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ORIGINAL ARTICLE

Characterization of mating-type idiomorphs suggests that *Morchella importuna, Mel-20* and *M. sextelata* are heterothallic

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Abstract Morels (*Morchella* spp.) are highly prized for their culinary qualities and intensively collected worldwide by mycophiles. Morels are divided into three clades by phylogenetic analyses: black morels, yellow morels and the rufobrunnea clade. *Morchella importuna*, *Mel-20* and *M. sextelata* are included in the black morel clade and are widely distributed in Yunnan province, China. *M. importuna* and *M. sextelata* have been artificially cultured in recent years, but their life cycles and reproductive systems are still poorly understood, which delays the progress of morel cultivation. In this study, the genomes of two ascospore isolates of *M. importuna* with opposite mating-type were sequenced and two idiomorphs, *MAT1–1* and *MAT1–2*, were identified. The *MAT1–2* idiomorph was 6.7 kb in length containing a single *MAT1–2-1* gene, and the *MAT1–1* idiomorph was 10.5 kb containing a *MAT1–1-1* gene and two other open reading frames (ORFs), GME3123 and GME3124. These ORFs differed greatly from the homologues of previously published mating-type genes; therefore, we speculate that they are novel mating genes found only in morels. Single-ascospore populations of *M. importuna*, *M. sextelata* and *Mel-20* were analysed, and the result indicated that the ratios of *MAT1–1*- and *MAT1–2*-harbouring idiomorphs were not significantly different from a 1:1 ratio. The results suggest that these three black morels are heterothallic.

Keywords Morel · *MAT1–1-1* · *MAT1–2-1* · Single-ascospore population

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Introduction

As a group of edible mushrooms with excellent flavour and high medicinal value (Fu et al. 2013; Su et al. 2013; Hu et al. 2013), morels (Pezizomycetes, Ascomycota) are found in most parts of the world and are extensively traded (Pilz et al. 2007; Pildain et al. 2014). In recent years, morels have been commercially cultivated to meet the ever-growing need in China.

Phylogenetic analyses indicate that *Morchella* consists of three clades: the Elata Clade (black morels), the Esculenta Clade (yellow morels) and the rufobrunnea Clade (O'Donnell et al. 2011). *Morchella importuna*, *Mel-20* and *M. sextelata* are included in the Elata Clade (Du et al. 2014; Richard et al. 2015). In a previous study, some black morel species were confirmed to form secondary ectomycorrhizal symbioses with spruce (Buscot 1994), while *M. importuna* and *M. sextelata* are considered to be saprophytic fungi that can be cultivated artificially (Peng et al. 2016).

Although several species of *Morchella* have been successfully cultivated (Ower et al. 1986; Miller 2005; Masaphy 2010; Peng et al. 2016), their genetic information, life cycles and reproductive systems remain poorly understood. Based on SNP, SCAR, and AFLP markers, Pagliaccia et al. (2011) consider members of the *Morchella* sp. *Mel*-12 phylogenetic lineage to be heterothallic and to outcross in nature. Multiple nuclei are present in a single ascospore or one hyphal segment

of *Morchella* (Hervey et al. 1978; Volk and Leonard 1990). However, whether the ascospores are heterokaryotic or homokaryotic remains unclear.

The sexual reproduction of Ascomycota fungi is controlled by a single mating-type locus (*MAT-1*) (Coppin et al. 1997) with two highly divergent nonhomologous idiomorphs (Metzenberg and Glass, 1990), designated *MAT1–1* and *MAT1–2* (Turgeon and Yoder 2000). The *MAT1–1* idiomorph is characterized by the *MAT1–1-1* gene, which encodes protein with an alpha-box domain, while the gene *MAT1–2-1*, which encodes a high-mobility group (HMG) domain, is generally located on the *MAT1–2* idiomorph (Debuchy and Turgeon 2006). Individual isolates usually contain either the *MAT1–1* or *MAT1–2* idiomorph in heterothallic species. In contrast, in homothallic species, both mating types are contained in the haploid genome, usually tightly linked, although exceptions exist (Nelson 1996; Debuchy and Turgeon 2006). In Pezizomycetes, edible mushrooms that are as highly praised as morels, *Tuber melanosporum* and *T. indicum*, have been identified as heterothallic (Rubini et al. 2011; Belfoori et al. 2013).

In our previous studies, a draft genome database of the *Mes-15* strain YAAS2689 included in the Esculenta Clade was obtained (data unpublished), and the sequences of the genes *MAT1–1-1* (KP776983) and *MAT1–2-1* (KP776984) were verified. In this study, the genomes of two single ascospore isolates from *M. importuna* (YPL6) with opposite mating-type were sequenced. The aim was (1) to identify the *MAT* locus structure of *M. importuna*, (2) to clone and characterise the *MAT1–1-1* and *MAT1–2-1* mating-type genes of *M. importuna*, *Mel-20* and *M. sextelata*, and (3) to analyse whether these three black morel species are heterothallic or homothallic.

