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Hybridization, characterization and transferability of SSRs in the genus *Morchella*

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ABSTRACT

Recently, *Morchella importuna*, *M. sextelata*, *M. eximia*, *M. exuberans*, *Mel*-13, and *Mel*-21 have been successfully cultivated in China and planting areas rapidly expanded because of their economic importance. Effective molecular markers are urgently needed for accurately identifying morel cultivars. Microsatellites are widely utilized for strain authentication in many fungal groups. To our knowledge, for the first time we characterized the distribution of microsatellites (simple sequence repeats, SSRs) in the *M. importuna* genome with 12902 SSRs and reported the first set of SSRs developed for *Morchella* species. Mono-nucleotides (66.2 %) were the most frequent motifs, followed by tri- (15.4 %), di- (12.1 %), tetra- (3.7 %), penta- (1.3 %) and hexa-nucleotides (1.3 %). We tested the cross-species amplification of 180 SSRs on 24 samples from the six species and high cross-species transferability of SSRs (87.7 %) was found. Among twenty-two microsatellites selected for genetic diversity analysis on 127 samples from the six species, fifteen to twenty polymorphic loci were identified in *M. importuna*, *M. sextelata*, *M. eximia*, *M. exuberans*, *Mel*-13 and *Mel*-21. Interspecific hybridization events were detected among morel species, indicating the potential application of morel crossbreeding. Ninety-one cultivated samples were characterized as new cultivars with different genotypes, but cultivar names used for these by farmers was confusing, with misnaming, synonyms and homonyms. Our results are not only helpful for cultivar identification and morel breeding programs in China, but also provide molecular tools for genetic studies in morels.

1. Introduction

Morel mushrooms, a globally distributed ascomata belonging to the genus *Morchella*, are regarded by gourmets as a delicacy because of their desirable flavor (Litchfield, 1967) and are marketed worldwide (Pilz et al., 2007). Their important economic value has resulted in rising market demand and led to world-wide over-harvest of wild morels (Du et al., 2016). Successful cultivation of morels under controlled conditions has helped alleviate the market pressure. *Morchella esculenta* was the first species reported to be cultivated in USA (Ower, 1982), followed by *Morchella rufobrunnea* in Israel (Masaphy, 2010). Recently, *Morchella importuna*, *Morchella sextelata*, *Morchella eximia*, *Morchella exuberans*, *Mel*-13 and *Mel*-21 have also been successfully cultivated in China. The technological progress and vigorous government agricultural support have promoted the rapid expansion of the morel production areas in China, which were reported to cover more than 1600 ha in 2016 according to a recent Chinese survey (http://mp.weixin.qq.com/s/y4N_AdTXFFIeFOgUpbZAQQ). Plentiful spawns used for morel cultivation are needed every year. China has been reported to be the modern species diversity centre of *Morchella*, with more than 30 species (Du et al., 2012), and the resulting abundant wild germplasm resources facilitate the breeding of morel cultivars.

However, our investigation has revealed that many morel breeding lines have been developed without identification. On the one hand, it is difficult to distinguish morels species according to morphology (Du et al., 2012), on the other hand, the DNA markers (ITS rDNA, *TEF*1a, *RPB*1 and *RPB*2) used for identifying species in the genus *Morchella* (Du et al., 2012) are not informative enough to distinguish morel culitvars within each species. As a result, misnaming, synonymies (different names for the same cultivar) and homonyms (the same name for different cultivars) are frequent at morel-growing sites. Spawn is an important factor for edible fungi cultivation, and the quality of spawn is directly related to the

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development of edible fungi production (Zhang and Zhao, 2016). Therefore, mixing spawns and unknown cultivars will have a negative impact on morel cultivation and the formation of high-quality spawns. It is urgent to use suitable molecular markers to exactly identify morel cultivars and breeding lines, especially before production testing and for sustainable use in the future.

Microsatellites, also known as simple sequence repeats (SSRs) or short tandem repeats (STRs), have emerged as one of the most powerful genetic markers for cultivar discrimination, fingerprinting and genetic diversity because of their high levels of allelic variation, codominant nature and extensive abundance in genomes, which allows them to deliver more information than other markers (Madhou et al., 2013; Chen et al., 2014). In fungi, low numbers of microsatellite repetitions were described (usually fewer than eight), with a high proportion of mononucleotides and only rare high-polymorphic SSRs (Dutech et al., 2007). Additionally, cross-species transferability of SSRs has also been found in many species (Reid et al., 2012; Maduna et al., 2014; Pirog et al., 2016; Rai et al., 2017), allowing for genetic studies in related species, but in total it seems to be lower in fungi than other organisms (Barbara et al., 2007; Schoebel et al., 2013a, b). Under the rapid development of the genome sequencing technique, more polymorphic SSRs were extracted from the genome of economically important mushrooms and proved powerful in the accurate identification of strains, for example, *Pleurotus ostreatus*, *Lentinula edodes*, *Flammulina velutipes* and *Auricularia auricula-judae* (Kyung-Ho et al., 2009; Zhang et al., 2012; Qu et al., 2016; Xiang et al., 2016; Liu et al., 2016, 2017). However, despite the fact that morel cultivation has been extremely popular and developing at a great rate in the emerging and booming mushroom industry, no SSRs have been developed in *Morchella* for spawn discrimination until now.

In this study, based on the unpublished draft genome of the strain M5 of *M. importuna* sequenced in Chongqing Normal University, we aimed to (1) characterize SSR patterns in the draft genome of *M. importuna*; (2) develop an effective SSR set for culitvar authentication in six cultivated *Morchella* species, *M. importuna*, *M. sextelata*, *M. eximia*, *M. exuberans*, *Mel*-13 and *Mel*-21; (3) evaluate the genetic diversity of these species; and (4) test cross-species transferability of SSRs and potential hybridization.

2. Materials and methods

2.1. Strain selection, DNA extraction

In order to assess the transpecific amplification pattern and explore comprehensive SSR markers used across-species, samples from six cultivated species were harbored including cultivars that were collected from morel farms by cooperating with growers, and wild samples. Geographical locations and sample distribution are summarized in Table 1. Genomic DNA was isolated from the silica gel-dried materials or mycelia using the CTAB-based method (Doyle and Doyle, 1987). Each strain was inoculated in a 90 mm Petri dish containing PDA medium at 23 °C for 10 d and then mycelium was harvested for DNA extraction. DNA concentration and purity were measured using a NanoDrop 2000 spectrophotometer. The DNA solution for each sample was diluted to 100 ng/μl.

2.2. Species identification

Because it is difficult to distinguish morels species according to morphology (Du et al., 2012), and considering that cultivar names used by farmers were always confusing and not very accurate, species identification of all the samples used in this study was previously performed based on molecular phylogenetic analysis of the ITS rDNA dataset according to Du et al. (2012). The phylogenetic analysis via a Maximum Likelihood (ML) were conducted using RAxML v.7.2.6 (Stamatakis, 2006). The best ML tree is shown in Supplementary file 1 (Fig S1). The results of species identification for each sample are shown in Table 1.

2.3. SSRs genotyping, primer design and PCR amplification

Microsatellite loci were retrieved from the genome sequences of *M. importuna* by using the software MISA (http://pgrc.ipk-gatersleben.de/misa/). Depending on the purity and complexity of the motifs, SSRs can be perfect (single motif in an uninterrupted array), imperfect (two or more motifs in interrupted arrays) or compound (two or more motifs in uninterrupted arrays) (Weber, 1990). Careful prioritization should be given to perfect SSRs, which are known to have greater allelic variability than compound or imperfect SSRs (Buschiazzo and Gemmell, 2006; Kelkar et al., 2008). The SSR motif detection criterion in this study was that only perfect SSRs $\geq$12 nucleotides long were selected. Primers were designed for 180 identified microsatellite loci 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). Twenty-four fruiting bodies from six morel species were used to evaluate the successful amplification ratio of the 180 primer pairs. Eighty-nine SSRs could be successfully amplified, of which 22 were selected for the following analysis (Table 2). For each of twenty-two primer pairs, the forward primer was fluorescently labeled with 6 - Carboxyfluorescein (6-FAM) on the 5′ end. PCR was carried out on an ABI 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) using the following program: predenaturation at 94 °C for 3 min, then followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C/48 °C/52 °C (Table 2) for 30 s and elongation at 72 °C for 30 s; this was followed by a final elongation at 72 °C for 10 min. PCR products were sent to Kunming Shuoqing Biotech Ltd to carry out capillary electrophoresis and length scoring. Alleles of each locus were scored in base pairs by GeneMapper v.3.2 (Applied Biosystems, USA) and the size of the PCR products for each SSR was reported in an Excel spreadsheet.

2.4. Statistical analysis

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 v.6.5 (Peakall and Smouse, 2006). The informativeness of the SSR markers was estimated in the analyses by calculating the polymorphic information content (PIC) of each locus, which was used to measure the information content of molecular markers and computed from the frequency of alleles detected per locus according to Bostein et al. (1980).

