Table S1). Then, we resolved all 74 genotypes, using a genotype accumulation curve that reached a plateau at more than 13 loci (Supplementary Fig. S1). Of the 74 genotypes, 61 were identified from wild-type strains and eight were from sawdust-cultivated P. microspora, mainly in Japan but also in China. Additionally, one genotype was from a hybrid between a wild-type strain and sawdust-cultivated strain in Japan, one genotype was from a sawdust-cultivated strain in China, and the remaining three were from log-cultivated strains. We also observed identical genotypes among the wild strains collected from the same sampling locations, indicating putative clones. Moreover, overlapping genotypes among the cultivated strains existed, some of which were distinct cultivated spawn strains (Table 1). All genotypes from commercial mushroom products in Japan exactly matched the spawn strains used for sawdust cultivation in Japan. Besides, the genotype of a commercial mushroom from China (ID: CP10) also exactly matched that found in cultivated spawn strains and commercial samples in Japan (Supplementary Table S1). Hence, we then used the 74 unique genotypes to assess the genetic diversity of our samples.

3.2. Genetic diversity

Summarized statistics of genetic diversity (Table 3) showed a moderate level of genetic diversity across all the genotypes ($H_e = 0.542$). As shown, the cultivars for sawdust cultivation in Japan ($A = 1.4$, $AR = 1.4$, $H_e = 0.159$) were significantly less genetically diverse than the wild strains ($A = 6.6$, $AR = 3.3$, $H_e = 0.564$; all $P < 0.05$ by Kruskal–Wallis tests). Additionally, the cultivars for sawdust cultivation in Japan had one or two alleles per locus. Both alleles were also found in the wild strains (Supplementary Table S1). In contrast, the bottleneck test detected a bottleneck signal in the sawdust-cultivated samples from Japan ($P = 0.03$) but not in the wild strains ($P = 0.99$) and other cultivars ($P = 0.19$) (Table 3).

The neighbor-joining dendrogram (Fig. 1) showed sawdust-cultivated samples in Japan clustered in a single subgroup whose branch lengths are shorter than those of other subgroups. The wild strains did not cluster according to geographical regions. Alternatively, the PCA plot (Fig. 2) shows the relationships among the genotypes along the first two PCA axes that explained 10.2% and 8.8% of the total variance, respectively. Therefore, the PCA result agrees with the dendrogram, i.e., that the sawdust-cultivated samples in Japan were tightly clustered, while the wild strains and the other cultivars were scattered and do not group according to source origin. The sawdust-cultivated samples in Japan were also more genetically related ($r_{xy} = 0.799 \pm 0.112$ SD) compared with the wild strains ($r_{xy} = -0.040 \pm 0.190$) and other cultivars ($r_{xy} = 0.035 \pm 0.368$) ($P < 0.001$ by randomization test; Fig. 3). Besides, all but one relatedness within the sawdust-cultivated samples in Japan had $r_{xy}$ values greater than 0.5 (i.e., indicative of full-sib relationship).

4. Discussion

4.1. Domestication footprints of sawdust-cultivated strains in Japan

Sawdust-cultivated strains of *P. microspora* in Japan ($H_e = 0.16$) are significantly less genetically diverse than wild strains ($H_e = 0.56$) (Table 3), and the former display a genetic bottleneck signature (Table 3). Analysis of the genetic relationships among all genotypes shows the sawdust-cultivated samples in Japan clustering into a monophyletic subgroup (Figs. 1, 2). Using SNP genotyping at 252 loci, Kanno et al. (2016) showed that among 25 sawdust-cultivated and 3 wild strains of *P. microspora* in Japan, all 25 cultivated strains are monophyletic (however, the authors did not address their genetic diversity). This study demonstrated that the genetic relatedness among sawdust-cultivated samples in Japan ($r_{xy} = 0.799 \pm 0.112$ SD) exceeds that of full-sib relationships, indicating that the sawdust-cultivated strains in Japan are derived from self-breeding and/or within-family mating. These results agree with the single founder hypothesis stating that commercial nameko mushrooms produced by sawdust cultivation in Japan are the descendants of a single ancestor. Therefore, we conclude that the genetic diversity of commercial nameko mushrooms in Japan is remarkably low because they have originated from a single domestication event.

