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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

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), where this mushroom was traditionally consumed. Modern sawdust-based cultivation of nameko mushroom was established in 1930 by Hi-

kosaburo 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

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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, Matsuki, Shigihara, Kimura, & Suyama, 2016), that was collected from Kawairi, Ichinoki, Yamato-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, & Castaglione, 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 strains 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., Foulongne-Oriol, Spataro, & 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 & Matsuki, 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 (T_a) 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 μM of each primer. Each forward primer was labeled with either FAM, VIC, PET, or NED fluorescent dye. We also prepared unlabeled forward primers and mixed them with the fluorescent ones, as recommended by Suyama (2012). Five reverse primers were appended with the "PIG-tail" sequence (GTTTCTT) at the 5' end to promote non-templated A addition and facilitate subsequent genotyping (Brownstein, Carpten, & 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

Table 1. Collections of *Pholiota microspora* used in this study.

ID	MLG [a]	Type	Organ	Date [b]	Collector [c]	Location / Source
218	G14	Wild strain	Hypha	1995/10/01	SG	Etanbetsu, Asahikawa, Hokkaido, Japan
242	G03	Wild strain	Hypha	1998/10/15	KB	Kamui-yama, Okushiri, Hokkaido, Japan
243	G38	Wild strain	Hypha	1998/10/15	KB	Kamui-yama, Okushiri, Hokkaido, Japan
171	G30	Wild strain	Hypha	1996/10/24	AK	Nisimeya-mura, Aomori, Japan
172	G12	Wild strain	Hypha	1996/10/24	AK	Ammonnotaki, Nisimeya-mura, Aomori, Japan
173	G39	Wild strain	Hypha	1996/10/24	AK	Ammonnotaki, Nisimeya-mura, Aomori, Japan
174	G34	Wild strain	Hypha	1996/10/24	AK	Ammonnotaki, Nisimeya-mura, Aomori, Japan
192	G25	Wild strain	Hypha	1996/11/06	KB	Yagen-onsen, Ohata-machi, Aomori, Japan
K32	KG62	Wild strain	DNA extract	2014	KX	26-99 strain, Hirosaki, Aomori, Japan
K07	KG45	Wild strain	DNA extract	—	KX	62-2 strain, Mt. Hakkoda, Aomori, Japan
216	G04	Wild strain	Hypha	1997/10/21	KB	Hayasaka-kogen, Morioka, Iwate, Japan
217	G06	Wild strain	Hypha	1997/10/21	KB	Hayasaka-kogen, Morioka, Iwate, Japan
121	G33	Wild strain	Hypha	1994/11/01	AK	Yuri-gun, Akita, Japan
K27	KG06	Wild strain	DNA extract	2006	KX	19-25 strain, Towada, Akita, Japan
K28	KG60	Wild strain	DNA extract	2009	KX	21-48 strain, Mt. Funagata, Miyagi, Japan
K29	KG07	Wild strain	DNA extract	2014	KX	26-59 strain, Miyagi, Japan
K30	KG07	Wild strain	DNA extract	2014	KX	26-60 strain, Miyagi, Japan
K31	KG07	Wild strain	DNA extract	2014	KX	26-61 strain, Miyagi, Japan
111	G46	Wild strain	Hypha	1993/10/30	AK	Gassan, Yamagata, Japan
112	G26	Wild strain	Hypha	1993/10/30	AK	Gassan, Yamagata, Japan
248	G49	Wild strain	Hypha	1999/10/29	KB	Nishikawa-machi, Yamagata, Japan
