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Research paper

Genetic diversity and breeding history of Winter Mushroom (*Flammulina velutipes*) in China uncovered by genomic SSR markers

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ABSTRACT

Flammulina velutipes is one of the most widely cultivated mushroom species in China. However, its genetic background remains poorly understood due to the limited sampling and poor molecular markers used. In this study, 124 *F. velutipes* strains were employed, including 110 cultivars and 14 wild strains, and 25 new SSR markers were developed based on the genome of *F. velutipes*. A total of 153 alleles were detected in 124 strains to investigate the improper cultivar naming, genetic diversity and breeding history of *F. velutipes* in China. Our fingerprinting analyses indicated that 65 strains can be differentiated from the total of 124 strains, and over 53% of the strains are labeled with improper commercial names. The genetic diversities of wild strains are higher than those of the cultivars, suggesting that wild strains may harbor a large "arsenal" gene pool in nature available for strain breeding. The white cultivars in China were originally introduced from Japan, while the yellow cultivars were directly domesticated from wild strains isolated from southeastern China or hybridized between the white cultivars and yellow strains.

1. Introduction

Flammulina velutipes, known as the Winter Mushroom, is one of the most popular cultivated mushrooms in East Asia (Mizuno et al., 2009; Van Peer et al., 2011; Shi et al., 2012). This species contains immune regulation, antitumor, antibiotic and antioxidant bioactive compounds (Wang et al., 2004; Ng et al., 2006; Wang et al., 2012; Yang et al., 2012), and is also known as an effective curative for liver and allergic airway diseases (Pang et al., 2007; Chu et al., 2015).

China is the largest producer of *F. velutipes* with an annual production of 2.4 million tons (Li and Li, 2014). However, the inconsistent nomenclature of *F. velutipes* cultivars in circulation has led to much confusion in the cultivar names (Su et al., 2008a, 2008b; Zhang et al., 2011). Therefore, an accurate identification of the currently available strains on the market is essential for the cultivation and breeding of strains in China. During the last 10 years, researchers have conducted a number of studies on the strain identification of *F. velutipes* with different genetic markers, e.g., PCR-RFLP, ISSR and SCAR (Palapala et al., 2002; Su et al., 2008a, 2008b). However, these marker technologies may be limited by weak polymorphism and unstable reproducibility (Xu, 2006; Kumar et al., 2009; Singh et al., 2013).

In contrast, simple sequence repeats (SSRs) are randomly repeated DNA sequences generally 1–6 base pairs in length per unit. SSRs can spread extensively throughout a genome. They are typically co-dominant, with high polymorphism, reproducible and easy to score (Zane et al., 2002; Freeland, 2005; Selkoe and Toonen, 2006). Studies on strain identification based on SSRs have been conducted on other economically important mushrooms, for example *Pleurotus ostreatus*, *Lentinula edodes* and *Auricularia auricula-judae*. Highly polymorphic SSRs in these mushrooms which can accurately identify the cultivars have been developed (Xiao et al., 2010; Kyung-Ho et al., 2009; Zhang et al., 2012; Xiang et al., 2016). Zhang et al. (2010) developed 10 SSRs to discriminate 14 strains of *F. velutipes*. However, the numbers of strains used in this study were limited, and were not enough to resolve the improper strain commercial naming in China. In addition, few SSRs were used in this study, due partly to the low efficiency of the SSRs that had been developed for experimental methods at that time. Rapid developments in fungal genome sequencing have accelerated the discovery of SSRs in *Agaricus bisporus*, *Pleurotus ostreatus*, *Ganoderma lucidum*, and other economically important mushrooms (Murat et al., 2011; Qian et al., 2013; Foulongne-Oriol et al., 2013; Qu et al., 2016; Xiang et al., 2016). Recently, the genome sequencing of *F. velutipes* has

Abbreviations: SSR, Simple Sequence Repeat; ISSR, inter simple sequence repeat; RFLP, restriction-fragment length polymorphism; AFLP, amplified fragment length polymorphism; SCAR, sequence characterized amplified regions; CTAB, cetyl-trimethyl ammonium bromide; MISA, MIcroSAtellite; Na, number of alleles; Ne, effective number of alleles; Ho, observed heterozygosity; He, expected heterozygosity; PIC, polymorphic information content; UPGMA, unweighted pair group method with arithmetic mean; MCMC, Markov Chain Monte Carlo; PCoA, principle coordinate analysis; FIASCO, fast isolation by AFLP of sequences containing repeats; SE, standard error.

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Table 1
Number of SSRs identified in the genome of *F. velutipes*.

Repeat motif	Number (%)
Mono	242 (18.3%)
Di	211 (16%)
Tri	824 (62.4%)
Tetra	23 (1.7%)
Penta	1 (0.8%)
Hexa	20 (1.5%)
Total	1321

been completed (Park et al., 2014), which aids the development of large numbers of SSRs to identify strains of *F. velutipes*.

In addition, genetic diversity is also important for strain breeding. Wang and Xian (2013) revealed abundant genetic diversity among 114 cultivated strains of *F. velutipes* using eight inter-SSR (ISSR) markers. In contrast, Lu et al. (2014) uncovered low genetic diversity among 20 strains of *F. velutipes* using 60 AFLP markers. Unfortunately, there is currently no available study concerning the genetic diversity of wild strains. Studies on *Lentinula edodes* have revealed the large genetic diversity of the wild strains in China, which are considered an important resource for strain breeding (Xu et al., 1997; Xiao et al., 2010; Xiang et al., 2016). During our field survey of mushrooms in different regions of China, we collected several specimens of *F. velutipes* and successfully made cultures for them. Therefore, these wild strains will be important samples in studying the genetic diversity of *F. velutipes* in China.

Furthermore, mycologists in China have contributed to the breeding work of *F. velutipes* since the beginning of the 1960s (Guo, 1997; Song et al., 2007). However, detailed information about the strains used in

Table 2
Characterizations of 25 SSR markers used for the identification of strains of *F. velutipes*.