Strain F27, collected approximately 60 years ago from Fukushima, Japan (Nakamoto et al., 1967), is the putative founder strain of *P. microspora* cultivars for sawdust cultivation in Japan; this strain was included in the microsatellite genotyping analysis, which shows that all the loci in strain F27 are fixed, i.e., one allele per locus for all the 14 loci analyzed. In contrast, in the sawdust-cultivated samples in Japan, five loci are polymorphic (*Pm08, Pm13, Pm14, Pm17*, and *Pm23*) with two types of alleles, and the nine remaining loci are monomorphic (Supplementary Table S2). Thus, strain F27 is not likely to be the original founder because the founder should have five heterozygous loci that are the sources of the five polymorphic loci in the descendant cultivars. In other words, it is possible to determine the genotype of the original founder strain at the time of collection based on the types of alleles found across all the cultivars (see detailed in Supplementary Table S2). We speculate that all loci in strain F27 have been fixed through inbreeding and/or dedikaryotization of the original F27 strain. In growing mycelia (especially in marginal hypheae) of *P. microspora*, dedikaryotization is a process that has often been observed (Arita, 1979).

It has been reported that the moderate genetic diversity level of wild *P. microspora* isolates collected from across Japan ($H_e = 0.56$) was equivalent to or lower than wild strains of other wood-decay mushroom species. For example, $H_e = 0.57$ in *Flammulina velutipes* (Liu, Feng, Li, Yan, & Yang, 2016); $H_e = 0.52$ in *Pleurotus ostreatus* (Li, Liu, Zhao, & Yang, 2019); and $H_e = 0.73$ in *L. edodes* (Lee, Moon, Ro, Chung, & Ryu, 2020). In this study, compared to these wild mushrooms, the sawdust-cultivated *P. microspora* strains in Japan were less genetically diverse ($H_e = 0.16$). Studies have also

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**Table 3. Genetic diversity in cultivated and wild strains of *Pholiota microspora***

| Category                        | $N$ | $G$ | $P$ (SE) | $A$ (SE) | $AR$ (SE) | $H_o$ | $H_e$ | Bottleneck test |
|---------------------------------|-----|-----|----------|----------|-----------|-------|-------|----------------|
| Wild strain                     |     |     |          |          |           |       |       |                |
| Cultivated strain               |     |     |          |          |           |       |       |                |
| Sawdust-cultivated strains in Japan | 41  | 8   | 0.36 (0.1) | 1.4 (0.1) | 1.4 (0.1) | 0.179 | 0.159 | 0.031†         |
| The other cultivated strains    | 9   | 5   | 086       | 2.8 (0.3) | 2.8 (0.3) | 0.429 | 0.536 | 0.190          |
| Overall                         | 123 | 74  | 1.00      | 6.6 (0.9) | 3.2 (0.3) | 0.339 | 0.539 | —              |

$N$: number of samples analyzed; $G$: number of multi-locus genotypes; $P$: percent of polymorphic loci; $A$: number of alleles; $AR$: allelic richness per five individuals; $H_o$: observed heterozygosity; $H_e$: expected heterozygosity. Bottleneck test: probability of significant heterozygosity excess was calculated using the program BOTTLENECK ver.1.2.02 (Piry et al., 1999). †: $P < 0.05$. 

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shown that the cultivars of the other mushroom species all had multiple domestication origins, i.e., $H_e = 0.36$ in *F. velutipes* (Liu et al., 2016); $H_e = 0.50$ in *P. ostreatus* (Li et al., 2019); and $H_e = 0.76$ in *L. edodes* (Lee et al., 2020). Thus, we propose that the natural populations of *P. microspora* can serve as a new genetic resource to develop effective strains for sawdust cultivation. Specifically, although the Fukushima-N2 strain as a hybrid of the wild and sawdust-cultivated *P. microspora* was established at the Fukushima Prefecture Forest Research Center, it was rarely produced in the Fukushima Prefecture, Japan (A. Kumata, personal communication). Hence, we did not find its genotype in the mushroom products analyzed. Our results confirm the result of Obatake et al. (2002), who reported the absence of geographic influence on the population genetic structure of *P. microspora* across Japan (Figs. 1, 2), suggesting that genetic resource management at the regional scale is not critical for the mushroom breeding strategy. Nevertheless, the genetic signatures of local adaptations to heterogeneous environments may play an important role in breeding for sources of diversity. In the genomics era, the isolation and characterization of functional genetic diversity will play important roles in the breeding and cultivation of *P. microspora*.

4.2. Genetic characteristics of the other cultivars

The log-bed cultivation of nameko mushrooms accounts for only less than 1% of the total production of the mushroom in Japan (Forestry Agency, 2019). We analyzed eight spawn strains for log-bed cultivation—seven and one from Japan and China, respectively, to identify three multi-locus genotypes (MLGs) existed among them (Table 1; Supplementary Table S1). The three distinct genotypes were scattered on the neighbor-joining dendrogram (Fig. 1) and PCA diagram (Fig. 2), implying multiple origins of domestication. Results also showed that one of the three genotypes (MLG: KG02) was frequently discovered in several spawns from Japan and China, suggesting that one of the preferred spawn strains with a common ancestor was introduced from Japan to China.

