Heterothallism and potential hybridization events inferred for twenty-two yellow morel species

Xi-Hui Du1*, Dongmei Wu2, Heng Kang3*, Hanchen Wang1, Nan Xu1, Tingting Li1 and Keliang Chen1

Abstract

Mating-type genes are central to sexual reproduction in ascomycete fungi and result in the establishment of reproductive barriers. Together with hybridization, they both play important roles in the evolution of fungi. Recently, potential hybridization events and MAT genes were separately found in the Elata Clade of *Morchella*. Herein, we characterized the *MAT1*–1–1 and *MAT1*–2–1 genes of twenty-two species in the Esculenta Clade, another main group in the genus *Morchella*, and proved heterothallism to be the predominant mating strategy among the twenty-two species tested. Ascospores of these species were multi-nuclear and had many mitochondrial nucleoids. The number of ascospore nuclei might be positively related with the species distribution range. Phylogenetic analyses of *MAT1*–1–1, *MAT1*–2–1, intergenic spacer (IGS), and partial histone acetyltransferase ELP3 (*F1*) were performed and compared with the species phylogeny framework derived from the ribosomal internal transcribed spacer region (ITS) and translation elongation factor 1-alpha (*EF1-a*) to evaluate their species delimitation ability and investigate potential hybridization events. Conflicting topologies among these genes genealogies and the species phylogeny were revealed and hybridization events were detected between several species. Different evolutionary patterns were suggested for MAT genes between the Esculenta and the Elata Clades. Complex evolutionary trajectories of *MAT1*–1–1, *MAT1*–2–1, *F1* and IGS in the Esculenta Clade were highlighted. These findings contribute to a better understanding of the importance of hybridization and gene transfer in *Morchella* and especially for the appearance of reproductive modes during its evolutionary process.

Keywords: *Morchella*, Esculenta clade, Mating type, Conflict, Phylogeny, Reproductive mode, IGS, F1, Gene transfer

INTRODUCTION

In fungi, sexual reproduction is regulated by genomic regions called mating-type (MAT) loci (Kronstad and Staben 1997; Debuchy et al. 2010; Casselton and Feldbrügge 2010), which operate on the establishment of reproductive barriers, determine mating compatibility and result in speciation (Coppin et al. 1997; Lee et al. 2010; Palumbi 2009; Swanson and Vacciquer 2002). In filamentous ascomycetes, the mating-type system is bipolar and has a single MAT locus called *MAT1* with two alternate forms as master regulators of sexual reproduction: the *MAT1*–1–1 and the *MAT1*–2–1 genes, respectively, encoding for alpha-box and a high mobility group (HMG) domain proteins (Metzenberg and Glass 1990; Debuchy et al. 2010). Due to their dissimilar sequences, the two forms of the MAT locus are referred to as idiomorphs instead of alleles (Metzenberg and Glass 1990). Filamentous ascomycetes have two main sexual reproductive modes: homothallism and heterothallism. Homothallic fungi are self-fertile and can complete the sexual cycle without a mating partner. Typically, a single homothallic strain harbors both MAT idiomorphs (linked or unlinked) in the same haploid nucleus (Debuchy et al. 2010). On the contrary, heterothallic fungi are self-sterile, contain only one of the two MAT genes, and need the participation of an opposite mating type partner to reproduce.

Reproductive modes of fungi are assumed to have a great influence on the evolutionary trajectory of their genomes (Burt 2000). In recent years, MAT genes have gained significant attention in evolutionary biology: on the one hand because of the general relationship between the
evolution of reproductive genes and the reproductive modes of individuals (Paolletti et al. 2006; Walters and Harrison 2008; Wik et al. 2008), and on the other hand because MAT genes are known to be evolutionarily dynamic with high evolutionary rates (Gioti et al. 2012; Martin et al. 2013; Sun and Heitman 2015). Therefore, MAT gene sequences have often been used to study evolutionary trends of mating systems (Fraser et al. 2007; Wik et al. 2008) and population genetics (Zhan et al. 2007; Groenenwald et al. 2007). Because of the high evolutionary rates of MAT genes, some studies inferred a phylogenetic relationship suggesting that the phylogenies of MAT genes were consistent with other genes (Groenenwald et al. 2006; Yokoyama et al. 2006; Lopes et al. 2017; O’Donnell et al. 2004; Duong et al. 2013; Du et al. 2017) whereas others found conflicting topologies (Wik et al. 2008; Strandberg et al. 2010).