2.5. Cluster analysis

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 v.2.10e (Rohlf, 2000). The number of genetic clusters was inferred using the program STRUCTURE v.2.3.4 (Pritchard et al., 2000). As suggested by the software manual, we applied the admixture model and used sampling locations as prior (LOCPRIOR). The allele frequency model was set to “Allele Frequencies Correlated” (Falush et al., 2003). The simulation was run with the number of clusters (K) from one to ten, and each K was repeated ten times. Each run consisted in a burn-in period of 10^6 iterations, followed by

Table 1
Details regarding the analyzed *Morchella* species and cultivars.

Sample No.	Species[b]	Location	Type	Unreliable cultivar names[c]	Sample Depository[a]
M1	*M. importuna*	France	Wild	/	Chongqing Normal University
M2	*M. importuna*	Chongqing, China	Wild	/	Chongqing Normal University
M3	*M. importuna*	Germany	Wild	/	Chongqing Normal University
M4	*M. importuna*	Pakistan	Wild	/	Chongqing Normal University
M5	*M. importuna*	Sichuan, China	Wild	/	Chongqing Normal University
M6	*M. importuna*	Sichuan, China	Wild	/	Chongqing Normal University
M7	*M. importuna*	Yunnan, China	Wild	/	Chongqing Normal University
M8	*M. importuna*	Yunnan, China	Wild	/	Chongqing Normal University
M9-M13	*M. importuna*	Chongqing, China	Cultivated	Morel	Chongqing Normal University
M14-M16	*M. importuna*	Hebei, China	Cultivated	Morel	Chongqing Normal University
M17-M23	*M. importuna*	Henan, China	Cultivated	Morel	Chongqing Normal University
M24-M49	*M. importuna*	Sichuan, China	Cultivated	Chuan one	Chongqing Normal University
M50	*M. importuna*	Sichuan, China	Cultivated	Yang one	Chongqing Normal University
M51-M53	*M. importuna*	Shanxi, China	Cultivated	Morel	Chongqing Normal University
M54-M62	*M. importuna*	Xinjiang, China	Cultivated	Chuan one	Chongqing Normal University
M63-M67	*M. importuna*	Yunnan, China	Cultivated	Yun one	Chongqing Normal University
M68-M72	*M. importuna*	Zhejiang, China	Cultivated	Black pearl	Chongqing Normal University
M73	*M. sextelata*	Yunnan, China	Wild	/	Chongqing Normal University
M74	*M. sextelata*	Yunnan, China	Wild	/	Chongqing Normal University
M75-M78	*M. sextelata*	Sichuan, China	Cultivated	Morel	Chongqing Normal University
M79-M80	*M. sextelata*	Sichuan, China	Cultivated	*M. importuna*	Chongqing Normal University
M81-M84	*M. sextelata*	Sichuan, China	Cultivated	*M. sextelata*	Chongqing Normal University
M85-M86	*M. sextelata*	Hubei, China	Cultivated	Black pearl	Chongqing Normal University
M87-M89	*M. sextelata*	Sichuan, China	Cultivated	Morel	Chongqing Normal University
M90	*M. eximia*	USA	Wild	/	Chongqing Normal University
M91-M94	*M. eximia*	Yunnan, China	Wild	/	Chongqing Normal University
M95-M96	*M. eximia*	France	Wild	/	Chongqing Normal University
M97-M98	*M. eximia*	Yunnan, China	Wild	/	Chongqing Normal University
M99-M102	*M. eximia*	Sichuan, China	Cultivated	Morel	Chongqing Normal University
M103-M109	*M. exuberans*	Yunnan, China	Wild	/	Chongqing Normal University
M110	*M. exuberans*	Yunnan, China	Cultivated	Morel	Chongqing Normal University
M111	*Mel*-13	Shaanxi, China	Wild	/	Chongqing Normal University
M112	*Mel*-13	Xinjiang, China	Wild	/	Chongqing Normal University
M113	*Mel*-13	Sichuan, China	Wild	/	Chongqing Normal University
M114	*Mel*-13	Xinjiang, China	Wild	/	Chongqing Normal University
M115	*Mel*-13	France	Wild	/	Chongqing Normal University
M116	*Mel*-13	Sichuan, China	Wild	/	Chongqing Normal University
M117	*Mel*-13	Sichuan, China	Wild	/	Chongqing Normal University
M118	*Mel*-13	Sichuan, China	Cultivated	Morel	Chongqing Normal University
M119	*Mel*-21	Hubei, China	Wild	/	Chongqing Normal University
M120	*Mel*-21	Sichuan, China	Wild	/	Chongqing Normal University
M121	*Mel*-21	Sichuan, China	Wild	/	Chongqing Normal University
M122-M127	*Mel*-21	Sichuan, China	Cultivated	Morel	Chongqing Normal University

[a] All the samples are kept in Chongqing Normal University.
[b] Species identification for all the samples was performed by molecular phylogenetic analysis of ITS nrDNA dataset.
[c] Unreliable culitvar names were used by farmers.

10^6 Markov Chain Monte Carlo (MCMC) steps. The obtained results were analyzed in Structure Harvester (Earl, 2012), with the most likely value for K determined by Evanno's delta K (ΔK) (Evanno et al., 2005). The results were visualized by using the software CLUMPP (Jakobsson and Rosenberg, 2007) and DISTRUCT (Rosenberg, 2004).

2.6. Assignment tests

Assignment tests of individuals were performed by Geneclass 2.0 softwares (Piry et al., 2004) using a Bayesian method (Evanno et al., 2005) and a simulation algorithm (Rannala and Mountain, 1997) with 10000 simulated individuals.

2.7. Principal co-ordinates analysis

In order to view the patterns of grouping more effectively, principal co-ordinates analysis (PCOA) was performed with GenAlex v.6 (Peakall and Smouse, 2006) based on distance matrix to visualize genetic relationships among strains and to confirm the usefulness of the selected SSR loci to differentiate among cultivars and wild samples.

3. Results

3.1. Characterization of SSRs in the genome of M. importuna

A total of 12902 SSRs made up of 490 repeat types were identified in the draft genome of *M. importuna*. The mono-nucleotides were the most abundant repeat motif, accounting for 66.2 % of all SSRs, followed by tri- (15.4 %), di- (12.1 %), tetra- (3.7 %), penta-nucleotide (1.3 %) and hexa-nucleotide repeats (1.3 %) (Table 3). Total 180 from the 12902 SSRs were tested for PCR amplification of 24 strains from six species. The results showed 153 of the 180 SSRs could generate amplification products from at least 1 and up to 6 species, among which 89 SSRs was successful in all species. Finally, 22 polymorphic SSRs were selected for subsequent analyses on all the 138 samples.

3.2. Strain fingerprinting of samples and genetic diversity of SSRs among M. importuna, M. sextelata, M. eximia, M. exuberans, Mel-13 and Mel-21

Given that *M. sextelata*, *M. eximia*, *M. exuberans*, *Mel*-13 and *Mel*-21 have also been successfully domesticated in China, cultivars and

Table 2
List of 22 SSR primers used for this study with primer sequences and characteristics of these markers.