255	G17	Wild strain	Hypha	1999/10/29	AK	Nishikawa-machi, Yamagata, Japan
257	G29	Wild strain	Hypha	1999/10/29	AK	Nishikawa-machi, Yamagata, Japan
262	G50	Wild strain	Hypha	1999/10/29	AK	Nishikawa-machi, Yamagata, Japan
H01	GH1	Wild strain	Fruit body	2020/10/25	AH	Nukumidaira, Oguni-machi, Yamagata, Japan
H02	GH2	Wild strain	Fruit body	2020/10/25	AH	Nukumidaira, Oguni-machi, Yamagata, Japan
202	G27	Wild strain	Hypha	1997/10/29	AK	Tengendai, Yonezawa, Yamagata, Japan
K04	KG32	Wild strain	DNA extract	2013/07/19	KX	Iide, Yamagata, Japan
K33	KG23	Wild strain	DNA extract	2015/10/22	KX	27-111 strain, Yamagata, Japan
K34	KG36	Wild strain	DNA extract	2015/10/22	KX	27-122 strain, Yamagata, Japan
K35	KG56	Wild strain	DNA extract	2015/10/26	KX	27-140 strain, Mogami-gun, Yamagata, Japan
K36	KG52	Wild strain	DNA extract	2015/10/26	KX	27-141 strain, Mogami-gun, Yamagata, Japan
K37	KG08	Wild strain	DNA extract	2015/10/26	KX	27-144 strain, Mogami-gun, Yamagata, Japan
222	G22	Wild strain	Hypha	1998/11/11	AK	Mt. Kinpoku-san, Sado, Niigata, Japan
224	G48	Wild strain	Hypha	1998/11/11	AK	Mt. Kinpoku-san, Sado, Niigata, Japan
226	G10	Wild strain	Hypha	1998/11/11	AK	Mt. Kinpoku-san, Sado, Niigata, Japan
290	G40	Wild strain	Hypha	2000/10/17	TK	Akiyamago, Tsunan-machi, Niigata, Japan
291	G42	Wild strain	Hypha	2000/10/17	TK	Akiyamago, Tsunan-machi, Niigata, Japan
114	G51	Wild strain	Hypha	1993/10/30	AK	Mori-toge, Koriyama, Fukushima, Japan
012	G23	Wild strain	Hypha	1982/10/21	MW	Yaheishiro, Nishiaizu, Fukushima, Japan
013	G13	Wild strain	Hypha	1982/10/21	MW	Yaheishiro, Nishiaizu, Fukushima, Japan
022	G18	Wild strain	Hypha	1982/10/21	MW	Yaheishiro, Nishiaizu, Fukushima, Japan
015	G19	Wild strain	Hypha	1982/10/21	MW	Yaheishiro, Nishiaizu, Fukushima, Japan
020	G19	Wild strain	Hypha	1982/10/21	MW	Yaheishiro, Nishiaizu, Fukushima, Japan
021	G19	Wild strain	Hypha	1982/10/21	MW	Yaheishiro, Nishiaizu, Fukushima, Japan
017	G41	Wild strain	Hypha	1982/10/21	MW	Yaheishiro, Nishiaizu, Fukushima, Japan
018	G37	Wild strain	Hypha	1982/10/21	MW	Yaheishiro, Nishiaizu, Fukushima, Japan
294	G20	Wild strain	Hypha	2000/11/09	AK	Hinoemata-mura, Fukushima, Japan
237	G43	Wild strain	Hypha	1998/10/08	AK	Furaisawa, Kaneyama-machi, Fukushima, Japan
270	G44	Wild strain	Hypha	1999/11/10	AK	Tadami-machi, Fukushima, Japan
199	G47	Wild strain	Hypha	1997/10/19	AK	Tenei-mura, Iwase-gun, Fukushima, Japan
188	G31	Wild strain	Hypha	1995/11/08	KM	Gokayama, Taira-mura, Toyama, Japan
116	G28	Wild strain	Hypha	1993/10/28	KM	Izumi-mura, Onogun, Fukui, Japan
117	G28	Wild strain	Hypha	1993/10/28	KM	Izumi-mura, Onogun, Fukui, Japan
118	G28	Wild strain	Hypha	1993/10/28	KM	Izumi-mura, Onogun, Fukui, Japan
119	G28	Wild strain	Hypha	1993/10/28	KM	Izumi-mura, Onogun, Fukui, Japan
115	G36	Wild strain	Hypha	1993/10/28	KM	Izumi-mura, Onogun, Fukui, Japan
283	G02	Wild strain	Hypha	2000/10/18	KB	Akiyamago, Sakae-mura, Nagano, Japan
284	G02	Wild strain	Hypha	2000/10/18	SO	Akiyamago, Sakae-mura, Nagano, Japan
287	G05	Wild strain	Hypha	2000/10/18	KM	Akiyamago, Sakae-mura, Nagano, Japan