Materials and methods

Strains and isolations of ascospores

The fruiting body WXLBD7 of *Mel-20* was collected from the forests of Weixi (Yunnan province). YPL2 and YPL6 of *M. importuna* and HL1 of *M. sextelata* were obtained from morel cultivation fields in Chuxiong (Yunnan province) and Huili (Sichuan province), respectively (Table 1). The corresponding strains were obtained by tissue isolation. Single-ascospore strains were isolated from the fruiting bodies after spore dilution. Every ascospore isolate was numbered with an Arabic numeral suffix corresponding to the ascocarp. For example, YPL6–3 refers to the 3rd ascospore isolate from ascocarp YPL6. All the cultures were identified by phlogenetic analyses (Table S1; Figs. S1, S2) and deposited in Mushroom Center of Yunnan Crops Genebank (in Yunnan Academy of Agricultural Sciences, YAAS), Kunming, China.

DNA extraction and PCR amplification

Each isolate was incubated on PDA at 23 °C for 7 days. Mycelia were scraped and ground in liquid nitrogen. DNA extraction was performed using an EZgene™ Fungal gDNA Kit (BIOMIGA Inc.). All primer pairs were designed by Primer Premier v.5 and synthesized by Beijing Tsingke Biotechnology. The PCR amplifications were performed in a 25-μL mixture containing 12.5 μL of PCR Mix (TSINGKE), 1 μL of primer (10 μM/L) and 10 ng of DNA. Long-range PCR amplification was performed using LA Taq DNA polymerase (TaKaRa Biotechnology (Dalian)). The primer sequences and main PCR cycling parameters are listed in Table 2.

Genome sequencing

According to the conserved amino acid sequences of the *MAT1–2-1* gene HMG domain of ascomycetes, primer pair p1 was designed to amplify the corresponding gene fragments from the single-ascospore isolate group which contained 105 isolates of *M. importuna* YPL6. The isolates that contained the predicted amplification bands were considered to contain the *MAT1–2* idiomorph, while the isolates that lacked detectable bands were hypothesised to contain the *MAT1–1* idiomorph.

Table 1 Ascocarps used in this study

Ascocarp	Species	Origin	Genbank acc.Nr			
			ITS	RPB2	EF1-a	LSU
HL1	*Morchella sextelata*	Huili, Sichuan	KX809732	KX809721	KX809716	–
WXLBD7	*Mel-20*	Weixi, Yunnan	KX809734	KX809723	KX809718	–
YPL2	*M. importuna*	Chuxiong, Yunnan	KX809735	KX809724	KX809719	–
YPL6	*M. importuna*	Chuxiong, Yunnan	KX809736	KX809725	KX809720	–
YAAS2689	*Mes-15*	Shanggri-La,Yunnan	KM485942	–	KM527910	KM485975

Genomic DNA from the isolates YPL6–1 with the *MAT1–2* idiomorph and YPL6–3 with the *MAT1–1* idiomorph were submitted to the Beijing Genomics Institute (BGI). Genomic libraries were constructed and paired-End sequences were produced using Illumina HiSeq™ 2000. The clean reads were assembled into contigs by the SOAPdenovo assembly programme (http://soap.genomics.org.cn/soapdenovo.html), and the scaffolds were built by analysing the information of paired reads that covered different contigs. Genes were predicted using GeneMark-ES (v.2.3e)software and corresponding gene annotations were conducted by BLASTx analysis with the KEGG, KOG, SwissProt, NR and GO databases.

Identification and verification of the *MAT1–1* and *MAT1–2* idiomorphs of *M. importuna*

The *MAT1–2* and *MAT1–1* idiomorphs were identified by alignment analyses of two scaffolds containing *MAT1–2-1* or *MAT1–1-1*. Ten PCR primer pairs (p2–1, p2–2, p2–3, p2–4, p2–5, p3–1, p3–2, p3–3, p3–4, and p3–5) (Table 2) were employed to verify the two idiomorphs based on their genome sequences. PCR was performed under standard conditions (Table 2). The products were recovered from agarose gels and purified using a Gel/PCR Extraction Kit (BIOMIGA), then cloned into the pGM-T vector and sequenced.