Locus name	Primer sequence (5′-3′)	Repeat motif	PIC[a]	Na[b]	Ne[c]	Ho[d]	He[e]
SSR1	F-CCAGCTTCAGGATACCTTCG R-ACGGGTATGAGATGGAATCG	(CCT)8	0.49	7	1.83	0.37	0.46
SSR2	F-GCACGCTCCTAAACCTATCG R-CGACTTGTCGAGGAAGATCC	(GAA)10	0.61	8	2.85	0.62	0.65
SSR4	F-AGGAGGAGGAGGAAGGCATA R-TCGAACCTGCAATCTTCTGA	(GAG)7	0.1	4	1.05	0.04	0.05
SSR5	F-CTACACGTCGAGCCTGTCAC R-ACTCGAATCGTCGGAGAGTG	(ACG)10	0.05	3	1.03	0.03	0.02
SSR7	F-ACGCTAATCCGCTTGTTCAT R-TCGCGTCACTCGTGTAAGTC	(CCT)8	0.6	5	2.95	0.72	0.66
SSR15	F-GATGACGAGGACAGTGACGA R-CCTCCTTCCTCCTCCATAGC	(GAA)7	0.37	2	1.96	0.35	0.49
SSR19	F-CGGCGGTTTGTCAGAACTAT R-GCAAACCACAACCTTCCTTC	(CGA)8	0.4	5	1.84	0.58	0.46
SSR21	F-GAAGCTGATCCTTGGCTTTG R-CTAGACACACCGCCTCTTCC	(TGT)7	0.75	8	3.91	0.46	0.74
SSR22	F-TCTTCGGATGCTTTGGAATC R-TCGTTCTCTTTGCACACGTC	(GAT)7	0.68	11	3.62	0.44	0.72
SSR23	F-AAGTCATCGACGAGCCTAGC R-CGAACGAAGGGAATGTCTGT	(CGA)7	0.36	4	1.552	0.370	0.356
SSR24	F-ATGATGCTGCAATGCTCAAG R-TCGCTTTGTGGCACTATCTG	(TG)10	0.42	7	1.61	0.17	0.38
SSR25	F-CCGAAGGATCAGAGCAAGAG R-GACGTGAGTGCTTGGCATAC	(TGG)7	0.23	8	1.21	0.07	0.18
SSR26	F-AGCCGCATTGTATCATACCC R-AGTGGCCAAGACGGTTTCTA	(TCG)7	0.2	4	1.15	0.14	0.13
SSR32	F-CGTCGTTCAAGGTGTCGATG R-GACCGGTTGTTTGTTCCACT	(GTG)8	0.66	13	3.19	0.26	0.69
SSR45	F-CCCAACACCGGACATATACA R-GTTGGTTAGGGATGTGACGC	(ACC)5	0.52	6	2.39	0.48	0.58
SSR65	F-AGATGATGCTGCAATGCTCA R-CGTCGCTTTGTGGCACTATC	(TG)10	0.32	8	1.276	0.15	0.22
SSR87	F-CGCTTGTCCATGTCGTTCTC R-TGCTGACTGATCTGACAATGC	(TC)7	0.56	8	2.62	0.46	0.62
SSR95	F-ACAACTTAGCGGTTGGCTTG R-CTATCGAAGCGGACTTGCAT	(CA)8	0.41	3	1.90	0.61	0.47
SSR107	F-ACACGGACGTCTTCGGTTAC R-AGAGCCGTTGAAACACACCT	(GA)6	0.43	7	1.55	0.27	0.36
SSR119	F-TATTCACCGGAACATGCTGA R-CGAGGTCTGGTTAGCTGAGG	(CCTCAG)5	0.23	5	1.23	0.09	0.18
SSR124	F-AATTGAGAGGGTGCATGGAG R-GTGTTGGACGAATCCTCGTT	(ACA)5	0.52	6	2.38	0.48	0.58
SSR128	F-GAATGATCGGAAGCAAAGGA R-TTGCTGAGAGGTTCGTGTTG	(GAC)7	0.37	3	1.71	0.47	0.41
SSR132	F-ACGCTAATCCGCTTGTTCAT R-TCGCGTCACTCGTGTAAGTC	(CCT)8	0.61	6	3.02	0.67	0.67
SSR133	F-TGAACGTCGAGTGATTCAGG R-GGAGACGAGTCAATCCATCC	(TC)7	0.58	9	2.18	0.22	0.54
SSR136	F-ACGGAACCATCTTCGATGTC R-AGGAACGTGGCGTATACCAG	(GC)7	0.11	3	1.12	0.01	0.11
Mean			0.42	6.12	2.04	0.34	0.43

[a] Polymorphic information content.
[b] The number of alleles.
[c] The effective number of alleles.
[d] The number of observed heterozygosity.
[e] The number of expected heterozygosity.

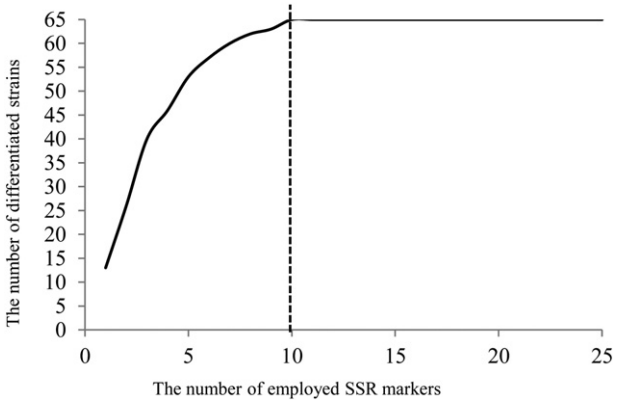


Fig. 1. Relationship between the SSRs used and the number of discriminated strains.

crossbreeding is scarce. Thus, uncovering the breeding history based on genetic evidence is necessary for further strain breeding.

In this study, we aimed to (i) explore SSRs in the genome of *F. velutipes* and compare their pattern with other species; (ii) select several powerful SSRs for the identification of strains of this fungal species; (iii) evaluate the genetic diversity of both cultivars and wild strains of *F. velutipes*; and (iv) uncover the breeding history of *F. velutipes* in China.