288	G01	Wild strain	Hypha	2000/10/18	KB	Akiyamago, Sakae-mura, Nagano, Japan
106	G09	Wild strain	Hypha	1993/10/17	AK	Otari-mura, Nagano, Japan
107	G16	Wild strain	Hypha	1993/10/28	AK	Otari-mura, Nagano, Japan
108	G32	Wild strain	Hypha	1993/10/28	AK	Otari-mura, Nagano, Japan
109	G32	Wild strain	Hypha	1993/10/28	AK	Otari-mura, Nagano, Japan
105	G45	Wild strain	Hypha	1993/10/28	AK	Otari-mura, Nagano, Japan
189	G21	Wild strain	Hypha	1996/10/24	KB	Daisen, Tottori, Japan
190	G08	Wild strain	Hypha	1996/10/24	KB	Daisen, Tottori, Japan
204	G11	Wild strain	Hypha	1997/10/30	AK	Ishizuchi-yama, Kochi, Japan
205	G00	Wild strain	Hypha	1997/10/30	AK	Ishizuchi-yama, Kochi, Japan
209	G00	Wild strain	Hypha	1997/10/30	AK	Ishizuchi-yama, Kochi, Japan
210	G00	Wild strain	Hypha	1997/10/30	AK	Ishizuchi-yama, Kochi, Japan
211	G00	Wild strain	Hypha	1997/10/30	AK	Ishizuchi-yama, Kochi, Japan
F27	CG1	Cultivated strain for sawdust cultivation	DNA extract	—	KX	Putative original strain for indoor sawdust cultivation
Tohoku-N104	CG1	Cultivated strain for sawdust cultivation	DNA extract	—	KX	Tohoku N104 strain, Kinokkusu Co., Miyagi, Japan
KX-N006	CG2	Cultivated strain for sawdust cultivation	DNA extract	—	KX	KX-N006 strain, Kinokkusu Co., Miyagi, Japan—
KX-N007	CG3	Cultivated strain for sawdust cultivation	DNA extract	—	KX	KX-N007 strain, Kinokkusu Co., Miyagi, Japan
KX-N008	CG3	Cultivated strain for sawdust cultivation	DNA extract	—	KX	KX-N008 strain, Kinokkusu Co., Miyagi, Japan
KX-N009	CG4	Cultivated strain for sawdust cultivation	DNA extract	—	KX	KX-N009 strain, Kinokkusu Co., Miyagi, Japan
KX-N010	CG2	Cultivated strain for sawdust cultivation	DNA extract	—	KX	KX-N010 strain, Kinokkusu Co., Miyagi, Japan
Tohoku-N123	CG5	Cultivated strain for sawdust cultivation	DNA extract	—	KX	Tohoku N123 strain, Kinokkusu Co., Miyagi, Japan
Tohoku-N127	CG6	Cultivated strain for sawdust cultivation	DNA extract	—	KX	Tohoku N127 strain, Kinokkusu Co., Miyagi, Japan
007	CG1	Cultivated strain for sawdust cultivation	Hypha	—	FPFRC	Inoculum for wooden tray cultivation, Japan
008	CG6	Cultivated strain for sawdust cultivation	Hypha	—	FPFRC	Inoculum for wooden tray cultivation, Japan
N103	KG02	Cultivated strain for log cultivation	DNA extract	—	KX	N103 strain, Kinokkusu Co., Miyagi, Japan
KX-N109	KG58	Cultivated strain for log cultivation	DNA extract	—	KX	KX-N109 strain, Kinokkusu Co., Miyagi, Japan
Mori-13	CG6	Cultivated strain for sawdust cultivation	DNA extract	—	KX	Mori-13 strain, Mori Sangyo Co. Ltd, Gunma, Japan
N245	CG7	Cultivated strain for sawdust cultivation	DNA extract	—	KX	N245 strain, Kawamura-shokuyokin-kenkyujo Co., Yamagata, Japan
N258	CG5	Cultivated strain for sawdust cultivation	DNA extract	—	KX	N258 strain, Kawamura-shokuyokin-kenkyujo Co., Yamagata, Japan
Hokken-N108	CG5	Cultivated strain for sawdust cultivation	DNA extract	—	KX	N108 strain, Hokken Co. Ltd, Tochigi, Japan
Hokken-N160	CG6	Cultivated strain for sawdust cultivation	DNA extract	—	KX	N160 strain, Hokken Co. Ltd, Tochigi, Japan
Fukushima-N1	CG8	Cultivated strain for sawdust cultivation	DNA extract	—	KX	Fukushima N1 strain, FPFRC, Fukushima