Table 2 primer sequences and PCR cycling parameters used in this study

Specified use	Code	Sequence(5′-3′)	Annealing temperature (°C)	Extending time (s)
HMG domain conservation region	p1f	CCGGAATTGGGAACAACGATG	56	30
	p1r	GTTTTCGGGGTGTATACTTATAC		
Verify the *MAT1–2* idiomorph of *M. importuna*	p2–1f	GGGGTGTGTGTCGTCACTGGGGT	62	120
	p2–1r	CCGTCTGCTTTCATACTTTGGGT		
	p2–2f	ACAGAATTGTTGCCAGTAGA AGTGA	60	90
	p2–2r	CGATGAACTGATAGGGTGGT AAGAG		
	p2–3f	ACACGGACCGCACCTTGAAATACTT	60	360
	p2–3r	CGACAACAGACATTGGGCTACTTAC		
	p2–4f	TAGTAGACCATCACCGCACAGCAG	64	120
	p2–4r	ATACGCAATAATCAAGCACCCAGG		
	p2–5f	TCACTACCGACCACTCTTACCCGC	64	120
	p2–5r	CGACATTCATTGTCAACACCATGC		
Verify the *MAT1–1* idiomorph of *M. importuna*	p3–1f	GTCGTCACCACAAACCACCCACC	61	180
	p3–1r	TCTCCCAACTTCTCAACAATCCA		
	p3–2f	TCTACCAGCCATGTGAAACA AGCAA	64	120
	p3–2r	GCTCTCTTGTGCCCCTTTTGACTAT		
	p3–3f	ACTCTTGTAGTGTTCACCATAAA	51	180
	p3–3r	GGAAAAATCTCAAAAATCGTCTC		
	p3–4f	AGTCAATAGTCAATGGAGCGTTA	56	100
	p3–4r	AGGATAGAAGTAGAAGAGGGGGG		
	p3–5f	ATGTATTATTCTCATCGTATTTT	51	180
	p3–5r	CTTTAGGTGGTAGTCTGGGTTTC		
MAT1–1-1 gene conservation region	p7f	CCGGTTTATCTTACTGGACTGGTTC	62	40
	p7r	GCTTTCCTCTTCTCTCGTTGCCATA		
Verify the *MAT1–1-1* gene coding region	p8f	ATGTCACTCCGTCCGGTTTACCTTA	65	90
	p8r	TGGAATGTCTGTGATTGAGGCTGTG		
Walking for the total sequence of *MAT1–1-1* gene	p9-1f	TCTTTGTCTTCGTAACGCCACTTTG	65	120
	p9-2f	CCAGGAGGGTCGGTATTTCAGGTGC	65	120
	p9-3f	CCGATTGTTGATGAACCGTTGACTA	65	120
	p9-1r	CCTAGCCCTCCCAGGAATTTTGATA	65	120
	p9-2r	TAGTCAACGGTTCATCAACAATCGG	65	120
	p9-3r	GTGGGGGCACAATATATCGACAGTC	65	120
Verify the *MAT1–2-1* gene coding region	p10f	GGCCAGAACAGATGCTCGAA GAAGC	64	60
	p10r	CTCCCAAAGCATGATCAAATCCCTC		

Sequence comparison and dotplot analysis were conducted to verify the two idiomorphs of *M. importuna*.

Isolation of *MAT1–2-1* and *MAT1–1-1* from *Mel-20* and *M. sextelata*

Conserved sequences could be found by analysis of the flanking regions of *MAT1–2-1* gene in *M. importuna* (KY782629) and *Mes-15* (KY782632). A pair of specific primers (p2–3f and p2–3r) were designed based on the conserved regions to amplify the complete sequences of the *MAT1–2-1* gene in *Mel-20* and *M. sextelata*. The PCR amplicons (approximately 5 kb) were cloned and sequenced.

Because no conserved portions could be located in the 10-kb flanking regions from the upstream to the downstream region of the *MAT1–1-1* gene in *M. importuna* (KY782630) and *Mes-15* (KY782631), a primer pair (p7f and p7r) was designed to sequences within the *MAT1–1-1* gene to amplify the corresponding DNA fragments. To obtain the complete sequence of the *MAT1–1-1* gene, the DNA walking method was performed using the Genome Walking Kit (TaKaRa Biotechnology (Dalian)) with SP Primers (p9-1f, p9-2f, p9-3f, p9-1r, p9-2r, and p9-3r) based on *MAT1–1-1* fragments of *Mel-20* and *M. sextelata*.

Verification of the coding regions of *MAT1–1-1* and *MAT1–2-1* genes

Total RNA was extracted from the mycelia of YPL6, YPL6–1, YPL6–3, HL1, HL1–1, HL1–47, WXLBD7, WXLBD7–11 and WXLBD7–23 using a TaKaRa MiniBEST Plant RNA Extraction Kit. The first-strand cDNAs were synthesized using Oligo dT primer (TaKaRa PrimeScript™ II 1st Strand cDNA Synthesis Kit). The primer pairs p8 for the *MAT1–1-1* gene and p10 for the *MAT1–2-1* gene were designed using predicted sequences adjacent to the start site and end site of the two genes. The design strategy also took advantage of conserved regions, so the primers could be used in all three morel species. Introns were verified manually by comparing the RT-PCR sequences with the corresponding DNA sequences.

Mating type ratios of ascospores from the same ascocarp

Single-ascospore populations of WXLBD7, YPL2, YPL6 and HL1 were obtained to determine the occurrence and frequency of the mating type idiomorphs. Standard PCR tests with p8 and p10 primer pairs were conducted to examine the four single-ascospore populations. A chi-square test was applied to compare the *MAT1–1*/*MAT1–2* ratio at the significance level $P = 0.05$.

Results

MAT loci structures of *M. importuna*

The results of genome assembly and gene annotation of isolations YPL6–1 and YPL6–3 showed that the genome sizes and components of two isolations are similar (Tables S2, S3). The *MAT1–1-1* gene was observed only on Scaffold 152 (KY782630) of the *M. importuna* isolate YPL6–3 genome, which was 80 kb long and contained 18 predicted genes. The *MAT1–2-1* gene was found on Scaffold 91 (KY782629) of the YPL6–1 genome, which was 75 kb long and contained 26 predicted genes. Sequence alignment of the two scaffold regions revealed that the *MAT1–1* and *MAT1–2* idiomorphs were 10.5 and 6.7 kb long, respectively.