2. Materials and methods

2.1. Strains and DNA extraction

In total, 124 strains of *F. velutipes* were analyzed in this study, including 110 cultivars and 14 wild strains (Table S1). Genomic DNA was extracted from mycelia for each strain using the CTAB-based method (Doyle and Doyle, 1987). To prepare mycelia for DNA isolation, each strain was inoculated in a 90 mm Petri dish containing PDA medium at 23 °C for 10 days and then harvested. DNA concentration and purity were measured using a NanoDrop2000 spectrophotometer. The DNA solution for each sample was diluted to 100 ng/μl.

2.2. SSR mining and primer design

The genome sequence of *F. velutipes* (KACC 42780) was downloaded from the GenBank under accession numbers CM002695 to CM002705. SSRs were scanned using MIcroSAtellite (MISA) software (http://pgrc.ipk-gatersleben.de/misa/). Only perfect SSRs, including mono-, di-, tri-, tetra-, penta-, and hexa-nucleotide repeats with numbers of repeat units greater than or equal to 10, 6, 5, 5, 5, 5 and 5, respectively, were selected. Subsequently, primer pairs were designed using the online software Primer 3 with the following criteria: primer length ranging from 18 to 23 bp, product size from 100 to 300 bp and GC content averaging 50–55% (Untergasser et al., 2012; Koressaar and Remm, 2007).

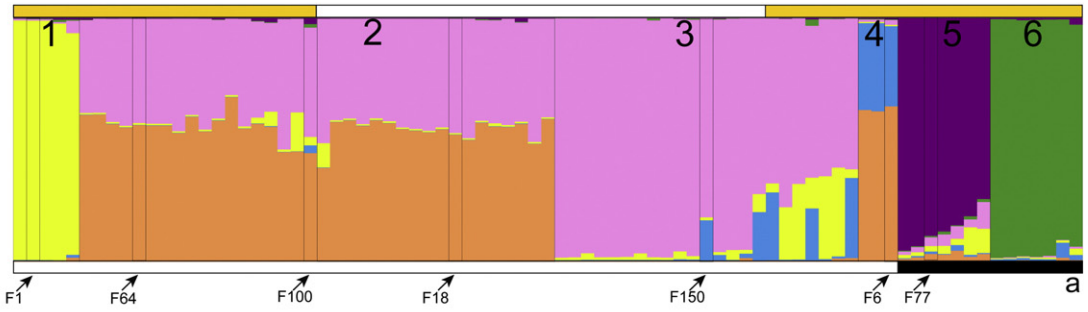
2.3. PCR amplification and SSR detection

For a preliminary test of the designed primer pairs, the SSRs were used for PCR amplification on eight randomly selected strains. PCR reactions were carried out in a total volume of 25 μl, containing 1 μl template DNA, 2.5 μl reaction buffer, 2.5 μl dNTP (200 μM), 1 μl for each primer (5 μM), 0.3 μl Taq DNA polymerase, and 17.7 μl ddH_2O. PCRs were conducted on an ABI 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) or an Eppendorf Master Cycler (Eppendorf, Netheler-Hinz, Hamburg, Germany) under the following parameters: 94 °C for 4 min, then 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, followed by a final extension step of 72 °C for 8 min. PCR products were checked using electrophoresis on a gel with 1% concentration. Then, one to four SSRs per chromosome (except chromosome 5, because SSRs in this chromosome are difficult to amplify), with a total of 25 SSRs, were used for a second round of PCR on all strains. In this procedure, the forward primer of each SSR was labeled with a fluorescent dye (FAM) at its 5′ end (TSINGKE, Kunming, China). The PCR products were analyzed with an ABI 3730 Genetic Analyzer using GeneScan500 Rox as a size

Table 3
Genetic diversity parameters in cultivars and wild strains.

Locus name	Na[a]		Ne[b]		Ho[c]		He[d]	
	cultivar	wild	cultivar	wild	cultivar	wild	cultivar	wild
SSR1	4	6	1.49	4.17	0.33	0.57	0.33	0.76
SSR2	4	8	2.45	4.04	0.58	0.79	0.59	0.75
SSR4	3	3	1.03	1.16	0.02	0.14	0.03	0.14
SSR5	2	2	1.01	1.07	0.02	0.07	0.02	0.07
SSR7	4	5	2.51	3.96	0.76	0.50	0.60	0.75
SSR15	2	2	1.77	1.07	0.40	0.07	0.44	0.07
SSR19	2	5	1.72	2.45	0.60	0.50	0.42	0.59
SSR21	7	7	3.39	4.26	0.48	0.36	0.71	0.77
SSR22	5	9	2.95	6.03	0.48	0.29	0.66	0.83
SSR23	3	3	1.57	1.45	0.43	0.07	0.36	0.31
SSR24	2	7	1.40	2.93	0.10	0.50	0.28	0.66
SSR25	3	6	1.05	2.67	0.05	0.21	0.04	0.63
SSR26	3	3	1.11	1.34	0.10	0.29	0.10	0.26
SSR32	7	11	2.34	8.91	0.25	0.29	0.57	0.89
SSR45	3	6	2.10	4.08	0.51	0.36	0.52	0.76
SSR87	3	8	1.95	3.84	0.46	0.43	0.49	0.74
SSR65	3	7	1.03	3.38	0.03	0.71	0.03	0.70
SSR124	4	6	2.13	2.24	0.46	0.57	0.53	0.55
SSR133	4	8	1.61	7.13	0.15	0.57	0.38	0.86
SSR95	3	2	1.89	1.91	0.60	0.64	0.47	0.48
SSR107	6	5	1.32	3.27	0.27	0.29	0.25	0.69
SSR119	3	4	1.11	1.90	0.05	0.29	0.10	0.47
SSR128	2	3	1.69	1.78	0.51	0.29	0.41	0.44
SSR132	5	5	2.55	3.47	0.72	0.43	0.61	0.71
SSR136	2	3	1.03	1.64	0	0.07	0.03	0.39
Mean(SE)	3.56(0.30)	5.36(0.48)	1.77(0.13)	3.21(0.39)	0.33(0.05)	0.37(0.04)	0.36(0.05)	0.57(0.05)