Fukushima-N2	KG42	Cultivated strain for sawdust cultivation	DNA extract	—	KX	Fukushima N2 strain, FPFRC, Fukushima
KM-86	KG02	Cultivated strain for log cultivation	DNA extract	2012	KX	KM-86 strain, Kagawashiitake Co., Miyagi, Japan
KM-88	KG09	Cultivated strain for log cultivation	DNA extract	2012	KX	KM-88 strain, Kagawashiitake Co., Miyagi, Japan
K10	KG02	Cultivated strain for log cultivation	DNA extract	2013/07/19	KX	5-126 strain, Miyagi, Japan
N133	CG6	Cultivated strain for sawdust cultivation	DNA extract	—	KX	N133 strain, Sakegawa, Yamagata, Japan
K25	KG02	Cultivated strain for log cultivation	DNA extract	—	KX	Middle-maturing strain, Sakata-Kawamura, Japan
K26	KG02	Cultivated strain for log cultivation	DNA extract	—	KX	Middle-maturing strain, Amarume-Kawamura, Japan
CP06	CG3	Commercial product	Fruit body	2019/11	AH	Donguri farm Coop., Miyagi, Japan
K17	CG5	Commercial product	DNA extract	2021/07/06	KX	Fresh-nameko, SugawaraReizou Co., Ltd., Yamagata, Japan
K18	CG5	Commercial product	DNA extract	2014/05/08	KX	Okki-nameko, SugawaraReizou Co., Ltd., Yamagata, Japan
K19	CG5	Commercial product	DNA extract	2014/05/08	KX	Chokai-nameko, SugawaraReizou Co., Ltd., Yamagata, Japan
K40	CG5	Commercial product	Fruit body	2021/08/04	AH	Chokai-nameko, SugawaraReizou Co., Ltd., Yamagata, Japan
K41	CG3	Commercial product	Fruit body	2021/07/11	AH	JA-Oishii-mogami Coop., Yamagata, Japan
CP09	CG3	Commercial product	Fruit body	2019/11	AH	Oak farm Coop., Yamagata, Japan
CP01	CG4	Commercial product	Fruit body	2019/11	AH	JA-Fukushima-mirai Coop., Fukushima, Japan
K24	CG5	Commercial product	DNA extract	2014	KX	Suzuki farm Co., Ltd., Fukushima, Japan
CP03	CG5	Commercial product	Fruit body	2019/11	AH	Suzuki farm Co., Ltd., Fukushima, Japan
CP04	CG5	Commercial product	Fruit body	2019/11	AH	Suzuki farm Co., Ltd., Fukushima, Japan
CP05	CG5	Commercial product	Fruit body	2019/11	AH	Suzuki farm Co., Ltd., Fukushima, Japan
CP07	CG3	Commercial product	Fruit body	2019/11	AH	An individual farmer, Koriyama, Fukushima, Japan
CP08	CG3	Commercial product	Fruit body	2019/11	AH	Happy farm Co., Ltd., Fukushima, Japan
CP02	CG3	Commercial product	Fruit body	2019/11	AH	JA-Tokamachi Coop., Niigata, Japan
K43	CG3	Commercial product	Fruit body	2021/12/12	AH	JA- Tanofuji Coop., Gunma, Japan
K44	CG3	Commercial product	Fruit body	2021/07/11	AH	JA-Nagano Coop., Nagano, Japan
K46	CG3	Commercial product	Fruit body	2021/12/18	TT	Nameko farm Hida Coop., Gifu, Japan
K22	CG3	Commercial product	DNA extract	2010/11/29	KX	Akaike farm Coop., Shizuoka, Japan
K45	CG3	Commercial product	Fruit body	2021/12/16	AH	Kamiyukawa kinoko-seisan Coop., Nara, Japan
K42	CG3	Commercial product	Fruit body	2021/12/16	AH	Oyamacho-nokyo Coop., Oita, Japan
CP10	CG5	Commercial product	Fruit body	2021/07	SK	Marukome Co., Ltd., a processed food made in China
K23	NA	Commercial product	DNA extract	2017/11/27	KX	Marukome Co., Ltd., a processed food made in China
K38	KG11	Cultivated strain for sawdust cultivation	DNA extract	2013	KX	25-1 strain, China
No137	KG02	Cultivated strain for log cultivation	DNA extract	—	KX	No137 strain, China