The *MAT1–2* idiomorph contained only a single *MAT1–2-1* gene. However, in addition to the *MAT1–1-1* gene, there were two predicted coding sequences GME3123 and GME3124 on the *MAT1–1* idiomorph (Fig. 1). They were not homologues to any of the mating genes that have been published; therefore, we speculated that they are novel mating genes unique to morels. GME3123 was found to be 1688 bp containing four introns and encoding 488 amino acids, and GME 3124 was 1825 bp containing five introns and encoding 512 amino acids. BLASTx analysis revealed that GME3123 had 24% and GME3124 21% amino acids similarity to hypothetical protein (AIU38081.1) of *Tuber borchii* (Belfiori et al. 2016).

To compare this analysis with the published morel genome databases, a sequence completely identical to the *MAT1–1* idiomorph was found on the Scaffold 48 of the *M. conica* genome (http://genome.jgi.doe.gov/Morco1/Morco1.home.html) between 239.6 kb and 250.1 kb. Three genes, 10317, 10316 and 10315, were in this area on the Scaffold 48 of the *M. conica* genome, and they were completely identical with the genes *MAT1–1-1*, GME3123 and GME3124, respectively. No sequences identical to the *MAT1–2* idiomorph were founded in this genome database. Sequences completely identical to the *MAT1–2* idiomorph were located on the Scaffold 20 of the *M. importuna* strain SCYDJ1-A1 genome (http://genome.jgi.doe.gov/Morimp1/Morimp1.home.html) between 230.6 kb and 237.3 kb. Only an ORF known as fgenesh1_pg.20_#_79 was found in this area, and its sequence was the same as the *MAT1–2-1* gene. The *MAT1–1* idiomorph could not be found in this genome database.

The coding sequences flanking the two idiomorphs were highly conserved. The 5′ flanking region of both idiomorphs contained *APN2*, which is connected to the *cox13* gene, and there were four additional predicted ORFs between the *cox13* gene and the *MAT* idiomorphs (Fig. 1). The *APN2* gene displayed 66% amino acid

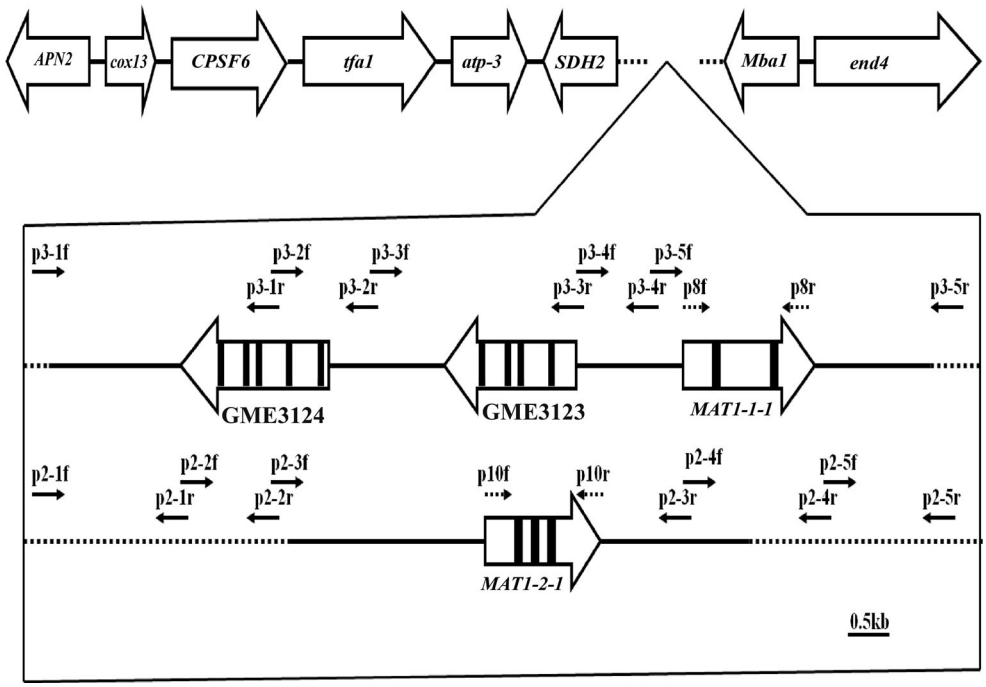

Fig. 1 Organization of *MAT* locus in *M. importuna*. The *scale bar* indicates sizes of only the *MAT* idiomorphs, the distance and sizes of other genes are not to scale. Introns in the *MAT* genes are indicated by *black boxes*. Thick and thin *arrows* indicate the orientation of genes and prime pairs respectively. Gene or superfamily names refer to a gene encoding a protein showing the highest percentage identity to the putative gene product following BLASTx analysis. *APN2* DNA-(apurinic or apyrimidinic site) lyase 2; *cox13* cytochrome c oxidase subunit 6A, mitochondrial; *CPSF6*: cleavage and polyadenylation specificity factor subunit 6; *tfa1* transcription initiation factor IIE subunit alpha; *atp-3* ATP synthase subunit 4, mitochondrial; *SDH2* succinate dehydrogenase (ubiquinone) iron-sulfur subunit, mitochondrial; *Mba1* mitochondrial inner membrane protein; *end4* (also known as *SLA2*) endocytosis protein end4

sequence similarity to CCX30259.1 (*Pyronema omphalodes*), and the *cox13* gene displayed 62% similarity to GAP92444.1 (*Rosellinia necatrix*). The *end4* (also known as *SLA2*) gene was the second gene that located on the 3′ flanking region of two idiomorphs, and it displayed 66% amino acid sequence similarity to CCX33915.1 (*Pyronema omphalodes*).