Loci	Repeat motif	Primer sequence (5′–3′)	Size range	Tm
C1	$(AT)_9$	F-AGGAGCACCAATCAATACC; R-CTTCAATTGCCCGCTTACTT	251–265	50 °C
C2	$(GA)_7$	F-GCACTAAGGCGGATTCAGTC; R-ATGCGGAGATTCTACATCCG	207–245	50 °C
C3	$(CA)_6$	F- GGTCCAATGGAGGTTGAGAA; R-TTAACCTGCATGCTGCACTC	259–275	50 °C
C4	$(TC)_6$	F-CAGAGGGTATCGTCATCGGT; R-TTCCGGAAGTTGCCGTATAG	115–131	50 °C
C5	$(AT)_6$	F-GACAGAACCAAGCTGCAACA; R-GAGGCCAGTGATCTGAGGAG	260–278	50 °C
C6	$(AT)_8$	F-TGAACCTAGGATAATCGAGGG; R-CATGCCATTCTTGTTGAGGA	218–228	50 °C
C7	$(AG)_8$	F-GCGATCTGATCCCTCAACTC; R-TTGCCAATGATATGCGGTTA	229–263	48 °C
C8	$(CAC)_7$	F-ACGGAATCAGGGCTAGGTTT; R-AGCGGCACGGATTCATAATA	266–278	50 °C
C9	$(GAA)_8$	F-CCTTGAGACTGCACCACAGA; R-AAGATCCGAGAACACGCACT	167–191	50 °C
C10	$(CTG)_6$	F-TGACCGAGTTTGTTTCCCAT; R-AGCTACTCATGCTGGGAGGA	151–166	50 °C
C11	$(GAG)_9$	F-AGCGATGAGGAGGAAGATGA; R-TCTTCGTCGCTGTCTGAGTG	234–252	50 °C
C12	$(ACC)_5$	F-ATGATGGAAGATGCCCTGTC; R-TGTGCGAATTGGTCAGACAT	214–232	48 °C
C13	$(TGC)_8$	F-GCGAATCAATCTCAGCAACA; R-GTGGACCTGGTACGCCTAAA	263–293	48 °C
C14	$(TGC)_6$	F-TACCTTACCGGCTCTGTGCT; R-CCCTTGTGGTTGTGTAGGCT	217–229	52 °C
C15	$(CAT)_5$	F-TTCAAGCCATTCGTAAAGCC; R-TGGTCGTAGGTGTTTCCACA	251–260	48 °C
C16	$(TTC)_5$	F-TGAGTTTCTCTGTGGCAACG; R-GGCTGAAGCTCTTGAAGGTG	177–192	50 °C
C17	$(AT)_6$	F-ATGGTGCCGCTGAACTCTAT; R-TGATCGATGCAGGGACTGTA	265–281	50 °C
C18	$(CT)_6$	F-TGATGTTCTGCTGCCTTGTC; R-AGATCGGTATCGGTTGCAGA	247–259	50 °C
C19	$(TG)_6$	F-TACTTCGGCTCCAAATCACC; R-ATATTGTCCCACCGTTTCCA	202–218	50 °C
C20	$(CT)_7$	F-GTCGGCAGGTAAAGGTGAAA; R-TCAGTGACGACAGCTTCCTG	126–146	50 °C
C21	$(AT)_6$	F-ACTCCGTGATCTTGTGCTCC; R-AAGAAGGCAGCTTATTGCCA	145–163	50 °C
C22	$(CATGG)_5$	F-ACTGTGCAAGGGTGATCCTC; R-ATCAACCCACCTTGCAACTC	258–282	50 °C

Table 3
Number of SSRs identified in the genome of *M. importuna*.

Repeat motif Number (%)	Number (%)
Mono	8541 (66.2 %)
Di	1564 (12.1 %)
Tri	1986 (15.4 %)
Tetra	486 (3.8 %)
Penta	163 (1.3 %)
Hexa	162 (1.3 %)
Total	12,902

wild strains from these five species were therefore used in our study. All 138 strains, including 102 cultivars and 36 wild samples selected from six morel species, were evaluated using twenty-two SSR primer pairs. Results showed that the 138 samples were divided into 127 kinds of cultivars with different genotypes based on 22 SSR loci. Among the 127 kinds of cultivars, eleven cultivated samples from *M. importuna* were assigned into five kinds of cultivars, each of which contained identical allelic patterns. So, only one sample from each of the five kinds of cultivars was selected in the following analysis. All the cultivated and wild samples in the other five species could be clearly distinguished by twenty-two SSRs. Therefore, a total of 127 samples were used for subsequent analysis.

The genetic structure of the 127 samples was evaluated using STRUCTURE v.2.3.4 based on the alleles resulting from the 22 SSR markers. Admixture model-based simulations computed that the most suitable ΔK was six, which means it was reasonable to divide total strains into six groups (Fig. 1A). The PCoA was conducted as an alternative measure of the relationships among 127 strains and suggested the first two axes explained 45.70 % and 36.37 % of the total variance separately (Fig. 1B). Six clades were also clustered in the dendrogram constructed using the UPGMA method (Fig. 1C), respectively: *M. importuna*, *M. sextelata*, *M. eximia*, *M. exuberans*, *Mel*-13 and *Mel*-21. However, among the 127 identified strains, two unexpected assignments of M110 and M50 were recognized. According to the molecular phylogenetic analysis of ITS rDNA data (Fig S1), the former belonged to *M. exuberans* although it was assigned to *M. eximia* by 22 SSR loci, and the latter belonged to *M. importuna* but clustered in *Mel*-21. In genetic assignment test (data not shown) 98.4 % of the samples have been allocated correctly to their original groups except M50 and M110, respectively grouping into *Mel*-21 and *M. eximia*. Both samples were not included for the genetic analysis of each species.

3.3. *Genetic diversity of SSRs within* M. importuna, M. sextelata, M. eximia, M. exuberans, Mel-*13 and* Mel-*21*

In *M. importuna*, twenty of the newly developed microsatellite markers proved to be polymorphic except for C10 and C19 (Table 4). The analyses of genetic diversity of 71 individuals, including cultivars and wild strains (M50 excluded), revealed that the 20 SSRs yielded 67 alleles ranging from 2 to 6 per locus, with an average of 3.4 per locus. The mean PIC value of the 20 SSRs was 0.26, ranging from 0.02 to 0.55. Based on the criterion of Botstein et al. (1980), two SSR markers (C12 and C20) in this species were shown to be highly informative with PIC > 0.5 (Table 4), and C12 was the highest informative locus. The observed heterozygosity ranged from 0.00 to 0.51 and the expected heterozygosity ranged from 0.01 to 0.62 per locus (Table 4). The parameters Na, Ne, Ho and He of the wild strains, cultivars and total strains are respectively indicated in Table 4 by GenAlEx v.6.5 analyses. A PCoA was conducted as an alternative measure of the relationships among eight wild samples and 63 cultivars. The first two axes explained 37.19 % and 24.16 % of the total variance, respectively (Fig. 2A). The genetic structure of the 71 *M. importuna* strains was inferred using STRUCTURE v.2.3.4 based on the alleles stemmed from 20 SSR loci. Admixture model-based simulations supported that the most suitable ΔK was five, which means it was reasonable to divide these 71 *M. importuna* strains into five groups (Fig. 2A). Strains in groups 2 to 5 were cultivars, and those in groups 1 were wild strains (Fig. 2A).

In *M. sextelata*, twenty-one of the newly developed microsatellite markers proved to be polymorphic, except for C11 (Table 5). Due to the limitation of small sample size (seventeen individuals), genetic diversity of cultivars and wild strains in this species were evaluated together. The analyses of genetic diversity revealed that the number of alleles per locus ranged from 2 to 6 per locus. The mean PIC value of the 21 SSRs was 0.39, ranging from 0.11 to 0.71. Six SSR markers (C12 and C20) in this species were indicated to be highly informative, with PIC > 0.5 (Table 5), according to the criterion of Botstein et al. (1980) and C1 was the highest informative