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[a]	Multiplex combination	A
Phmi01	F-AAAGCTTATTTCACGCCAGG R-CGGCGTTAGTCGTTACATTG	$(ATC)_{10}$	59	85–94	FAM	1:7	A	4
Phmi02	F-TGTGCCCTTTCTCCTTGTCT R-TCCGAAACGCGATTTCTTAC**	$(AG)_{10}$	60	99–107	VIC	1:7	A	5
Phmi03	F-AGCTGGCGATCCCTGAAC R-GCCTCACCACCAGCCATC*	$(AGC)_9$	61	106–115	PET	1:3	A	4
Phmi05	F-GGGTTATCTCGATAATTTAACGGC R-GAGTCCATAAGTTTATCATCTAGCAAA	$(AG)_9$	61	102–110	NED	1:7	A	4
Phmi07	F-TACCTCAGCCACACTGGCA R-TGCCTTCAGTTCCGGAATAC**	$(AC)_9$	61	120–159	FAM	1:3	B	11
Phmi08	F-TTAACGGGTTTCTAGACCGC R-AAATCAATCTCAGAGCGCC	$(AC)_9$	59	135–147	PET	1:3	B	5
Phmi09	F-ACATGCGCATCATCGAAGT R-GCGCTGAGCTACAGTGAGACT*	$(AT)_9$	60	146–154	VIC	1:7	B	5
Phmi10	F-TAACACCACAGTTTGCATGG R-GACTTATGGTGCTGGCCC	$(AAC)_9$	58	133–145	NED	1:5	B	4
Phmi13	F-GGTTCCATGTCCGTGTTCTT R-GGGCTACCTTGCCTTCATCT	$(ACC)_9$	60	151–175	PET	1:3	A	8
Phmi14	F-GATGCCAGCCATTAGACGAT R-TGTATCCGCTTTCCTCTTGC**	$(AGC)_{10}$	60	142–183	VIC	1:7	A	8
Phmi17	F-CCGAGTGAACATATCCAGCA R-TTGTCTTCCTCCCTTCCTGA	$(AC)_{12}$	60	213–238	FAM	1:3	A	16
Phmi20	F-CGCTTAGAGGCACCAATACG R-GCTGAACGTGGACACTCTGA	$(AT)_9$	61	282–292	FAM	1:3	B	7
Phmi23	F-ACAAGACCCAACGTACAGGG R-ACAGGTGAAAGGGTCATGGA	$(AGG)_9$	60	286–313	NED	1:7	B	5
Phmi24	F-GCAATCTCATGAGCCTTTCA R-GAGATCCTGAAGGTTGTGACG	$(AT)_9$	59	309–321	FAM	1:3	A	7