Structure of the *MAT1–1-1* gene of three morel species

The lengths of the *MAT1–1-1* gene sequences of *M. importuna*, *M. sextelata* and *Mel-20* were 1694, 1730, and 1651 bp, respectively. RT-PCR confirmed that the *MAT1–1-1* ORFs contained two introns and encoded proteins of 525, 537 and 511 amino acids, respectively (Table 3). Sequence alignments and dotplot analyses indicated that the *MAT1–1-1* gene in *M. importuna* displayed 92.3 and 87.2% sequence identity to those in *M. sextelata* and *Mel-20*, respectively.

The deduced amino acid sequences of these three morel species contained a conserved alpha-box motif of the MAT1–1-1 protein of ascomycetes (Fig. 2a). In addition, the three proteins shared more than 20% amino acid identities to those in *Tuber indicum* (AHE80942), *T. melanosporum* (ADU56595) and *T. borchii* (AIU38080).

Table 3 The variance of *MAT1–1-1* and *MAT-2-1* gene in three morel species

Strain	Species	*MAT1–1-1*					*MAT1–2-1*					
		DNA (bp)	Intron (bp)		Amino acids (aa)	GenBank accession number	DNA (bp)	Intron(bp)			Amino acids (aa)	GenBank accession number
			1	2				1	2	3		
YPL6	*M. importuna*	1694	58	61	525	KX809728	1197	59	93	58	329	KX809731
HL1	*M. sextelata*	1730	58	61	537	KX809726	1199	59	94	59	329	KX809729
WXLBD7	*Mel-20*	1651	57	61	511	KX809727	1247	59	94	62	344	KX809730