Additionally, two sawdust-cultivated samples from China were successfully genotyped. From the results, while the genotype of a commercial nameko mushroom produced in China (ID: CP10) exactly matched that of some sawdust-cultivated samples from Japan (MLG: CG5), the other genotype of a spawn strain from China (ID: K38) did not uniquely match any sample (Table 1; Supplementary Table S1). These results indicated that although a descendant of the founder strain F27 from Japan partly contributed to the production of nameko mushrooms in China, at least a part of Chinese cultivars underwent different domestication processes. In this study, the Chinese sample of a commercial product was collected from a freeze-dried food product. Thus, its DNA components had suffered considerable damage due to high-temperature pressurization during food processing. Despite the damage to its DNA, the processed food sample was successfully genotyped using the microsatellite marker system. Therefore, microsatellite analysis can be ap-

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**Fig. 1.** A neighbor-joining dendrogram showing the 74 genotypes of *Pholiota microspora*. Samples are colored and symbol-coded according to their sources. Cultivar.sawdust.JPN: sawdust-cultivated samples from Japan; Cultivar.log.JPN: log-cultivated samples from Japan; Cultivar.sawdust.x.wild.JPN: a cultivated sample derived from the hybrid between a sawdust-cultivated strain and a wild strain; Cultivar.CHN: cultivated samples from China; The other samples: wild strains.

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**Table 1.** Multi-locus genotypes (MLGs) of *Pholiota microspora* from Japan and China. C: Cultivar; S: Sawdust; Wild: Wild; X: Hybrid; JPN: Japan; CHN: China; Size: Length of the marker. The underlined typeface indicates that a spawn strain was successfully genotyped. DOI: 10.47371/mycosci.2022.03.002
plied to natural samples and food products such as mushrooms. It should be noted that the microsatellite markers for *P. microspora* were designed from a genome assembled from a nameko mushroom collected from a local fresh market in China (Li et al., 2018). The geographic separation between China and Japan should lead to genetic differentiation between the wild strains of *P. microspora* from the two regions. Thus, some level of mismatch is expected when applying a specific set of genetic markers on strains from both countries. However, the microsatellite markers designed from the Chinese sample allowed the successful genotyping of all the samples from Japan. Additionally, while developing the markers, we achieved high rates of success in amplifying the primary candidate markers (21/24 = 0.85) as well as the final set of selected markers (14/24 = 0.58). This result was in contrast to the usual process of our microsatellite marker development, where the ratios of the final selected markers to the primary candidate ones were several to dozens in percentages: 18/45 = 0.40 in *Gastrodia takeshimensis* (Kishikawa et al., 2019); 13/102 = 0.13 in *Phraortes elongatus* (Nozaki et al., 2021); 15/293 = 0.05 in *Pyrola japonica* (Shutoh, Izuno, Isagi, Kurosawa, & Kaneko, 2017); and 26/238 = 0.11 in *Cypripedium japonicum* (Yamashita, Izuno, Isagi, Kurosawa, & Kaneko, 2016). Our results imply that the Chinese sample used for genome assembly is genetically similar to Japanese samples. Specifically, the cultivation of nameko mushroom was introduced from Japan to China in 1970s (Meng et al., 2019). Thus, the Chinese sample used for genome assembly may have the same origin as Japanese cultivars. However, we genotyped only two sawdust-cultivated and one log-cultivated sample from China. Therefore, further investigation on the overall genetic diversity of cultivated nameko mushrooms in China is necessary to reveal its cultivation history outside Japan.

**Fig. 2.** Principal component analysis of 74 *Pholiota microspora* genotypes. Samples are colored and symbol-coded according to their sources. Cultivar.sawdust.JPN: sawdust-cultivated samples from Japan; Cultivar.log.JPN: log-cultivated samples from Japan; Cultivar.sawdust.x.wild.JPN: a cultivated sample derived from the hybrid between a sawdust-cultivated strain and a wild strain; Cultivar.CHN: cultivated samples from China; The other samples: wild strains.

**Fig. 3.** Genetic relatedness between individual genotypes of *Pholiota microspora* within the wild samples (Wild), within the sawdust-cultivated samples in Japan (Cultivar.sawdust), or within the other cultivars (Cultivar.others).
5. Conclusions

Cultivated *P. microspora* originated from a single domestication event that greatly reduced the genetic diversity of current commercial nameko mushrooms in Japan. Nameko mushroom cultivation has been spreading to China, South-East Asia, North America, and Europe. Clarifying the genetic diversity of nameko mushrooms worldwide will provide important information on the sustainable use of this edible mushroom as well as insight into the evolutionary biology of this recently domesticated organism.

Disclosures

No conflicts of interest among the authors. All the experiments undertaken in this study complied with the current laws of the country where they were performed.

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