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

[a] 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.

Biosystems) and GeneMaker ver. 1.6 (SoftGenetics, State College, PA, USA). To confirm the power and accuracy of the microsatellite markers, we plotted a genotype accumulation curve with 1000 iterations, then calculated the pairwise linkage disequilibrium between loci, and finally determined the multi-locus genotypes (MLGs) with the genetic distance threshold set at 5%, using the "Poppr" package (Kamvar, Tabima, & Grünwald, 2014) in the R 4.1.2 software environment (R Core Team, 2021).

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_o and H_e, 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 Tohoku-N127 strain (PVP Office of Japan, 2022). Thus, to test the evidence accounting for recent bottleneck events in each group of strains, we used the Wilcox signed-rank test of heterozygosity excess (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 individual genotypes using principal component analysis (PCA), using the "adegenet" package. We also evaluated the pairwise relatedness (r_{xy}) (Queller & Goodnight, 1989) among genotypes within wild strains, sawdust-cultivated strains from Japan, and other cultivated strains, after which the r_{xy} estimate was calculated using the "related" 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 microsatellite 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 microsatellites with a minimum of nine repetitions per unit. Twenty-one out of the 24 candidate markers were amplified, and we selected 14 polymorphic microsatellites with easy-to-score profiles for subsequent analyses (Table 2).

One sample with missing alleles (ID: K23 from China) was excluded 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% genetic distance threshold to identify 74 multi-locus genotypes (MLGs)

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 hyphae) 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

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

	Category	N	G	P	A (SE)	AR$_s$ (SE)	Ho	He	Bottleneck test
Wild strain		73	61	1.00	6.6 (0.9)	3.3 (0.3)	0.352	0.564	0.999
Cultivated strain	Sawdust-cultivated strains in Japan	41	8	0.36	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$_s$*: allelic richness per five individuals; *Ho*: observed heterozygosity; *He*: expected heterozygosity. Bottleneck test: probability of significant heterozygosity excess was calculated using the program BOTTLENECK ver.1.2.02 (Piry et al., 1999). [*]: $P < 0.05$.