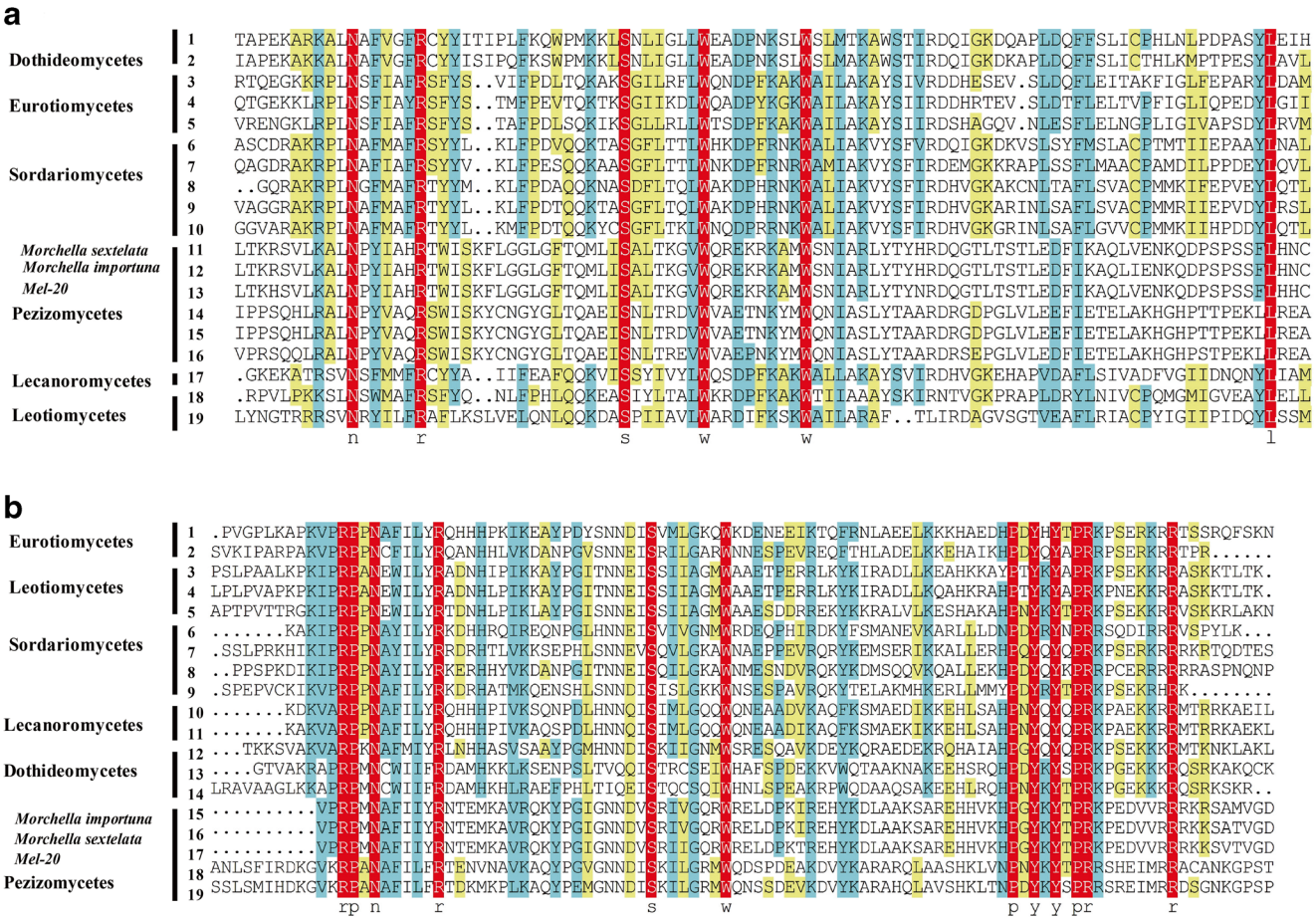

Fig. 2 Amino acid alignment of conserved regions of *MAT* proteins of *Morchella* spp. with those of other ascomycetes. **a** Amino acid alignment of the conserved alpha-box domain of the following species: (*1*) *Stemphylium callistephi*(AAR04468); (*2*) *Alternaria brassicicola*(AAK85542); (*3*) *Aspergillus fischeri*(XP_001263836); (*4*) *Talaromyces marneffei* (ABC68484); (*5*) *Histoplasma capsulatum* (ABO26868); (*6*) *Ophiocordyceps sinensis* (ALH25059); (*7*) *Cordyceps militaris* (AKM95188); (*8*) *Fusarium poae* (CAD59610.3); (*9*) *Colletotrichum musae* (CAD59611.3); (*10*) *Fusarium avenaceum* (CAD59608.4); (*11*) *Morchella sextelata*; (12) *Morchella importuna*; (*13*) *Mel-20*; (*14*) *Tuber indicum* (AHE80942); (*15*) *Tuber melanosporum* (ADU56595); (*16*) *Tuber borchii* (AIU38080); (*17*) *Xanthoria polycarpa* (CAI59771); (*18*) *Pyrenopeziza brassicae* (CAA06844); (*19*) *Sclerotinia homoeocarpa* (AJW31369). **b** Amino acid alignment of the conserved HMG domain of following species: (*1*) *Aspergillus fumigatus* (XP_754989.2); (*2*) *Penicillium chrysogenum* (CAP17333); (*3*) *Sclerotinia sclerotiorum* (AGB05594); (*4*) *Sclerotinia trifoliorum* (ANN44262); (*5*) *Sclerotinia homoeocarpa* (AJW31335); (*6*) *Sordaria fimicola* (CAB63226); (*7*) *Cordyceps militaris* (AKM95197); (*8*) *Ophiocordyceps sinensis* (AEH27625); (*9*) *Colletotrichum gloeosporioides* (AKO22190); (*10*) *Rusavskia elegans* (CAI59778.2); (*11*) *Dufourea flammea* (CAI59780.2); (*12*) *Passalora fulva* (ABG49507); (*13*) *Leptosphaeria maculans* (AAO37761); (*14*) *Bipolaris sorokiniana* (AAF87724); (*15*) *Morchella importuna*; (*16*) *Morchella sextelata*; (*17*) *Mel-20*; (*18*) *Tuber borchii* (AIU38078); (*19*) *Tuber indicum* (AHE80950)

Structure of the *MAT1–2-1* gene of three morel species

The lengths of *MAT1–2-1* gene sequences of *M. importuna*, *M. sextelata* and *Mel-20* were 1197, 1199 and 1247 bp, respectively. The results of RT-PCR analyses confirmed that the *MAT1–2-1* ORFs contained three introns and encoded proteins of 329, 329 and 344 amino acids, respectively (Table 3). Sequence alignments and dotplot analyses indicated that the *MAT1–2-1* gene in *M. importuna* shared 94.3 and 86.3% sequence identity with the corresponding genes in *M. sextelata* and *Mel-20*, respectively. The alignment of the MAT1–2-1 protein with corresponding sequences deposited in GenBank indicated that the HMG-box motifs of three morels were conserved (Fig. 2b).

Mating type ratios of three morel species based on single-ascospore populations

The primer pairs p8 for the *MAT1–1-1* gene and p10 for the *MAT1–2-1* gene could be used in all three morel species to examine the mating types of single-ascospore populations. Strains that contained only *MAT1–1-1* and not *MAT1–2-1* were amplified and scored as *MAT1–1*, and vice versa (Fig. 3). In the four ascospore populations (Table 4), the ratio of *MAT1–1* to