[a] The number of alleles.
[b] The effective number of alleles.
[c] The number of observed heterozygosity.
[d] The number of expected heterozygosity.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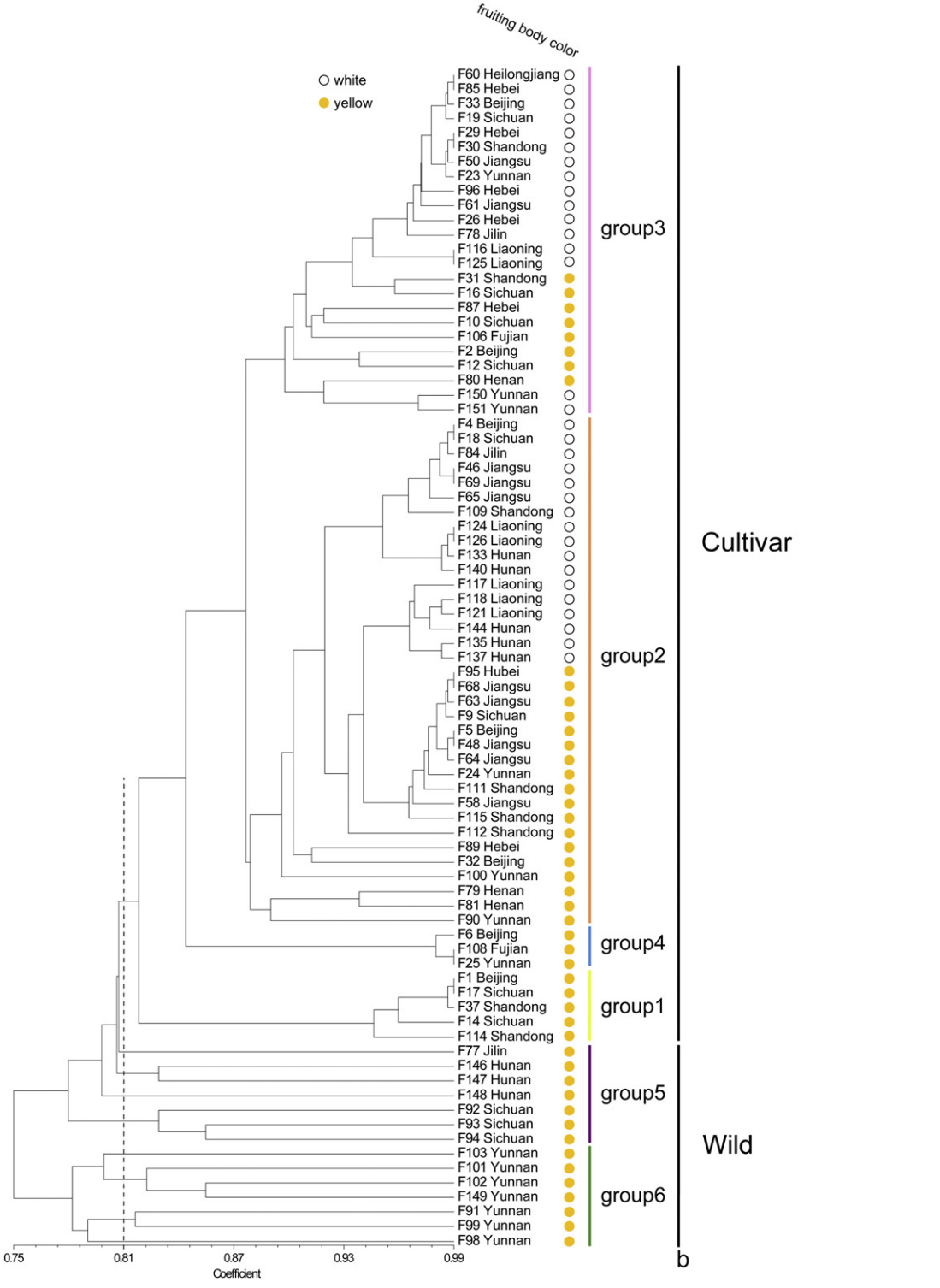
2
3
4
5
6
F1
F64
F100
F18
F150
F6
F77
a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white
yellow
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F85 Hebei
F33 Beijing
F19 Sichuan
F29 Hebei
F30 Shandong
F50 Jiangsu
F23 Yunnan
F96 Hebei
F61 Jiangsu
F26 Hebei
F78 Jilin
F116 Liaoning
F125 Liaoning
F31 Shandong
F16 Sichuan
F87 Hebei
F10 Sichuan
F106 Fujian
F2 Beijing
F12 Sichuan
F80 Henan
F150 Yunnan
F151 Yunnan
F4 Beijing
F18 Sichuan
F84 Jilin
F46 Jiangsu
F69 Jiangsu
F65 Jiangsu
F109 Shandong
F124 Liaoning
F126 Liaoning
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F95 Hubei
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F9 Sichuan
F5 Beijing
F48 Jiangsu
F64 Jiangsu
F24 Yunnan
F111 Shandong
F58 Jiangsu
F115 Shandong
F112 Shandong
F89 Hebei
F32 Beijing
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F94 Sichuan
F103 Yunnan
F101 Yunnan
F102 Yunnan
F149 Yunnan
F91 Yunnan
F99 Yunnan
F98 Yunnan
group3
group2
group4
group1
group5
group6
Cultivar
Wild
0.75
0.81
0.87
0.93
0.99
Coefficient
b

standard (Applied Biosystems, USA). Alleles of each locus were scored in base pairs by GeneMapper v3.2 (Applied Biosystems, USA). After manual correction of each migration, the size of the PCR products for each SSR was reported in an Excel spreadsheet.