True morels belong to the genus Morchella (Ascomycota, Pezizales, Morchellaceae), are widely known for their iconic edible ascomata and are among the world’s most prized edible fungi. The Morchella genus comprises the Esculenta Clade (yellow morels), the Elata Clade (black morels), and the Rufobrunnea Clade (blushing morels) with more than seventy species in total (Du et al. 2019b). Considering their important economic value, understanding the reproductive biology of these species is not only of fundamental but also of applied relevance, for example for artificial cultivation and cultivar breeding. However, knowledge about MAT genes in Morchella is currently limited to fourteen species from the Elata Clade. For these species, a heterothallic sexual cycle has been proposed while the phylogeny of MAT genes is consistent with other genes (Du et al. 2017). Species in the Esculenta Clade not only have a distinguished morphology from the Elata Clade, but also favor different ecological niches (Du et al. 2012; Kuo et al. 2012). Their reproductive modes are still unknown and the usefulness of MAT genes for species identification of MAT genes needs to be investigated. Additionally, considering that multiple hybridization events were detected in the Elata Clade (Du et al. 2016; 2019a), the F1 and IGS genes were chosen to assess whether they can be used for species identification and for the identification of potential hybridization events in the Esculenta Clade, based on previous studies focusing on the Elata Clade (Du et al. 2016) and the genus Fusarium (Mbofung et al. 2006).

The present study therefore sought: (i) to isolate and characterize the $MAT1–1–1$ and $MAT1–2–1$ genes of twenty-two yellow morel species; (ii) to illuminate the reproductive mode of these twenty-two species; (iii) to evaluate the $MAT1–1–1$ and $MAT1–2–1$ polymorphism intra- and interspecies; (iv) to infer the usefulness of the $MAT1–1–1$ and $MAT1–2–1$ genes as phylogenetic markers in the Esculenta Clade; and (v) to investigate the potential hybridization events among these species. Considering the increased interest in morel cultivation in China (Du et al. 2019a), description of the reproductive modes of these species is of high applied value.

**MATERIAL AND METHODS**

**Obtaining of $MAT1–1–1$ and $MAT1–2–1$ sequences**

The genome of Mes-21 was sequenced using an Illumina HiSeq 2500 Genome Analyzer (Illumina Inc., USA) by Genoseq Biotechnology Co., Ltd. (Wuhan, China) based on highly purified total genomic DNA isolated from the fungal mycelia culture. The genome was assembled by SOAPdeNovo2 (Zerbino et al. 2008) to first construct contigs based on the short insert libraries, then joining these to scaffolds using paired-end information, followed by local reassembly of unresolved gap regions. In order to detect the best assembly(s), kmer levels were respectively run at 41, 43, 45, 49 and 55, and an optimised k-mer size of 41 was suggested. The genome data of Mes-21 generated in this project has been deposited at DDBJ/EMBL/GenBank under the submission no. SUB6606995.

$MAT1–1–1$ and $MAT1–2–1$ were identified using BLASTn and BLASTx against the NCBI nucleotide and protein database by sequence similarity searches (http://www.ncbi.nlm.nih.gov/BLAST/) in the genome of Mes-21. The alpha-box- and HMG-containing sequences of M. importuna (KY508074 and KY508167) and M. sextelata (KY508145 and KY508228) from the Elata Clade were chosen for BLAST analysis of $MAT1–1–1$ and $MAT1–2–1$ in the Mes-21 genome.

**Fungal materials and species identification**

Eighty-three samples collected in China, Croatia and France between 2016 and 2019 were used in this study. Based on two-gene phylogenetic species recognition analysis via nuclDNA internal transcribed spacer region (ITS) and translation elongation factor 1-α (EF1-α) gene, the samples were characterized and belonged to twenty-two phylogenetic species of Esculenta Clade in the genus Morchella, namely M. steppicola, M. yangii, M. yishiuka, M. clivicola, M. dunensis, M. palazonii, M. americana, M. esculenta, M. galilaea, Mes-6, Mes-9, Mes-10, Mes-15, Mes-19, Mes-20, Mes-21, Mes-22, Mes-23, Mes-24, Mes-25, Mes-26, and Mes-27 (Table 1). At least two samples from each species were selected, except for M. steppicola and Mes-24 since only one sample was obtained of each. All the collections used in the present study were dried with silica and housed in the Fungal Herbarium of Chongqing Normal University, Chongqing City, China (FCNU). Information on these samples is detailed in Table 1.