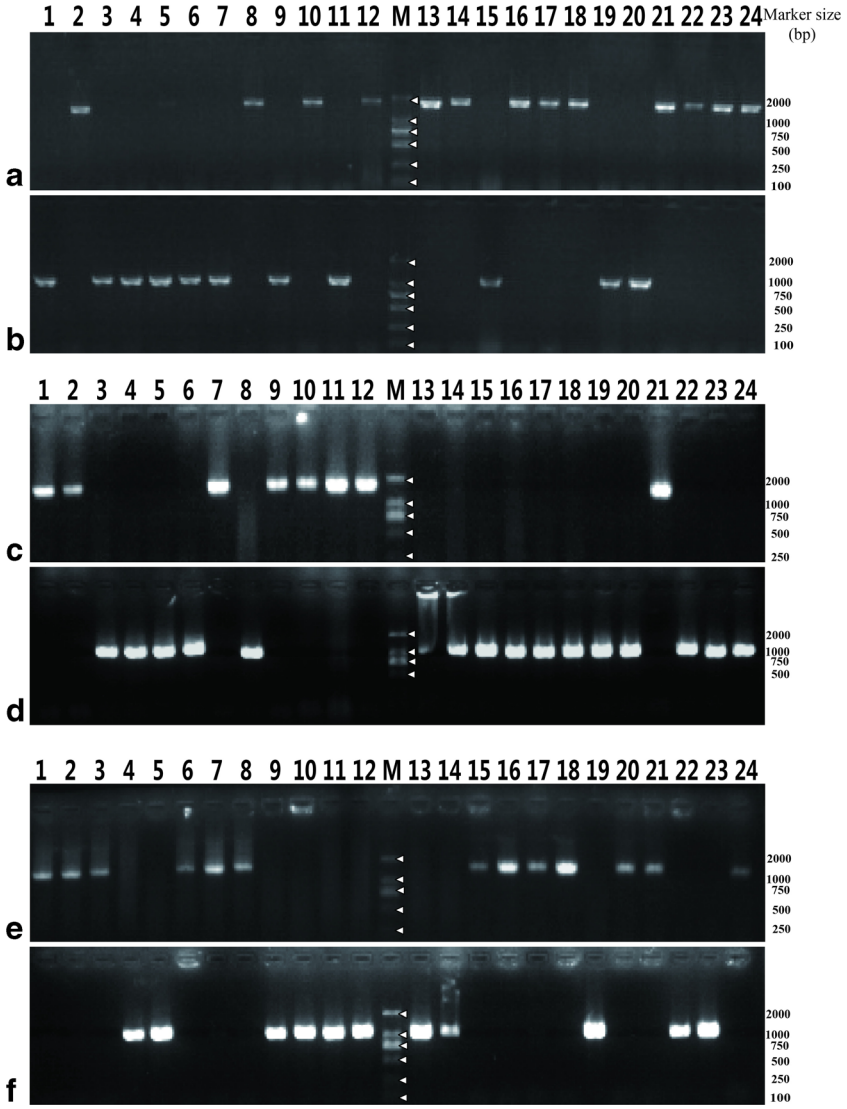

Fig. 3 *MAT* gene PCR products of single ascospore populations YPL2, HL1 and WXLBD7. **a** *MAT1–1-1* gene PCR products of YPL2–1 to YPL2–24. **b** *MAT1–2-1* gene PCR products of YPL2–1 to YPL2–24. **c** *MAT1–1-1* gene PCR products of HL1–1 to HL1–24. **d** *MAT1–2-1* gene PCR products of HL1–1 to HL1–24. **e** *MAT1–1-1* gene PCR products of WXLBD7–1 to WXLBD7–24. **f** *MAT1–2-1* gene PCR products of WXLBD7–1 to WXLBD7–24. *M* DNA marker 2000 (2000,1000, 750, 500, 250 and 100 bp)

MAT1–2 ranged from 0.44 to 0.96. Chi-square analysis indicated that the ratio was not significantly different from 1:1 except for the HL1 populations. No sequence difference was detected between the single-ascospore strains and the parent strains in either of the *MAT1–1-1* or *MAT1–2-1* genes.

Table 4 Summary of *MAT* type ratios of four ascospore populations

Population	*n*	*MAT1–1*	*MAT1–2*	Ratio[a]	Chi-Sq[b]	P-value[c]
HL1	46	14	32	0.44	7.043	0.00796
WXLBD7	53	26	27	0.96	0.019	0.89037
YPL2	66	32	34	0.94	0.061	0.80492
YPL6	105	47	58	0.81	1.152	0.28313

n number of single-ascospore populations per each ascocarp.

[a] Ratio of MAT1–1-1 to MAT1–2-1.

[b] Chi-square value for relative to the expected ratio of 1:1.

[c] Probability of chi-square values at $P \leq 0.05$ at 1 degree of freedom

Discussion

Heterothallism in *M. importuna, M. sextelata* and *Mel-20*

In ascomycetes, the mating type corresponds to two allelic forms of a single locus and, accordingly, heterothallism is bipolar (Whitehouse 1949). This property was first demonstrated in *Ascobolus magnificus* and *A. carbonarius* (Dodge 1920) and then in several *Neurospora* species (Shear and Dodge 1927). Molecular analyses of mating-type in filamentous ascomycetes began with the cloning of *A* and *a* mating-types from *Neurospora crassa* (Glass et al. 1988). It has since been confirmed that the mating-type locus contains one of two highly divergent sequences occupying the same chromosomal locus. Metzenberg and Glass (1990) used the word idiomorph to denote that these large sequences are not obviously related by structural similarity. In heterothallic ascomycetes, ascospore populations can be divided into two groups: those

harbouring the *MAT1–1* and *MAT1–2* idiomorphs. The sexual cycle occurs only between two individuals with opposite mating-type, rather than with self-fertilizing as in homothallic reproduction (Pöggeler 2001; Chilvers et al. 2014).

The genomic data of *M. importuna* YPL6 confirmed that the *MAT* locus is divided into two idiomorphs. In the YPL6–3 genome, only the *MAT1–1* idiomorph harbouring the *MAT1–1-1* gene was found. Similarly, the *MAT1–2* idiomorph with the *MAT1–2-1* gene was only located in the YPL6–1 genome. Furthermore, the conserved flanking sequences of the two idiomorphs confirmed that they occupy the same chromosomal locus. The characterization of the mating locus is consistent with heterothallic ascomycetes. Mating-type analysis of four ascospore populations from three species, *M. importuna, M. sextelata* and *Mel-20*, indicated that two sexual groups were present, and the ratios of *MAT1–1*- and *MAT1–2*-harbouring idiomorphs were not significantly different from a 1:1 ratio. These results suggested that these morels are all heterothallic.