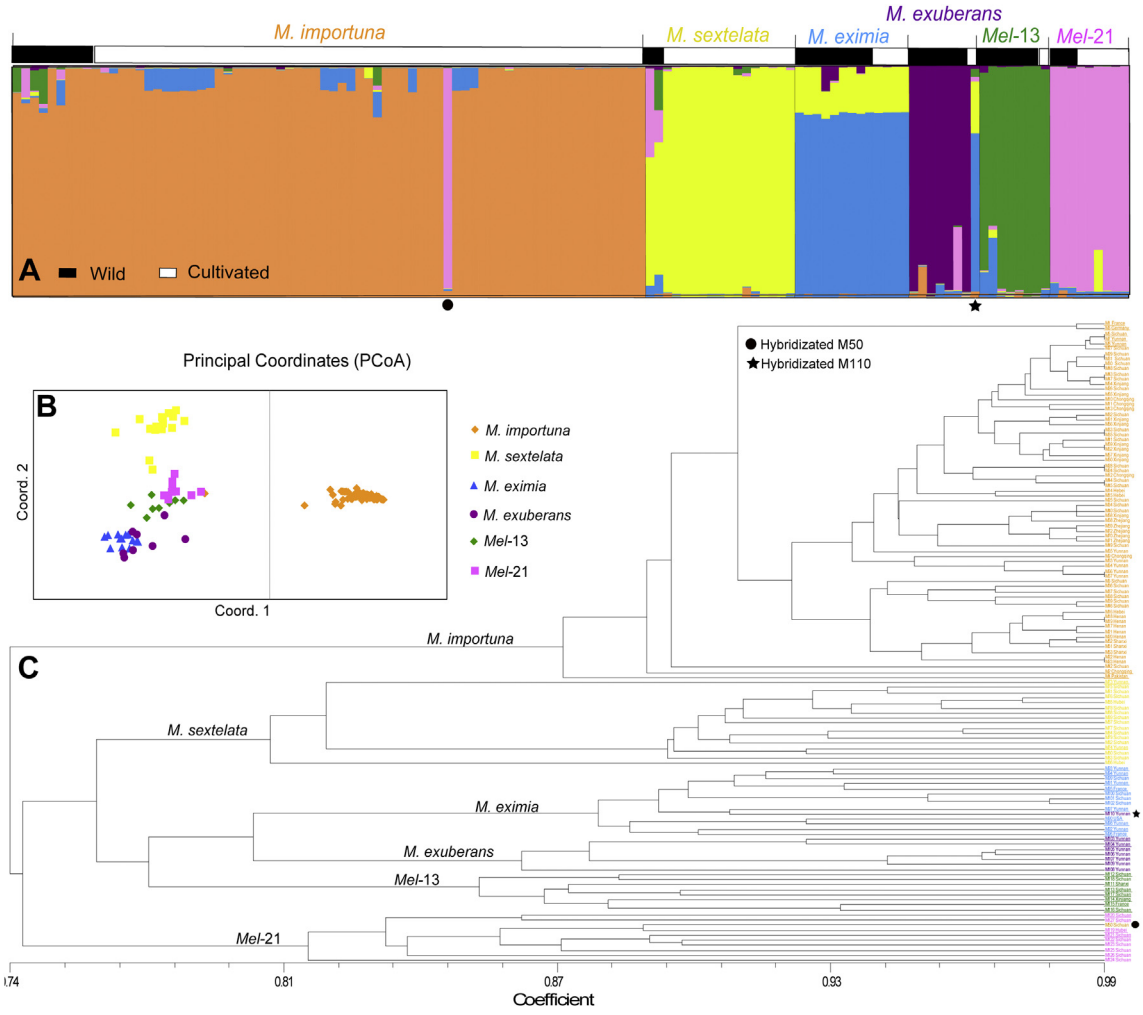

Fig. 1. Genetic relationship among 127 morel accessions from six species tested in this study. (A) Genetic structure analysis derived from 127 strains based on 22 SSR marker set by STRUCTURE v2.3.4 (Pritchard et al., 2000). (B) Principal coordinate analysis (PCoA) of 127 strains of six morel species based on 22 SSRs using GenAlEx v6.5 (Peakall and Smouse, 2006). (C) Dendrogram of 127 morel strains produced using DICE similarity coefficients and the UPGMA method by NTSYSpc v2.10e (Rohlf, 2000).

locus. The observed heterozygosity ranged from 0.00 to 0.71 and the expected heterozygosity ranged from 0.06 to 0.72 per locus (Table 5). The parameters Na, Ne, Ho and He are respectively indicated in Table 5. The PCoA analysis indicated that the first two axes explained 35.00 % and 20.23 % of the total variance, respectively (Fig. 2B). The genetic structure of the seventeen *M. sextelata* strains was inferred using STRUCTURE v.2.3.4 based on the alleles stemmed from 21 SSR loci (Fig. 2B).

In *M. eximia*, 17 SSR loci proved to be polymorphic except for C5, C13, C15, C19 and C22 (Table 5). Due to the limitation of small samples size (thirteen individuals), genetic diversity of cultivars and wild strains in this species was evaluated together. The analyses of genetic diversity revealed that the number of alleles per locus ranged from 2 to 5 per locus. The mean PIC value of the 21 SSRs loci was 0.40, ranging from 0.12 to 0.81. Five SSR markers (C3, C11, C12, C13 and C17) in this species were indicated to be highly informative, with PIC > 0.5 (Table 5) and C17 was the highest informative locus. The observed heterozygosity ranged from 0.00 to 0.50 and the expected heterozygosity ranged from 0.07 to 0.83 per locus (Table 5). The parameters Na, Ne, Ho and He are respectively indicated in Table 5. The PCoA analysis indicated the first two axes explained 41.19 % and 25.46 % of the total variance, respectively (Fig. 2C). The genetic structure of the thirteen *M. eximia* strains was inferred using STRUCTURE v.2.3.4 based on the alleles stemmed from 17 SSR loci (Fig. 2C).

In *M. exuberans*, 15 SSR loci proved to be polymorphic except for C3, C11, C12, C13, C17, C20 and C22 (Table 5). Due to the limitation of small samples size (seven individuals, M110 excluded), genetic diversity of wild strains in this species was evaluated together. The analyses of genetic diversity revealed that the number of alleles per locus ranged from 2 to 5 per locus. The mean PIC value of the 15 SSRs loci was 0.47, ranging from 0.18 to 0.66. Nine SSR markers (C1, C4, C5, C6, C8, C9, C14, C15 and C21) in this species were shown to be highly informative, with PIC > 0.5 (Table 5) and C8 was the highest informative locus. The observed heterozygosity ranged from 0.00 to 0.50 and the expected heterozygosity ranged from 0.12 to 0.70 per locus (Table 5). The parameters Na, Ne, Ho and He are respectively indicated in Table 5. The PCoA analysis indicated that the first two axes explained 61.11 % and 33.96 % of the total variance, respectively (Fig. 2D). The genetic structure of the seven *M. exuberans* strains was inferred using STRUCTURE v.2.3.4 based on the alleles stemmed from 15 SSR loci (Fig. 2D).

In *Mel*-13, 17 SSR loci proved to be polymorphic except for C3, C8, C15, C18 and C19 (Table 5). Due to the limitation of small samples size (eight individuals), the genetic diversities of cultivars and wild strains in this species were evaluated together. The

Table 4
Related parameters for 22 SSR loci of *M. importuna*.

Loci	*M. importuna*												
	Wild				Cultivar				Total				
	Na[a]	Ne[b]	Ho[c]	He[d]	Na[a]	Ne[b]	Ho[c]	He[d]	Na[a]	Ne[b]	Ho[c]	He[d]	PIC[e]
C1	2.00	1.28	0.00	0.22	2.00	1.10	0.00	0.09	2.00	1.12	0.00	0.11	0.09
C2	4.00	1.49	0.25	0.33	3.00	1.56	0.03	0.36	5.00	1.56	0.06	0.36	0.36
C3	1.00	1.00	0.00	0.00	3.00	1.21	0.08	0.17	3.00	1.19	0.07	0.16	0.23
C4	2.00	1.60	0.50	0.38	2.00	1.69	0.51	0.41	2.00	1.68	0.51	0.41	0.35
C5	2.00	1.88	0.00	0.47	3.00	1.76	0.21	0.43	3.00	1.90	0.18	0.47	0.48
C6	2.00	1.13	0.13	0.12	1.00	1.00	0.00	0.00	2.00	1.01	0.01	0.01	0.02
C7	2.00	1.28	0.00	0.22	1.00	1.00	0.00	0.00	2.00	1.22	0.00	0.18	0.2
C8	3.00	1.29	0.25	0.23	2.00	1.10	0.10	0.09	3.00	1.12	0.11	0.11	0.18
C9	3.00	1.47	0.13	0.32	1.00	1.00	0.00	0.00	3.00	1.04	0.01	0.04	0.08
C10	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]
C11	3.00	1.47	0.13	0.32	4.00	1.44	0.08	0.31	4.00	1.45	0.09	0.31	0.35
C12	2.00	1.88	0.00	0.47	4.00	2.73	0.33	0.63	4.00	2.66	0.23	0.62	0.55
C13	3.00	2.13	0.00	0.53	2.00	1.02	0.02	0.20	4.00	1.11	0.01	0.10	0.11
C14	1.00	1.00	0.00	0.00	2.00	1.02	0.02	0.02	2.00	1.01	0.01	0.01	0.02
C15	3.00	2.03	0.13	0.51	1.00	1.00	0.00	0.00	3.00	1.09	0.01	0.08	0.13
C16	2.00	1.75	0.13	0.43	3.00	1.12	0.02	0.11	3.00	1.19	0.03	0.16	0.11
C17	3.00	1.29	0.13	0.23	4.00	1.54	0.05	0.35	6.00	1.52	0.06	0.34	0.33
C18	3.00	1.47	0.13	0.32	4.00	2.15	0.05	0.54	4.00	2.15	0.06	0.54	0.47
C19	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]
C20	3.00	1.29	0.13	0.23	4.00	2.04	0.19	0.51	4.00	1.95	0.18	0.49	0.52
C21	2.00	1.13	0.13	0.12	4.00	1.21	0.13	0.18	4.00	1.21	0.13	0.17	0.25
C22	3.00	1.67	0.13	0.40	4.00	1.67	0.08	0.40	4.00	1.68	0.09	0.40	0.36

[a] The number of alleles.
[b] The effective number of alleles.
[c] The number of observed heterozygosity.
[d] The number of expected heterozygosity.
[e] Polymorphic information content.
[f] Monomorphic locus in the species.