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-
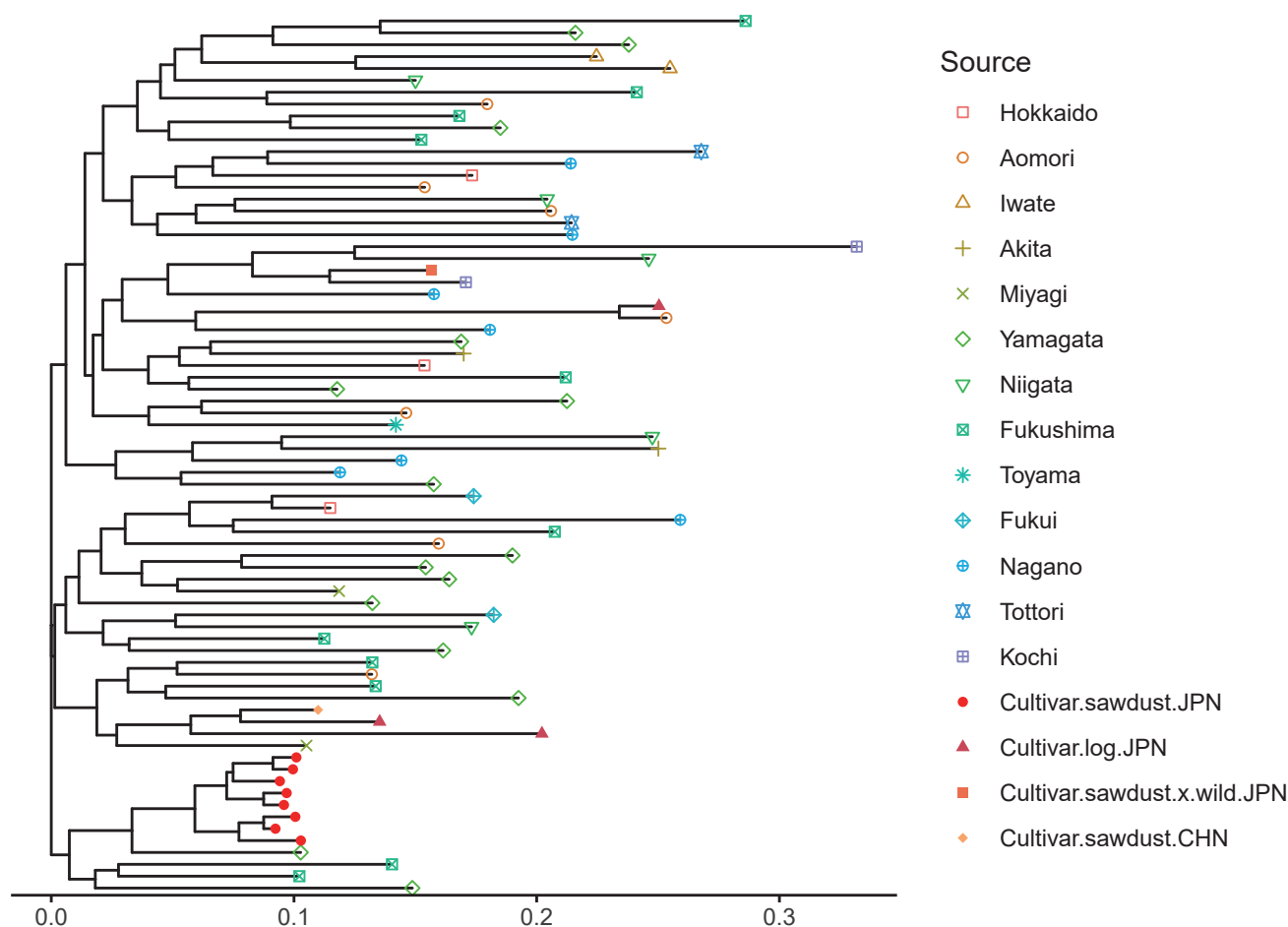