Because the lack of visible structures, such as the clamp connection in basidiomycetes precludes visible morphological analysis, these molecular data will facilitate crossbreeding to produce high-quality morel cultivars.

Structure of *MAT* idiomorphs

In heterothallic ascomycetes, the structure of the *MAT* idiomorph varies in different species. The mating idiomorphs of *Neurospora crassa* were the first to be investigated, and the *MAT1–1* idiomorph contains three ORFs: *MAT1–1-1*, *MAT1–1-2* and *MAT1–1-3* (*mat A-1*, *A-2* and *A-3* in *N. crassa*). The *MAT1–2* idiomorph contains the *MAT1–2-1* and *MAT1–2-2* ORFs (*mat a-1* and *a-2* in *N. crassa*) (Pöggeler and Kück 2000). Similar *MAT* structures have been found in many other species surveyed later (Debuchy and Turgeon 2006; Wilken et al. 2012; Hutchinson et al. 2016). In this study, *M. importuna* has been identified as possessing a similar *MAT* idiomorph structure similar to those previously described. The *MAT1–1* idiomorph contains three OFRs, and the *MAT1–2* idiomorph contains a single *MAT1–2-1* gene. However, two ORFs,GME3123 and GME3124,in the *MAT1–1* idiomorph were found to be highly divergent from the homologues of mating genes that have been published. At this point, we can only speculate that GME3123 and GME 3124 are new mating-type genes at the *MAT1–1* locus of *M. importuna*.

APN2, *SLA2* and *APC5* are ancestral companions of the *MAT* locus in euascomycetes (Debuchy and Turgeon 2006). Similarly, homologues of *APN2* and *SLA2* were found on each side of the *MAT* locus in Sordariomycetes, and the *ATG3*, *cox13* and *CWF24* genes were also conserved in the up- and downstream regions of *MAT* (Debuchy and Turgeon 2006; Xu et al. 2016; Lu et al. 2016). For *M. importuna*, the *APN2* gene was connected to a *cox13* gene on the 5′ flanking region of both *MAT* idiomorphs. However, four predicted genes were found between the *cox13* gene and the *MAT* idiomorphs. The *SLA2* (*end*4) gene was located on the 3′ flanking region of two idiomorphs. These results indicated that the *MAT* locus structure of *M. importuna* had some conserved sequences but was highly divergent from other species.

Based on the conserved flanking sequences, *MAT1–1* and *MAT1–2* idiomorphs have been cloned from many species (Martin et al. 2011; Belfiori et al. 2013). In this study, the coding sequences flanking the two idiomorphs of *M. importuna* were highly conserved, but the cloning of the whole *MAT* idiomorph of *M. sextelata* and *Mel-20* using long-distance PCR amplification was not successful (data not shown), possibly due to the variability of the sequence in the different species. Unfortunately, we were not able to identify the entire mating idiomorphs of these two morel species.

Expression of mating genes

In *Neurospora crassa* and *Tuber melanosporum*, mating genes are constitutively expressed during the vegetative as well as the sexual phases (Ferreira et al. 1996; Rubini et al. 2011). In this study, we did not analyse the expression of mating genes specially in different cultivation periods and conditions. However, we verified the coding region by successfully amplifying the *MAT1–1-1* or *MAT1–2-1* gene with cDNA from all the tested strains incubated on PDA for 10 days. For the single-ascospore isolates, *MAT1–1-1* of YPL6–3, HL1–1 and WXLBD7–11, and *MAT1–2-1* of YPL6–1, HL1–47 and WXLBD7–13 were amplified with their cDNA, respectively. In addition, the expression of the two genes was detected in the mycelia from the YPL6, WXLBD7 and HL1 tissues. These results implied that the mating genes in morels are constitutively expressed.

Mating locus and phylogenetic relationship

In ascomycetes, as throughout the eukaryotic kingdom, genes controlling sex determination are evolving more rapidly than other protein-coding genes (Whitfield et al. 1993; Ferris et al. 1997; Pöggeler 1999), which enables the study of the phylogenetic relationship of closely related fungal species. The sequences from the *gpd* (glyceraldehyde-3-phosphate dehydrogenase gene), *mat A-1* and *mat a-1* genes produced phylogenetic trees with a similar topology and strict separation of homothallic and heterothallic species within the genera *Neurospora* and *Sordaria* (Pöggeler 1999). Belfiori et al. (2013) revealed the existence of cryptic species of *Tuber indicum* by comparing the idiomorph structure and sequences of *MAT* genes.

Morchella importuna and *M. sextelata* had a closer phylogenetic relationship than did either to *Mel-20* (Fig. S1). Based on comparing the DNA sequences of the *MAT1–1-1* and

MAT1–2-1 genes, *M. importuna* showed greater identity to *M. sextelata* than to *Mel-20*, which was consistent with the molecular evolution relationship based on the analysis of ITS, *RPB1*, *RPB2* and *TEF1* (Du et al. 2012; Richard et al. 2015). Acquiring more sequence information on the mating loci will help to investigate the phylogenetic diversity of morel species, thereby producing new insights.

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