2.4. Data analyses

The genetic diversity parameters, including the number of alleles (Na), effective number of alleles (Ne), observed heterozygosity (Ho) and expected heterozygosity (He), were calculated with GenAlEx v6.5 (Peakall and Smouse, 2012). A dendrogram of the genetic relationship among the strains was constructed based on the Simple Matching coefficient by applying the unweighted pair group method with arithmetic mean (UPGMA) using NTSYSpc v2.10e (Rohlf, 2000). Genetic structure was inferred by STRUCTURE v2.3.4 based on an admixture model. Models were tested for K-values ranging from 2 to 10, with 10 independent runs per K value. Each run consisted of a burn-in period of 10^6 iterations, followed by 10^6 Markov chain Monte Carlo (MCMC) steps. To determine the most probable value of K, the deltaK method (Evanno et al., 2005) was used as implemented in Structure Harvester (Earl and vonHoldt, 2012). Furthermore, we conducted a principal coordinate analysis (PCoA) using GenAlEx v6.5.

3. Results

3.1. Characterization of SSRs in the genome of F. velutipes and strain fingerprinting

A total of 1321 SSRs made up of 102 repeat types were identified in the genome of *F. velutipes*. The relative abundance of SSRs was 37 per Mbp. The tri-nucleotides were the most abundant repeat motif (62.4%), followed by mono- (18.3%), di- (16%), tetra- (1.7%), hexa- (1.5%) and penta- (0.8%) nucleotides (Table 1). The most frequent repeat type was the tri-nucleotide ACG/CGT, accounting for 24.8% of the total SSRs, followed by A/T and AG/CT, accounting for 14% and 6.7% of the total SSRs, respectively (Table S2). Among the 1321 SSRs, 115 were selected for PCR amplification on all of the 124 strains, 109 of which could be successfully amplified, and 25 polymorphic SSRs were selected for subsequent analyses.

The 25 SSRs yielded 153 alleles, ranging from 2 to 13 per locus with an average of 6.12 per locus. The mean polymorphic information content (PIC) value of the 25 SSRs was 0.42; further information is detailed in Table 2. Based on the criterion of Botstein et al. (1980), the 25 SSRs can be divided into three groups: highly informative, with PIC > 0.5 (10 SSR markers, namely SSR2, SSR7, SSR21, SSR22, SSR32, SSR45, SSR87, SSR124, SSR132 and SSR133), reasonably informative, with 0.5 > PIC > 0.25 (9 SSR markers, namely SSR1, SSR15, SSR19, SSR23, SSR24, SSR65, SSR95, SSR107 and SSR128), and slightly informative, with PIC < 0.25 (6 SSR markers, namely SSR4, SSR5, SSR25, SSR26, SSR119 and SSR136).

Of the 124 strains, 65 could be discriminated from other strains by using these 25 SSRs. Interestingly, a combination of 10 selected SSRs (Table 2, names in bold) could also clearly discriminate these 65 strains (Fig. 1). Specifically, we firstly selected the SSR marker that could discriminate the most strains among the 25 SSRs. Then, for the second SSR marker, we selected the one that could discriminate the most strains when combined with the first SSR marker. The subsequent eight SSRs were selected following this routine. Among the 65 identified strains, F5 and F135 shared the same commercial name "Jinzashi 9," while F29 and F89 shared the same commercial name "FV093." However, each of these two pairs shows distinct allelic patterns. The remaining 59 undiscriminated strains could be separated into 16 groups based on identical allelic patterns in each group. Strains in the same group are probably identical but with different commercial names. In the following analyses of genetic diversity and genetic structure, only a single strain from each of the 16 groups was selected.

3.2. Genetic diversity analyses

The 153 alleles identified from 25 SSRs were used to evaluate the genetic diversity of the 81 selected strains of *F. velutipes*, including 67 cultivars and 14 wild strains. GenAlEx v6.5 analyses indicated that the Na, Ne, Ho and He of the wild strains are higher than those of the cultivars (Table 3). Additionally, a total of 64 private alleles could be uncovered from the wild strains, whereas only 19 private alleles could be identified from the cultivars. Furthermore, the dendrogram constructed using the UPGMA method revealed that the cultivars are all grouped in one cluster with a similarity of 0.81, while the wild strains are separated into eight clusters (Fig. 2b). Therefore, the genetic diversity in wild strains is higher than in the cultivars.

Based on the cluster analyses, the 81 strains could be chiefly divided into 6 groups (Fig. 2a and b), 4 of which were formed mainly by cultivars, while the remaining 2 were dominated by wild strains. The genetic diversity of each group was subsequently analyzed using GenAlEx v6.5. Among the cultivar groups, group 2 possesses the highest genetic diversity, followed by group 1, group 4 and group 3. Of the wild groups, group 5 possesses higher genetic diversity than group 6 (Table 4).

3.3. Genetic structure analyses

The genetic structure of the 81 *F. velutipes* strains was inferred using STRUCTURE v2.3.4 based on the alleles detected from the 25 SSR markers. Admixture model-based simulations indicated that the most suitable ΔK is six, which means it is reasonable to divide these 81 *F. velutipes* strains into six groups (Fig. 2a). A similar result was also shown in the dendrogram constructed using the UPGMA method (Fig. 2b). Strains in groups 1 to 4 were cultivars, while those in groups 5 and 6 were wild strains (Fig. 2a and b). However, two unexpected assignments of F100 and F77 were detected. The strain F100, collected from the Kunming Botanic Garden and regarded as a "wild strain," was assigned to a cultivar group (group 2), while strain F77, purchased from a mushroom company and labeled as a "cultivar," was clustered into a wild group (group 5). Among the cultivars, strains from the same province were assigned to different groups, indicating that the genetic distances are not correlated with geographic origins. In regard to the wild strains, group 5 comprised strains from Sichuan and Hunan provinces, while group 6 comprised strains from Yunnan province. Both groups 5 and 6 possessed their own different genetic components. As regards the color of the pileus of the strains, groups 2 and 3 comprised both white and yellow strains, while the strains in the remaining groups (1, 4, 5 and 6) were exclusively yellow strains.