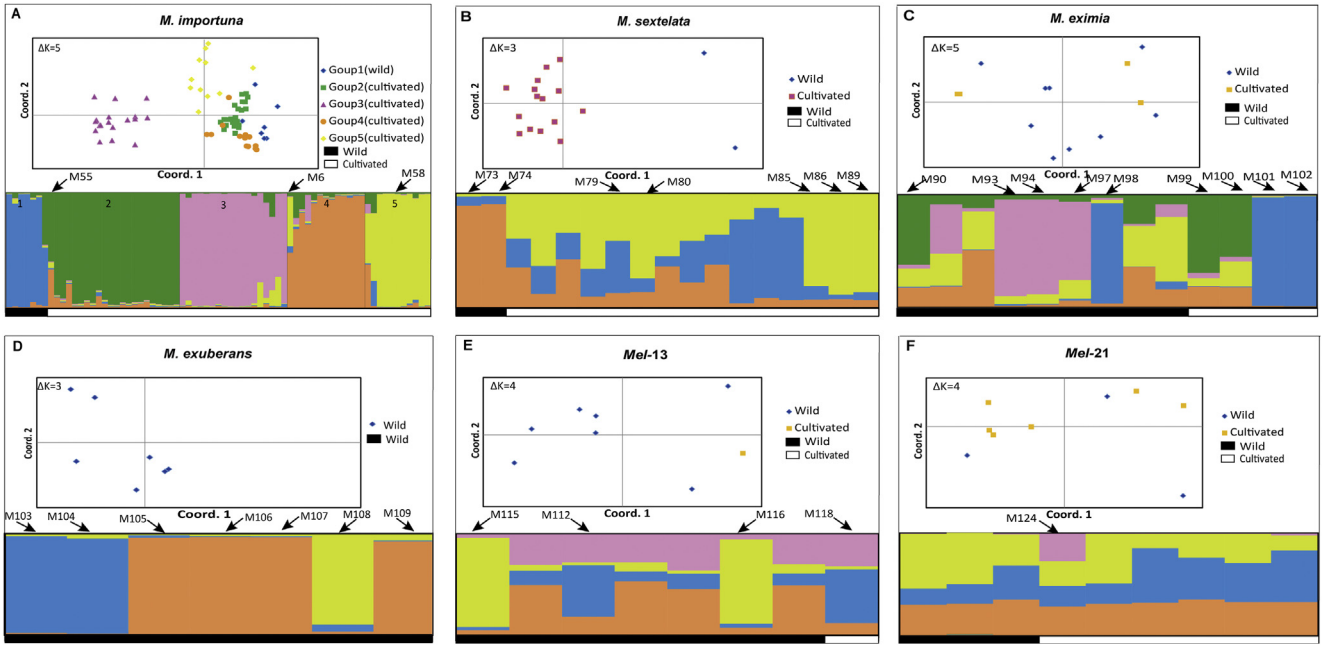

Fig. 2. Principal coordinate analysis (PCoA) by GenAlEx v6.5 (Peakall and Smouse, 2006) and genetic structure analysis by STRUCTURE v2.3.4 (Pritchard et al., 2000) respectively conducted in each species. (A) *M. importuna* (with results generated from 71 samples on 20 SSRs loci); (B) *M. sextelata* (with results generated from 17 samples on 21 SSRs loci); (C) *M. eximia* (with results generated from 13 samples on 17 SSRs loci); (D) *M. exuberans* (with results generated from 7 samples on 15 SSRs loci); (E) *Mel*-13 (with results generated from 8 samples on 17 SSRs loci); (F) *Mel*-21 (with results generated from 9 samples on 16 SSRs loci).

analyses of genetic diversity uncovered that the number of alleles per locus ranged from 2 to 7 per locus. The mean PIC value of the 17 SSRs loci was 0.49, ranging from 0.18 to 0.80. Eight SSR markers (C1, C6, C9, C10, C14, C17, C21 and C22) in this species were shown to be highly informative, with PIC > 0.5 (Table 5), and C17 was the highest informative locus. The observed heterozygosity ranged from 0.00 to 0.75 and the expected heterozygosity ranged from 0.12 to 0.82 per locus (Table 5). The parameters Na, Ne, Ho and He are

Table 5
Related parameters for 22 SSR loci of *M. sextelata*, *M. eximia*, *M. exuberans*, *Mel*-13 and *Mel*-21.

Loci	*M. sextelata*					*M. eximia*					*M. exuberans*					*Mel*-13					*Mel*-21				
	Na[a]	Ne[b]	Ho[c]	He[d]	PIC[e]	Na[a]	Ne[b]	Ho[c]	He[d]	PIC[e]	Na[a]	Ne[b]	Ho[c]	He[d]	PIC[e]	Na[a]	Ne[b]	Ho[c]	He[d]	PIC[e]	Na[a]	Ne[b]	Ho[c]	He[d]	PIC[e]
C1	6.00	3.61	0.71	0.72	0.71	3.00	1.91	0.08	0.48	0.43	5.00	4.00	1.58	0.29	0.37	5.00	4.70	0.75	0.79	0.76	6.00	5.16	0.86	0.81	0.76
C2	2.00	1.06	0.06	0.06	0.11	2.00	1.55	0.00	0.36	0.29	3.00	3.00	1.82	0.00	0.45	2.00	1.80	0.00	0.44	0.34	3.00	1.59	0.00	0.37	0.34
C3	3.00	1.44	0.00	0.30	0.29	2.00	1.17	0.00	0.14	0.14	2.00	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]
C4	3.00	1.74	0.29	0.42	0.42	3.00	1.86	0.15	0.46	0.44	3.00	3.00	2.65	0.43	0.62	2.00	1.28	0.25	0.22	0.27	N[f]	N[f]	N[f]	N[f]	N[f]
C5	2.00	1.06	0.06	0.06	0.11	N[f]	N[f]	N[f]	N[f]	N[f]	5.00	5.00	2.65	0.14	0.62	2.00	1.13	0.13	0.12	0.18	3.00	2.46	0.00	0.59	0.50
C6	5.00	2.99	0.47	0.67	0.69	2.00	1.45	0.08	0.31	0.28	4.00	4.00	2.13	0.29	0.53	4.00	3.77	0.00	0.74	0.68	6.00	4.46	0.57	0.78	0.77
C7	6.00	1.78	0.12	0.44	0.48	4.00	2.25	0.00	0.56	0.51	3.00	2.00	1.15	0.14	0.13	2.00	1.60	0.00	0.36	0.30	3.00	2.67	0.00	0.63	0.55
C8	5.00	4.13	0.44	0.76	0.63	2.00	1.55	0.15	0.36	0.28	4.00	4.00	3.16	0.43	0.68	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]
C9	2.00	1.06	0.06	0.06	0.11	4.00	2.62	0.23	0.62	0.60	4.00	4.00	2.51	0.29	0.60	5.00	3.92	0.29	0.75	0.73	5.00	4.80	0.50	0.79	0.73
C10	5.00	2.82	0.47	0.65	0.64	5.00	3.56	0.23	0.72	0.68	3.00	2.00	1.15	0.14	0.13	4.00	3.63	0.14	0.72	0.66	6.00	4.90	0.86	0.79	0.76
C11	N[f]	N[f]	N[f]	N[f]	N[f]	5.00	3.84	0.46	0.74	0.72	2.00	N[f]	N[f]	N[f]	N[f]	2.00	1.73	0.13	0.43	0.34	2.00	1.98	0.00	0.49	0.37
C12	2.00	1.19	0.06	0.16	0.18	4.00	1.38	0.08	0.28	0.34	2.00	N[f]	N[f]	N[f]	N[f]	3.00	1.29	0.13	0.23	0.34	4.00	1.80	0.22	0.44	0.50
C13	5.00	2.13	0.53	0.53	0.52	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]	3.00	1.47	0.13	0.32	0.34	5.00	4.50	0.50	0.78	0.75
C14	5.00	2.98	0.47	0.66	0.65	3.00	2.13	0.08	0.53	0.46	3.00	3.00	2.39	0.14	0.58	4.00	3.37	0.25	0.70	0.67	5.00	4.13	0.63	0.76	0.70
C15	2.00	1.99	0.00	0.50	0.37	N[f]	N[f]	N[f]	N[f]	N[f]	3.00	2.00	1.96	0.00	0.49	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]
C16	2.00	1.26	0.00	0.21	0.19	2.00	1.17	0.00	0.14	0.14	2.00	2.00	1.69	0.00	0.41	2.00	1.60	0.00	0.38	0.30	N[f]	N[f]	N[f]	N[f]	N[f]
C17	4.00	2.12	0.35	0.53	0.47	7.00	5.76	0.50	0.83	0.81	N[f]	Nf	Nf	Nf	Nf	7.00	5.57	0.38	0.82	0.80	5.00	4.67	0.14	0.79	0.75
C18	2.00	1.13	0.00	0.11	0.11	2.00	1.55	0.00	0.36	0.29	2.00	1.15	0.14	0.13	2.00	N[f]	N[f]	N[f]	N[f]	N[f]	3.00	2.31	0.00	0.57	0.49
C19	3.00	1.27	0.12	0.21	0.26	N[f]	N[f]	N[f]	N[f]	N[f]	3.00	1.56	0.29	0.36	3.00	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]	N[f]
C20	5.00	1.28	0.18	0.22	0.34	2.00	1.08	0.08	0.07	0.12	Nf	Nf	Nf	Nf	Nf	2.00	1.13	0.13	0.12	0.18	2.00	1.38	0.33	0.28	0.30
C21	3.00	2.17	0.18	0.55	0.44	2.00	1.35	0.00	0.26	0.22	2.00	1.32	0.29	0.25	2.00	7.00	5.57	0.38	0.82	0.78	6.00	4.92	0.38	0.80	0.78
C22	2.00	1.06	0.06	0.06	0.11	N[f]	N[f]	N[f]	N[f]	N[f]	Nf	Nf	Nf	Nf	Nf	4.00	2.91	0.00	0.66	0.60	4.00	3.06	0.33	0.67	0.56

[a] The number of alleles.
[b] The effective number of alleles.
[c] The number of observed heterozygosity.
[d] The number of expected heterozygosity.
[e] Polymorphic information content.
[f] Monomorphic locus in the species.

respectively indicated in Table 5. The PCoA analysis indicated that the first two axes explained 45.36 % and 24.97 % of the total variance, respectively (Fig. 2E). The genetic structure of the seven *M. exuberans* strains was inferred using STRUCTURE v.2.3.4 based on the alleles stemmed from 17 SSR loci (Fig. 2E).