Fig. 1. A neighbor-joining dendrogram showing the 74 genotypes of *Pholiota microspora*. Samples are colored and symbol-coded according to their sources. Cultivar. sawdust.JPN: sawdust-cultivated samples from Japan; Cultivar.log.JPN: log-cultivated samples from Japan; Cultivar.sawdust.x.wild.JPN: a cultivated 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.
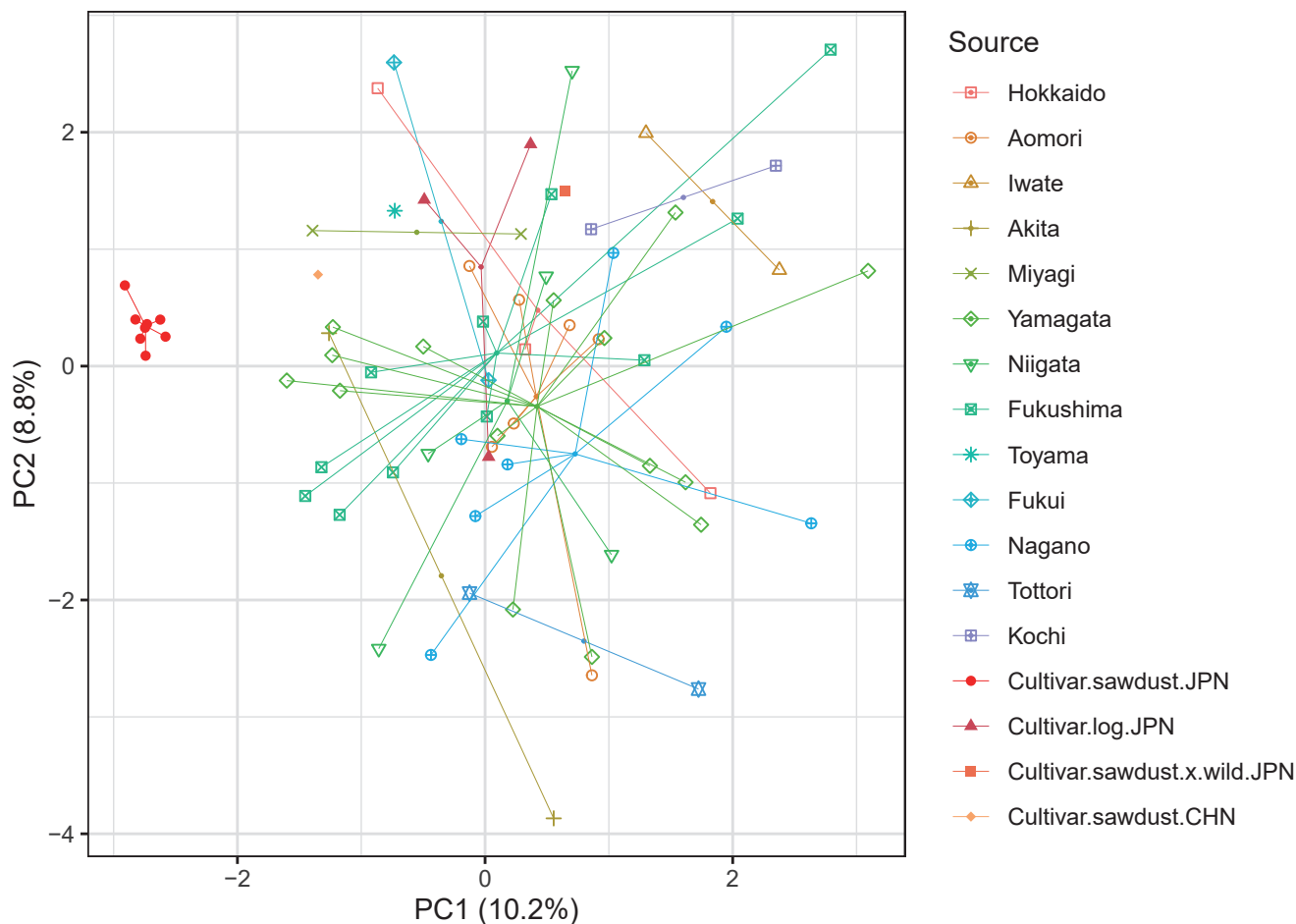
Fig. 2. Principal component analysis of 74 *Pholiota microspora* genotypes. Samples are colored and symbol-coded according to their sources. Cultivar.sawdust.JPN: sawdust-cultivated samples from Japan; Cultivar.log.JPN: log-cultivated samples from Japan; Cultivar.sawdust.x.wild.JPN: a cultivated sample derived from the hybrid between a sawdust-cultivated strain and a wild strain; Cultivar.CHN: cultivated samples from China; The other samples: wild strains.

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.
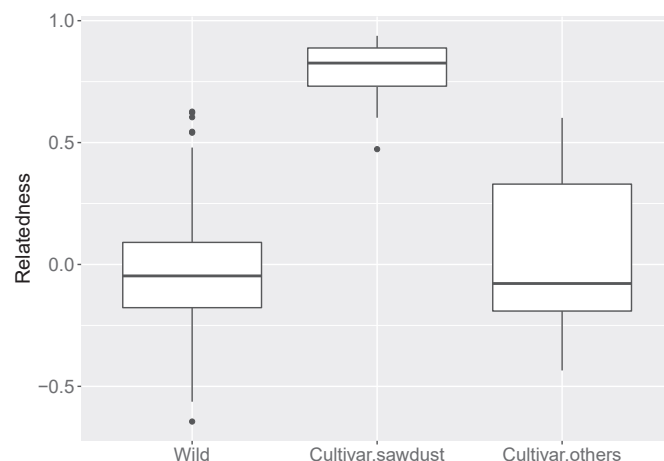

Fig. 3. Genetic relatedness between individual genotypes of *Pholiota microspora* within the wild samples (Wild), within the sawdust-cultivated samples in Japan (Cultivar.sawdust), or within the other cultivars (Cultivar.others).

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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