A principal coordinate analysis (PCoA) was conducted as an alternative measure of the relationships among the strains. The first two axes explained 21.89% and 36.52% of the total variance respectively (Fig. 3). The result roughly agreed with the structure and UPGMA analyses, i.e., the strains were mainly separated into 6 groups. As exceptions,

Fig. 2. STRUCTURE anaylses and UPGMA dendrogram of 81 *F. velutipes*. a Estimated genetic structure for K = 6. Each group is labeled in Arabic numerals. Each bar represents a single individual. The color of the pileus is indicated above the figure, yellow strain represented with yellow bar, white strain represented with white bar. Cultivars or wild strains are indicated below, cultivars represented with white bar, wild strain represented with black bar. F100 was collected from the field but clustered in the cultivar group. F77 was purchased from a mushroom company and labeled as "cultivars," yet clustered in the wild group. F64 is one of the hybridization progeny of "Xinnong 2" and "Sanming 1." F18 is a white strain introduced from Japan. F1, F150 and F6 are the strains mentioned in Fig. 4. b UPGMA dendrogram of 81 strains of *F. velutipes* based on the SSR genetic similarity coefficient of 81 strains.

Table 4
Genetic diversity in six groups.

Group	Mean Na[a] (SE)	Mean Ne[b] (SE)	Mean Ho[c] (SE)	Mean He[d] (SE)
1	2.60 (0.28)	1.31 (0.06)	0.17 (0.04)	0.20 (0.03)
2	2.72 (0.17)	1.69 (0.13)	0.43 (0.07)	0.33 (0.05)
3	1.48 (0.12)	1.46 (0.11)	0.43 (0.10)	0.22 (0.05)
4	1.64 (0.14)	1.43 (0.11)	0.34 (0.09)	0.22 (0.05)
5	3.80 (0.35)	2.63 (0.25)	0.41 (0.05)	0.52 (0.05)
6	3.48 (0.34)	2.52 (0.29)	0.34 (0.04)	0.48 (0.05)

[a] The number of alleles.
[b] The effective number of alleles.
[c] The number of observed heterozygosity.
[d] The number of expected heterozygosity.

F79 and F90, belonging to group 2, were separated near group 3 and group 5 respectively. F93 and F147 belonging to group 5, were separated near group 6 (Fig. 3).

4. Discussion

4.1. The abundance of SSRs in the genome of F. velutipes

In this study, 1321 SSRs were uncovered from the genome of *F. velutipes* (Park et al., 2014), and 115 SSRs were selected for PCR amplification. Among them, 109 SSRs could amplify single bands and 25 of the tested SSRs were polymorphic. In a previous study, Zhang et al. (2010) used a FIASCO method to construct a genomic library enriched for SSRs, and 100 positive clones were selected for detecting SSRs. Finally, they tested 26 SSRs, and only 10 were polymorphic among the 14 tested strains of *F. velutipes*. Therefore, our results suggest a higher efficiency of SSR mining through genomic data compared with the experimental efforts (Dutech et al., 2007; Zhang et al., 2010).

Microsatellites were relatively less abundant in the genome of *F. velutipes*, with 1321 SSRs and a relative abundance of 37 SSRs/Mb, than in the genomes of other fungi (Table 5). The number of SSRs in fungi ranges from 1973 to 21,889, and the relative abundance of SSRs ranges from 20 to 370 SSRs/Mb (Table 5) (Karaoglu et al., 2005; Murat et al., 2011; Qian et al., 2013; Foulongne-Oriol et al., 2013; Luo et al., 2015; Lim et al., 2004). For most of the fungal and other eukaryotic genomes, the most abundant motif is mono-nucleotide (Karaoglu et al., 2005; Murat et al., 2011; Qian et al., 2013; Foulongne-Oriol et al., 2013; Luo et al., 2015; Lim et al., 2004), while in most of the prokaryotic genomes, the most abundant motif is tri-nucleotide (Sharma et al., 2007; Tóth et al., 2000; Murat et al., 2011). Surprisingly, the most abundant SSR motif in the genome of *F. velutipes* is the tri-nucleotide, followed by the mono-nucleotide. Motifs with small repeat numbers were found to be predominant in the genome of *F. velutipes*, similar to previously reported fungal genomes (Karaoglu et al., 2005; Murat et al., 2011; Qian et al., 2013; Foulongne-Oriol et al., 2013; Luo et al., 2015; Lim et al., 2004).

4.2. Improper naming of commerical names for strains of F. velutipes in China

In this study, 65 out of 124 strains of *F. velutipes* could be clearly discriminated using 10 selected SSRs (Fig. 1). The remaining 59 strains could be roughly assigned into 16 groups, each of which contained identical allelic patterns. When the number of SSRs used for the identification of strains was increased from 10 to 25, the number of strains that could be identified did not change (Fig. 1), indicating that the combination of these 10 SSRs would be informative enough to identify most genetically different strains. Thus, the 59 strains that failed to be clearly identified could be well represented by 16 strains.

The labeling of the same cultivar with different commercial names could result from irregular management of the spawn market. For example, when spawn suppliers purchase a spawn labeled with an official commercial name, they may give a new commercial name to the spawn when selling it to others. Another possibility is that spawn suppliers give a famous spawn name to other genetically distinct spawns to increase their economic value. The use of wrong commercial names for spawns may result in economic loss for growers and intellectual property destruction for breeders (Palapala et al., 2002; Zhang et al., 2012). Thus, the nomenclature of the commercial strains of *F. velutipes* needs to be standardized.