In *Mel*-21, 16 SSR loci proved to be polymorphic except for C3, C4, C8, C15, C16 and C19 (Table 5). Due to the limitation of small samples size (nine individuals), the genetic diversities of cultivars and wild strains in this species were evaluated together. The analyses of genetic diversity revealed that the number of alleles per locus ranged from 2 to 6 per locus. The mean PIC value of the 16 SSRs loci was 0.60, ranging from 0.30 to 0.78. Twelve SSR markers (C1, C5, C6, C7, C9, C10, C12, C13, C14, C17, C21 and C22) in this species were shown to be highly informative, with PIC > 0.5 (Table 5) and C21 was the most informative locus. The observed heterozygosity ranged from 0.00 to 0.86 and the expected heterozygosity ranged from 0.28 to 0.81 per locus (Table 5). The parameters Na, Ne, Ho and He are respectively indicated in Table 5. The PCoA analysis showed that the first two axes explained 40.30 % and 24.50 % of the total variance, respectively (Fig. 2F). The genetic structure of the seven *M. exuberans* strains was inferred using STRUCTURE v.2.3.4 based on the alleles stemmed from 16 SSR loci (Fig. 2F).

3.4. *Cultivar identification within each of* M. importuna, M. sextelata, M. eximia, M. exuberans, Mel-*13 and* Mel-*21*

In *M. importuna* (Fig. 2A), 63 cultivars from main growth regions were collected. However, one strange assignment of M6, a wild sample collected from Sichuan, was assigned to the cultivated group (group 1). Among the cultivars, strains from the same province were clustered into different groups, indicating that the genetic distances are not correlated with geographic origins. For example, nine cultivars in the Xinjiang group, which were collected from the same cultivation site with the same commercial name (Table 1), showed nine distinct allelic patterns, separated into two gene pools represented by M55 and M58 (Fig. 2A). Every group possessed their own different genetic components (Fig. 2A). In *M. sextelata* (Fig. 2B), two cultivars (M79 and M80) from Sichuan that were mistaken for *M. importuna* (Table 1) by the growers according to morphology actually belonged to *M. sextelata* in terms of both current SSRs and the ITS dataset (Fig S1). Additionally, wild samples M73 and M74 had similar proportion of genetic components. The cultivars M85, M86 and M89, respectively from Hubei and Sichuan, had similar proportion of genetic components. In *M. eximia* (Fig. 2C), the wild sample M90 from the USA was similar to cultivars M99 and M100 from Sichuan, and the wild sample M98 from Yunnan was similar to cultivars M101 and M102 from Sichuan according to the proportion of genetic components. In *M. exuberans*, wild samples M103 and M104 from Dali, Yunnan, and M105, M106 and M107 from Chuxiong, Yunnan, had similar genetic components (Fig. 2D), but wild samples M108 and M109 from Weixi, Yunnan, had distinct gene pools and the latter was similar to samples from Chuxiong, Yunnan. In *Mel*-13 (Fig. 2E), wild samples M115 from France and M116 from Sichuan had similar proportions of genetic components, while wild sample M112 from Xinjiang was similar to the cultivar M118 from Sichuan in the proportion of genetic components. In *Mel*-21 (Fig. 2F), the cultivar M124 had a distinct gene pool from other samples.

3.5. *Transferability of the SSR marker set from* M. importuna *to its related species*

To test the transferability of novel SSR markers in different morel species, we selected 180 SSRs to genotype 24 samples from *M. importuna*, *M. sextelata*, *M. eximia*, *M. exuberans*, *Mel*-13 and *Mel*-21. Of these SSRs evaluated, 153 (87.7 %) were transferred to at least one of the studied species and 89 (49.4 %) successfully to all the studied species. Of the 22 SSRs analyzed on 127 samples from six species, several SSR loci failed to generate amplification products in some samples from *M. sextelata*, *M. eximia*, *M. exuberans*, *Mel*-13 and *Mel*-21. The percentage failure to generate amplification products for each locus and each species is shown in Fig. 3. The non-amplification of some microsatellite loci in the five species is most likely due to specific mutations in the primer binding sites.

4. Discussion

In this study, the microsatellite characteristics of *M. importuna*, cross-ability and cultivar identification among six *Morchella* species were analyzed based on the *M. importuna* draft genome.

4.1. *The abundance of SSRs in the draft genome of* M. importuna

The genome of *M. importuna* had the third most abundant SSRs (12902) following *Tuber melanosporum* and *Neurospora crassa*, with the number of SSRs ranging from 1973 to 21889 (Table 6) (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). In the genome of *M. importuna*, the most abundant SSR motif was the mono-nucleotide, consistent with the report that the most abundant motif was mono-nucleotide for most of the fungal and other eukaryotic 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), followed by the tri-nucleotide.

4.2. *Transferability of SSRs among* M. importuna, M. sextelata, M. eximia, M. exuberans, Mel-*13 and* Mel-*21*

SSRs have been often considered to be poorly transferable between fungal species (Dutech et al., 2007; Schoebel et al., 2013a,b; Mercière et al., 2015). It is noteworthy that high transferability of SSRs in the genus *Morchella* was observed, in which 158 of 180 SSRs (87.7 %) evaluated at present were transferred to at least one of the studied species and 89 (49.4 %) successfully to all the species. Peakall et al. (1998) suggested that cross-transferability of SSRs within a genus could vary from 50 to 100 %. The transferability percentage of SSRs among the current six species was obviously higher (87.7 %) than reported by other research groups (Davies et al., 2013; Dubé et al., 2017). High transferability of SSRs in this study presumed that microsatellites were kept conserved in *Morchella* during the evolutionary process. Barbara et al. (2007) proposed that transferability of SSRs among species of Bromeliaceae might result from large adaptive radiations and low-level divergence, which were also found in *Morchella* (Du et al., 2012). Among the 22 SSR loci analyzed on 127 samples from six species, although some failed to generate amplification product from individual samples (Fig. 3), it is speculated that variation at the primer binding sites may prevent annealing and subsequently result in no amplification of PCR products. In addition, the success amplification rate of microsatellites was closely related to genetic similarity and phylogenetic relationships among related species (Kalia et al., 2011; Barbara et al., 2007). In this study, *M. sextelata*, *M. eximia* and *M. exuberans* were more closed related to *M. importuna* than *Mel*-13 and *Mel*-21 (Du et al., 2012), and higher success rate was also found in the former species (Fig. 3). Many studies recommended a minimum of 8–16 SSR markers for performing population genetics analysis (Van et al., 2010; Schoebel et al., 2013a, b; Wang et al., 2014). The twenty-two SSR loci developed here could not only provide more options for DNA markers, but also reduce the

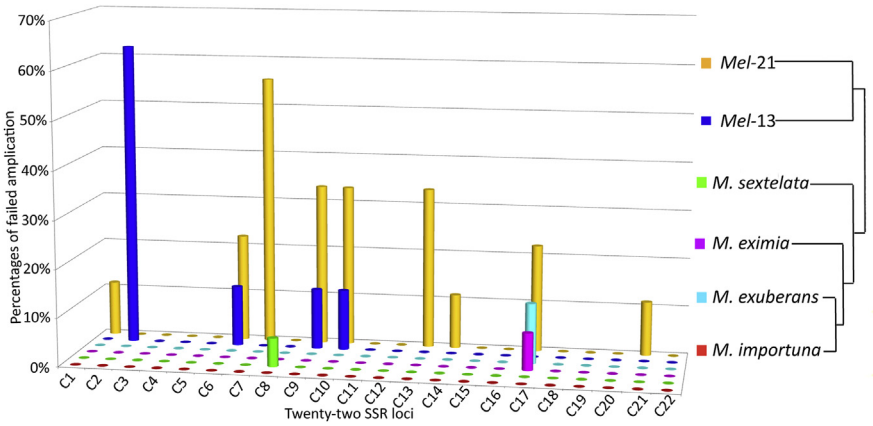

Fig. 3. The failed amplification loci considering all the samples analysed for each species. An outline phylogenetic tree representing the relationships between the six species is shown on the right, based on the phylogenetic analysis of *Morchella* proposed by Du et al., 2012.