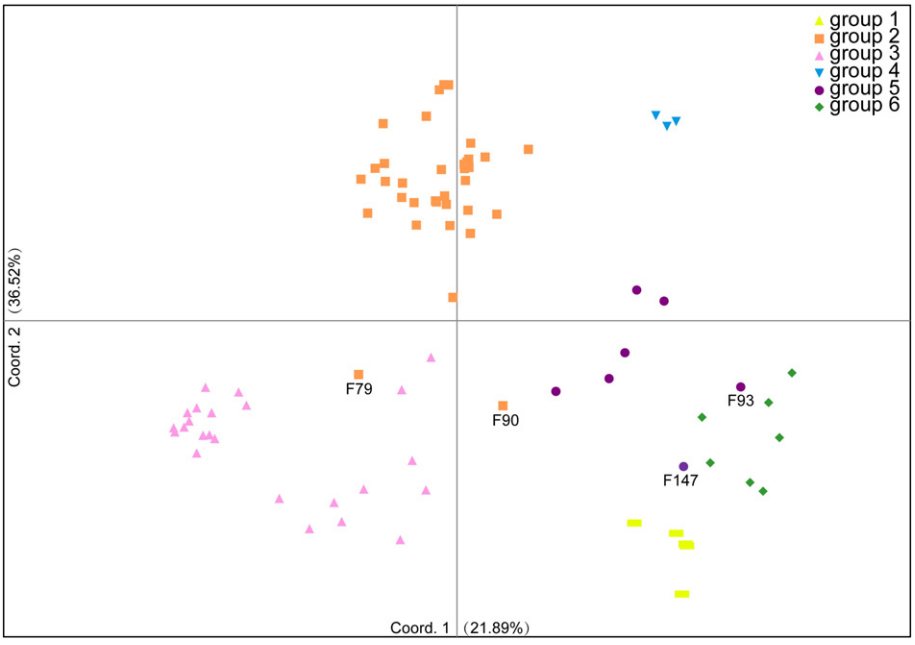

Fig. 3. Principal coordinate analysis (PCoA) of 81 strains of *F. velutipes* based on 25 SSR markers.

Table 5
Comparison of the number of SSRs and total relative abundance in 21 fungal genomes.

species	Repeat type						
	Mono-	Di-	Tri-	Tetra-	Penta-	Hexa-	Total (relative abundance)
Agaricus bisporus var. *bisporus*	2051	1244	717	22	10	18	4062 (133)
Agaricus bisporus var. *burnettii*	1897	1335	690	24	11	15	3972 (134)
Aspergillus nidulans	1249	753	325	36	28	19	2410 (80)
Coprinus cinereus	761	375	843	38	10	23	2050 (56)
Cryptococcus neoformans	855	671	401	20	3	23	1973 (104)
Fusarium graminearum	1109	1034	555	85	73	40	2896 (80)
Ganoderma lucidum	1090	485	806	58	29	206	2674 (62)
Laccaria bicolor	3375	740	1595	72	258	64	6104 (101)
Magnaporthe grisea	8042	1730	1573	219	33	45	11,642 (307)
Neurospora crassa	5943	3208	4084	758	192	134	14,319 (377)
Phanerochaete chrysosporium	386	379	598	14	3	13	1393 (40)
Postia placenta	505	489	642	55	76	49	1816 (20)
Saccharomyces cerevisiae	2363	817	396	14	9	19	3618 (255)
Schizophyllum commune	346	200	593	24	15	28	1206 (31)
Schizosaccharomyces pombe	2332	662	196	21	18	3	3232 (247)
Serpula lacrymans	1334	310	678	27	13	51	2413 (57)
Tuber melanosporum	16,733	1998	2007	707	262	112	21,889 (177)
Ustilago maydis	658	1162	865	76	76	196	3033 (154)
Flammulina velutipes	242	211	824	23	1	20	1321 (37)

4.3. Breeding history of *F. velutipes* in China

In China, mycologists have carried out a large number of studies on the domestication and crossbreeding of *F. velutipes* during the past 50 years (Guo, 1997; Liu, 2003; Song et al., 2007). Our study partially revealed the breeding history of Winter Mushroom (Figs. 2a and 4). The first cultivar of *F. velutipes* in China, "Sanming 1," was domesticated from a wild strain isolated from Fujian province in 1974 (Guo, 1997; Song et al., 2007). In 1983, breeders in Fujian introduced the first white strain, named "Xinnong 2," from Japan and hybridized it with "Sanming 1" (Guo, 1997). One of the hybridization progeny was selected as a new cultivar, which was used in this study as F64. Our genetic structure analysis indicated that the strain F64 was hybridized between the white strains in group 3 and another yellow strain (Fig. 2a). Thus, the white strains in group 3 are probably isolated and propagated from "Xinnong 2," while the yellow strains in group 3 possess gene flow between the yellow strains in group 1 and the white strains in group 3. In 1987, another white strain, F21 (named F18 in this study) was introduced to China from Japan by breeders in Zhejiang province. The white strains in group 2 probably came from the strain F21. In addition, the yellow strains in group 1 and group 4 were reported as hybridizations between the yellow strains or domestications from southeastern

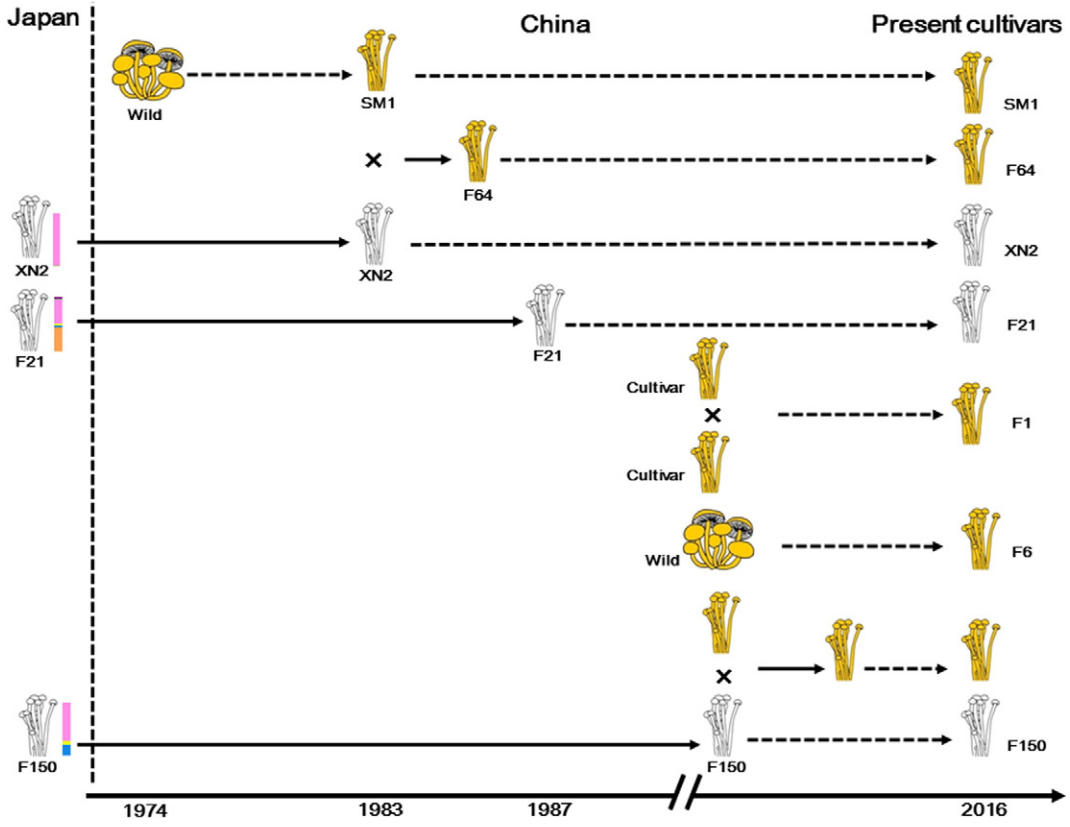

Fig. 4. Breeding history of Winter Mushroom in China.

China. Our genetic structure analyses revealed that the white strains in China were probably originally introduced from Japan, and the yellow strains may have directly been domesticated from the wild strains in southeastern of China or hybridized with the white cultivars or yellow strains.

4.4. Potential value of the wild strains for the improvement of cultivar spawns

Wild strains from different geographic origins possess different genetic components (Fig. 2a), and show significantly higher genetic diversity than the cultivars (Table 3). Similar results have also been found in *Lentinula edodes*, as revealed by SSR markers (Xiao et al., 2010; Xiang et al., 2016). This is probably partly due to evolution and adaptation to local environments. For example, the strains in group 5 were mainly collected from Hunan and Sichuan provinces. F92, F93 and F94 were collected in high-altitude areas (3100 m above sea level) in western Sichuan province, while F146, F147 and F148 were collected from areas of Hunan province with an altitude of 150–860 m. Therefore, environmental heterogeneity may play an important role in breeding the genetic diversity of these strains. Although the strains in group 6 possess a relatively high genetic diversity, they were collected solely from Yunnan province, from similar subtropical environmental conditions (five strains, namely F98, F99, F101, F102 and F149, collected from Longling, one strain, namely F103, collected from Kunming, and one strain, namely F91, collected from Dali). Therefore the genetic diversity of group 5 is higher than group 6.

The subjection of cultivars to human selection for over a half-century in China, the unitarily selected standardization and the common growth environment have caused a narrower genetic diversity and increased the inbreeding rate between the cultivars. This may lead to spawn degeneration in the cultivars (Xu, 1995). The high genetic diversity and distinctive genetic components in natural populations suggest that a large "arsenal" gene pool in nature is available for mushroom breeding. For example, some economically important traits, such as bacterial blotch resistance and resistance to degeneration, were previously identified in wild collections of *Agaricus bisporus* (Callac et al., 1993; Li et al., 1994). For *F. velutipes*, the strains used in cultivation at the large commercial scale are stringently controlled at low temperature (≤15 °C) to form the fruiting body, which costs large amounts of energy. Thus, strains tolerating relatively high temperature would be preferable because the use of such strains would reduce the costs, especially in summer. In our previous experiments, the wild isolate F98, collected from Longling, Yunnan in the summer, could grow more vigorously than the strains currently used by industry at 18 °C in the laboratory (Fig. 5). Therefore, wild strains are important for further strain breeding.

4.5. Spread of the cultivar spawns

Our clustering analysis (Fig. 2b) indicated that cultivars from the same province can be assigned into different groups, demonstrating that the genetic distances are not correlated with geographic origins. For such heavily cultivated mushrooms, exchanges of strains among different regions must be common, resulting in vanishing genetic differences in different locations. Moreover, the isolate F100, collected from Kunming Botanic Garden yet grouped with the cultivars, may have originated from basidiospores of the cultivars escaping from canteens, probably near the collecting site. The escape of basidiospores from cultivars to the wild is universal in the artificial cultivation of mushrooms (Hibbett and Donoghue, 1996; Xu et al., 1997; Kerrigan, 2005), and may cause the contamination of the wild gene pool. For example, in most rural areas in China, the outdoor log cultivation of *Lentinula edodes*, *Auricularia heimuer* and *Auricularia villosula* is common, which may produce a high density of the basidiospores around the cultivation areas and cause the escape of the cultivar spawns to the wild (Hibbett and Donoghue, 1996).

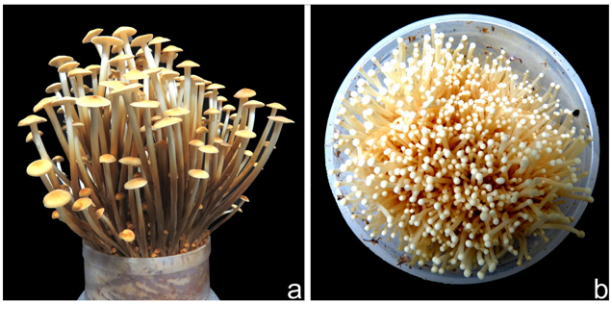

Fig. 5. Two strains of *F. velutipes* grown at 18 °C in the laboratory: a. F98 (wild strain); b. F3 (industrialized strain).

In conclusion, the SSRs discovered in this study showed that improper naming of the commercial names of the Winter Mushroom cultivars is common in China. Our data revealed the comprehensive genetic diversity of *F. velutipes* in China for the first time, and a higher genetic diversity in wild strains, which could be used as a valuable "arsenal" gene pool in future strain breeding. The breeding history of *F. velutipes* in China was uncovered based on genetic evidence. Finally, the genetic erosion of the wild germplasm by escaped cultivar spores should be taken into consideration for wild germplasm protection.

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