Table 6
Comparison of the SSR number of and total relative abundance in 19 fungal genomes.

Species	Repeat type						
	Mono-	Di-	Tri-	Tetra-	Penta-	Hexa-	Total
Agaricus bisporusvar.bisporus	2051	1244	717	22	10	18	4062
Aspergillus nidulans	1249	753	325	36	28	19	2410
Coprinus cinereus	761	375	843	38	10	23	2050
Cryptococcus neoformans	855	671	401	20	3	23	1973
Fusarium graminearum	1109	1034	555	85	73	40	2896
Flammulina velutipes	242	211	824	23	1	20	1321
Ganoderma lucidum	1090	485	806	58	29	206	2674
Laccaria bicolor	3375	740	1595	72	258	64	6104
Magnaporthe grisea	8042	1730	1573	219	33	45	11,642
Neurospora crassa	5943	3208	4084	758	192	134	14,319
Phanerochaete chrysosporium	386	379	598	14	3	13	1393
Postia placenta	505	489	642	55	76	49	1816
Saccharomyces cerevisiae	2363	817	396	14	9	19	3618
Schizophyllum commune	346	200	593	24	15	28	1206
Schizosaccharomyces pombe	2332	662	196	21	18	3	3232
Serpula lacrymans	1334	310	678	27	13	51	2413
Tuber melanosporum	16,733	1998	2007	707	262	112	21,889
Ustilago maydis	658	1162	865	76	76	196	3033
Morchella importuna	8541	1564	1986	486	163	162	12,902

cost of microsatellite isolation for the population studies of other morel species.

4.3. Hybridization among six morel species

High transferability indicated that SSRs were conserved among species in the genus *Morchella*. Conserved SSRs have been considered good candidates for analysis of interspecific hybrids (Ferreira-Ramos et al., 2014). That the strain M50 belongs to *M. exuberans* (Fig S1) although it is assigned to *M. eximia*, and that the strain M10 belongs to *M. importuna* (Fig S1) but clustered in *Mel*-21 (Fig. 2B) shown in our dendrogram and STRUCTURE plot indicated the multiple hybridization events within *Morchella*. In *M. sextelata*, the genepools of two wild samples collected from Yunnan province were derived from cultivated *M. sextelata* as well as wild *Mel*-21 (Fig. 2C). In *M. eximia*, three wild strains were found to be infiltrated with genepools from *M. exuberans* (Fig. 2C). Two wild samples of *M. exuberans* were in possession of genepools from not only *M. importuna* but also *Mel*-21 (Fig. 2C). The cultivar M124 clustered in *Mel*-21 was inferred to originate from a hybridization event between *M. sextelata* and *Mel*-21 (Fig. 2C). Notably, similarly potential hybridization in *Morchella* was already presented in Du et al. (2016), which implied that hybridization events likely happened among *M. exuberans*, *M. importuna*, *Mel*-13, *Morchella eohespera*, *Morchella eximioides*, *Mel*-21 and *Mel*-34 in DNA marker F1. Additionally, Our clustering analysis (Fig. 2) indicated that cultivars from the same cities 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. We believe that a broad survey involving more samples should provide stronger evidence to reveal the extent of hybridization and evolutionary relationship among the cultivar lineages in *Morchella*. The breeding history of morels in China has started to be explored in recent years, while current findings have disclosed the complexity of the genetic background of cultivated morels.

4.4. Authentication, synonyms and homonyms of cultivated morels in China

Accurate identification and authentication of culitvars are essential pre-requisites for breeding and exchanging spawns and research. The twenty-two SSRs developed here could serve as

effective novel molecular markers for the accurate identification and authentication of morel cultivars. In this study, we found that misnaming and mislabeling existed within these cultivated samples. Strains of *M. importuna* from Xinjiang had the same spawn name but actually belong to different breeds based on distinct allelic patterns. Cultivated samples M79 and M80 from Sichuan, belonging to *M. sextelata*, were mistaken for *M. importuna* by the growers according to morphology. Cultivated samples M122-127 belonged to different cultivar, but were mistaken for the same by farmers.

In terms of our investigation in many farms, various and confusing cultivar names were given by farmers based on the locality of origin, unreliable morphological traits, utility of production, personal preference or optionally replaced with famous names to increase their economic value. Due to the difficulty of distinguishing morels by morphology, many farmers just called all the cultivars 'morels' and could not identify to what species the morels they cultivated belonged. Inaccurate identification and errors arising from misnaming and mislabeling were common in the morel cultivating industry. The present complexity and confusion within the morel breeding field is mainly owing to the just-emerging cultivation industry lacking an effective and standardized management system on cultivars. We suggested that SSRs could be used in cultivar identification to avoid the extended confusion among denominations and true-to-type cultivar names in the morel cultivation industry and also for intellectual property protection.

In summary, our study characterized the distribution of microsatellites in the *M. importuna* genome and reported the first set of microsatellite markers developed for species of *Morchella*. Twenty-two polymorphic SSR loci discussed in this study could be useful and helpful for morel cultivar identification. Frequent transferability could allow these markers to be widely applied in the genetics and breeding studies of other species in the genus *Morchella*.

Author contributions

Xi-Hui Du designed the study. Xi-Hui Du, Hanchen Wang, Jingjing Sun, Lunyi Xiong and Jingjing Yu collected the samples and conducted the laboratory work. Xi-Hui Du analyzed the data and wrote the manuscript. All authors reviewed the manuscript.

Compliance with ethical standards

Conflict of interest

All the authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2019.05.005.

References

Barbara, T., Palma-Silva, C., Paggi, G.M., Bered, F., Fay, M.F., Lexer, C., 2007. Cross-species transfer of nuclear microsatellite markers: potential and limitations. Mol. Ecol. 16, 3759–3767.

Botstein, D., Whiter, L., Skolnick, M., Davis, R.W., 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am. J. Hum. Genet. 32, 314–331.

Buschiazzo, E., Gemmell, N.J., 2006. The rise, fall and renaissance of microsatellites in eukaryotic genomes. Bioessays 28, 1040–1050.

Chen, L.N., Ma, Q.G., Chen, Y.K., Wang, B.Q., Pei, D., 2014. Identification of major walnut cultivars grown in China based on nut phenotypes and SSR markers. Sci Hortic. Amst 168, 240–248.

Davies, S.W., Rahman, M., Meyer, E., Green, E.A., Buschiazzo, E., Medina, M., Matz, M.V., 2013. Novel polymorphic microsatellite markers for population genetics of the endangered Caribbean star coral, *Montastraea faveolata*. Mar. Biodivers. 43, 167–172.

Doyle, J.J., Doyle, J.L., 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. 19, 11–15.

Du, X.H., Zhao, Q., O'Donnell, K., Rooney, A.P., Yang, Z.L., 2012. Multigene molecular phylogenetics reveals true morels (*Morchella*) are especially species-rich in China. Fungal Genet. Biol. 49, 455–469.

Du, X.H., Zhao, Q., Xu, J., Yang, Z.L., 2016. High inbreeding, limited recombination and divergent evolutionary patterns between two sympatric morel species in China. Sci. Rep. 6, 22434.

Dubé, C.E., Planes, S., Zhou, Y.X., Berteaux-Lecellier, V., Boissin, E., 2017. Genetic diversity and differentiation in reef-building *Millepora* species, as revealed by crossspecies amplification of fifteen novel microsatellite loci. Peer J. 5, e2936. https://doi.org/10.7717/peerj.2936.

Dutech, C., Enjalbert, J., Fournier, E., Delmotte, F., Barres, B., Carlier, J., Tharreau, D., Giraud, T., 2007. Challenges of microsatellite isolation in fungi. Fungal Genet. Biol. 44 (10), 933–949.

Earl, D.A., 2012. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conserv. Genet. Resour. 4, 359–361.

Evanno, G., Regnaut, S., Goudet, J., 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol. Ecol. 14, 2611–2620.

Falush, D., Stephens, M., Pritchard, J.K., 2003. Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. Genetics 164, 1567–1587.

Ferreira-Ramos, R., Accoroni, K.A.G., Rossi, A., Guidugli, M.C., Mestriner, M.A., Martinez, C.A., Alzate-Marin, A.L., 2014. Genetic diversity assessment for *Eugenia uniflora* L., *E. pyriformis* Cambess., *E. brasiliensis* Lam. and *E. francavilleana* O. Berg neotropical tree species (Myrtaceae) with heterologous SSR markers. Genet. Resour. Crop Evol. 61, 267–272.

Foulongne-Oriol, M., Murat, C., Castanera, R., Ramírez, L., Sonnenberg, A.S., 2013. Genome wide survey of repetitive DNA elements in the button mushroom *Agaricus bisporus*. Fungal Genet. Biol. 55, 6–21.

Jakobsson, M., Rosenberg, N.A., 2007. CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. Bioinformatics 23 (14), 1801–1806.

Kalia, R.K., Rai, M.K., Kalia, S., Singh, R., Dhawan, A.K., 2011. Microsatellite markers: an overview of the recent progress in plants. Euphytica 177, 309–334.

Karaoglu, H., Lee, C.M.Y., Meyer, W., 2005. Survey of simple sequence repeats in completed fungal genomes. Mol. Biol. Evol. 22, 639–649.

Kelkar, Y.D., Tyekucheva, S., Chiaromonte, F., Makova, K.D., 2008. The genome-wide determinants of human and chimpanzee microsatellite evolution. Genome Res. 18, 30–38.

Koressaar, T., Remm, M., 2007. Enhancements and modifications of primer design program Primer3. Bioinformatics 23, 1289–1291.

Kyung-Ho, M., Lee, G.A., Lee, S.Y., Gwag, J.G., Kim, T.S., Kong, W.S., Seo, K.I., Park, Y.J., 2009. Development and characterization of new microsatellite markers for the oyster mushroom (*Pleurotus ostreatus*). J. Microbiol. Biotechnol. 19, 851–857.

Lim, S., Notley-McRobb, L., Lim, M., Carter, D.A., 2004. A comparison of the nature and abundance of microsatellites in 14 fungal genomes. Fungal Genet. Biol. 41, 1025–1036.

Litchfield, J., 1967. Morel mushroom mycelium as a food-flavoring material. Biotechnol. Bioeng. 9, 289–304.

Liu, X.B., Feng, B., Li, J., Yan, C., Yang, Z.L., 2016. Genetic diversity and breeding history of winter mushroom (*Flammulina velutipes*) in China uncovered by genomic SSR markers. Gene 591, 227–235.
Liu, X.B., Li, J., Yang, Z.L., 2017. Genetic diversity and structure of core collection of winter mushroom (*Flammulina velutipes*) developed by genomic SSR markers. Hereditas 155, 3.
Luo, H., Wang, X., Zhan, G., Wei, G., Zhou, X., Zhao, J., Huang, J., Kang, Z., 2015. Genome wide analysis of simple sequence repeats and efficient development of polymorphic SSR markers based on whole genome re-sequencing of multiple isolates of the wheat stripe rust fungus. PLoS One 10, e0130362.
Madhou, M., Normand, F., Bahorun, T., Hormaza, J.I., 2013. Fingerprinting and analysis of genetic diversity of litchi (*Litchi chinensis* Sonn.) accessions from different germplasm collections using microsatellite markers. Genomes 9 (2), 387–396.
Maduna, S.N., Rossouw, C., Roodt-Wilding, R., Bester-van, M.A.E., 2014. Microsatellite cross-species amplification and utility in southern African elasmobranchs: a valuable resource for fisheries management and conservation. BMC Res. Notes 7, 352.
Masaphy, S., 2010. Biotechnology of morel mushrooms: successful fruiting body formation and development in a soilless system. Biotechnol. Lett. 32, 1523–1527.
Mercière, M., Laybats, A., Carasco-Lacombe, C., Tan, J.S., Klopp, C., Durand-Gasselin, T., Alwee, S.S.R.S., Camus-Kulandaivelu, L., Breton, F., 2015. Identification and development of new polymorphic microsatellite markers using genome assembly for *Ganoderma boninense*, causal agent of oil palm basal stem rot disease. Mycol. Prog. 14, 1–11.
Murat, C., Riccioni, C., Belfiori, B., Cichocki, N., Labbé, J., Morin, E., Tisserant, E., Paolocci, F., Rubini, A., Martin, F., 2011. Distribution and localization of microsatellites in the Perigord black truffle genome and identification of new molecular markers. Fungal Genet. Biol. 48, 592–601.
Ower, R., 1982. Notes on the development of the morel ascocarp -*Morchella esculenta*. Mycologia 74, 142–144.
Peakall, R., Smouse, P.E., 2006. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research—an update. Bioinformatics 28, 2537–2539.
Peakall, R., Gilmore, S., Keys, W., Morgante, M., Rafalski, A., 1998. Cross-species amplification of soybean (Glycine max) simple sequence repeats (microsatellites) within the genus and other legume genera-implications for the transferability of microsatellites in plants. Mol. Biol. Evol. 15, 1275–1287.
Pilz, D., McLain, R., Alexander, S., Villarreal, R., Berch, S., Wurtz, T.L., Parks, C.G., McFarlane, E., Baker, B., Molina, R., Smith, J.E., 2007. Ecology and Management of Morels Harvested from the Forests of Western North America. General Technical Report PNW-GTR-710. US Department of Agriculture, Forest Service, Pacific Northwest Research Station, Portland, p. 161.
Pirog, A., Jaquemet, S., Blaison, A., Soria, M., Magalon, H., 2016. Isolation and characterization of eight microsatellite loci from *Galeocerdo cuvier* (tiger shark) and cross-amplification in *Carcharhinus leucas, Carcharhinus brevipinna, Carcharhinus plumbeus* and *Sphyrna lewini*. Peer J. 4, e2041.
Piry, S., Alapetite, A., Cornuet, J., Paetkau, D., Baudouin, L., Estoup, A., 2004. GeneClass2: a software for genetic assignment and first generation migrant detection. J. Hered. 95, 536–539.
Pritchard, J.K., Stephens, M., Donnelly, P., 2000. Inference of population structure using multilocus genotype data. Gene 155, 945–959.
Qian, J., Xu, H., Song, J., Xu, J., Zhu, Y., Chen, S., 2013. Genome-wide analysis of simple sequence repeats in the model medicinal mushroom *Ganoderma lucidum*. Gene 512, 331–336.
Qu, J., Huang, C., Zhang, J., 2016. Genome-wide functional analysis of SSR for an edible mushroom *Pleurotus ostreatus*. Gene 575, 524–530.
Rai, M.K., Shekhawat, J.K., Kataria, V., Shekhawat, N.S., 2017. Cross species transferability and characterization of microsatellite markers in *Prosopis cineraria*, a multipurpose tree species of Indian Thar. Desert 31 (4), 462–471.
Rannala, B., Mountain, J.L., 1997. Detecting immigration by using multilocus genotypes. Proc. Natl. Acad. Sci. U.S.A. 94, 9197–9221.
Reid, K., Hoareau, T.B., Bloomer, P., 2012. High-throughput microsatellite marker development in two sparid species and verification of their transferability in the family Sparidae. Mol. Ecol. Resour. 12, 740752.
Rohlf, F.J., 2000. Phylogenetic models and reticulations. J. Classif. 17, 185–189.
Rosenberg, N.A., 2004. DISTRUCT: a program for the graphical display of population structure. Mol. Ecol. Notes 4, 137–138.
Schoebel, C.N., Brodbeck, S., Buehler, D., Cornejo, C., Gajurel, J., Hartikainen, H., Keller, D., Leys, M., Ricanova, S., Segelbacher, G., Werth, S., Csencsics, D., 2013a. Lessons learned from microsatellite development for nonmodel organisms using 454 pyrosequencing. J Evol. Biol. 26 (3), 600–611.
Schoebel, C.N., Jung, E., Prospero, S., 2013b. Development of new polymorphic microsatellite markers for three closely related plant-pathogenic *Phytophthora* species using 454-pyrosequencing and their potential applications. Phytopathology 103 (10), 1020–1027.
Stamatakis, A., 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22, 2688–2690.
Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M., Rozen, S.G., 2012. Primer3-new capabilities and interfaces. Nucleic Acids Res. 40, e115.
Van, A.B., Pinheiro, R., Pereira, R., Alves, C., Pereira, V., Pereira, F., Gusmão, L., Amorim, A., 2010. A framework for the development of STR genotyping in domestic animal species: characterization and population study of 12 canine X–chromosome loci. Electrophoresis 31 (2), 303–308.
Wang, M., Xue, F., Yang, P., Duan, X.Y., Zhou, Y.L., Shen, C.Y., Zhang, G.Z., Wang, B.T., 2014. Development of SSR markers for a phytopathogenic fungus, *Blumeria graminis* f.sp *tritici*, Using a FIASCO Protocol. J Integr. Agric. 13 (1), 100–104.
Weber, J.L., 1990. Informativeness of human (dC-dA)n(dG-dT)n polymorphisms. Genomics 7, 524–530.
Xiang, X., Li, C., Li, L., Bian, Y., Kwan, H.S., Nong, W., Cheung, M.K., Xiao, Y., 2016. Genetic diversity and population structure of Chinese *Lentinula edodes* revealed by InDel and SSR markers. Mycol. Prog. 15, 1–13.
Zhang, J.X., Zhao, Y.C., 2016. Germplasm Resources of Edible Mushroom. Science Press, Beijing.
Zhang, R.Y., Hu, D.D., Gu, J.G., Hu, Q.X., Zuo, X.M., Wang, H.X., 2012. Development of SSR markers for typing cultivars in the mushroom *Auricularia auricula-judae*. Mycol. Prog. 11, 587–592.