**Selection of loci**

Two mating-type genes ($MAT1–1–1$ and $MAT1–2–1$), herein referred to as reproductive genes, were investigated in this study. The nuclDNA internal transcribed
| Taxon | Specimen voucher | Location | GenBank accession number | Pi of MAT1–1–1/2–1 (within each species) |
|-------|------------------|----------|--------------------------|------------------------------------------|
|       |                  |          | ITS | EF1-a | MAT1–1–1 | MAT1–2–1 | F1 | IGS |
| *M. americana* | FCNU1033 | France | MNS13710 | MNS13637 | MNS13853 | MNS13936 | MNS13445 | × | 0.0000/0.0012 |
| *M. americana* | FCNU1109 | France | MNS13706 | MNS13633 | MNS13849 | MNS13932 | MNS13442 | MNS13774 |
| *M. americana* | FCNU1110 | Croatia | MNS13664 | MNS13737 | MNS13883 | MNS13966 | MNS13470 | × |
| *M. americana* | FCNU1111 | France | MNS13708 | MNS13635 | MNS13851 | MNS13934 | × | × |
| *M. americana* | FCNU1112 | France | MNS13709 | MNS13636 | MNS13852 | MNS13935 | MNS13444 | × |
| *M. clivicola* | FCNU1019 | Sichuan, China | MK321870 | MK321918 | MNS13848 | MNS13931 | MNS13441 | MNS13773 | 0.0031/0.0012 |
| *M. clivicola* | FCNU1021 | Hubei, China | MK321871 | MK321919 | MNS13861 | MNS13944 | MNS13451 | MNS13782 |
| *M. dunensis* | FCNU1029 | Xinjiang, China | MK321872 | MK321920 | MNS13907 | MNS13948 | MNS13824 | × |
| *M. dunensis* | FCNU1030 | Xinjiang, China | MK321873 | MK321921 | MNS13906 | MNS13944 | MNS13823 | × |
| *M. esculenta* | FCNU1038 | Henan, China | MNS13752 | MNS13684 | MNS13904 | MNS13987 | MNS13861 | MNS13821 |
| *M. esculenta* | FCNU1039 | Henan, China | MNS13751 | MNS13665 | MNS13865 | MNS13905 | MNS13882 | × |
| *M. galilaea* | FCNU1061 | Kenya | MNS13758 | MNS13685 | MNS13905 | MNS13988 | × | MNS13822 |
| *M. galilaea* | FCNU1062 | Sichuan, China | MNS13733 | MNS13660 | MNS13877 | MNS13960 | MNS13465 | MNS13796 | 0.0018/0.0005 |
| *M. galilaea* | FCNU1063 | Yunnan, China | MNS13699 | MNS13626 | MNS13841 | MNS13924 | MNS13434 | MNS13766 |
| *M. galilaea* | FCNU1064 | Yunnan, China | MNS13700 | MNS13627 | MNS13842 | MNS13925 | MNS13435 | MNS13767 |
| *M. palazonii* | FCNU1031 | Xinjiang, China | MNS13759 | MNS13688 | MNS13909 | MNS13992 | MNS13825 | × |
| *M. palazonii* | FCNU1032 | Henan, China | MNS13758 | MNS13665 | MNS13884 | MNS13967 | × | MNS13826 |
| *M. galilaea* | FCNU1061 | Kenya | MNS13731 | MNS13646 | MNS13863 | MNS13946 | MNS13453 | × |
| *M. galilaea* | FCNU1062 | Sichuan, China | MNS13705 | MNS13632 | MNS13847 | MNS13930 | MNS13440 | MNS13772 |
| *M. galilaea* | FCNU1063 | Yunnan, China | MNS13699 | MNS13626 | MNS13841 | MNS13924 | MNS13434 | MNS13766 |
| *M. galilaea* | FCNU1064 | Yunnan, China | MNS13700 | MNS13627 | MNS13842 | MNS13925 | MNS13435 | MNS13767 |
| *M. palazonii* | FCNU1033 | Xinjiang, China | MNS13747 | MNS13674 | MNS13893 | MNS13976 | MNS13810 | × |
| *M. palazonii* | FCNU1034 | Henan, China | MNS13759 | MNS13688 | MNS13909 | MNS13992 | MNS13825 | × |
| *M. palazonii* | FCNU1035 | Henan, China | MNS13758 | MNS13665 | MNS13884 | MNS13967 | × | MNS13826 |
| *M. steppicola* | FCNU1036 | Qinghai, China | MNS13704 | MNS13631 | MNS13846 | MNS13929 | MNS13439 | MNS13771 |
| *M. yishuica* | FCNU1037 | Shandong, China | MNS13712 | MNS13639 | MNS13855 | MNS13938 | MNS13446 | MNS13776 |
| *M. yishuica* | FCNU1038 | Shandong, China | MNS13712 | MNS13639 | MNS13855 | MNS13938 | MNS13446 | MNS13776 |
| *M. yishuica* | FCNU1039 | Shandong, China | MNS13712 | MNS13639 | MNS13855 | MNS13938 | MNS13446 | MNS13776 |
| *M. yishuica* | FCNU1040 | Shandong, China | MNS13712 | MNS13639 | MNS13855 | MNS13938 | MNS13446 | MNS13776 |
| *M. yishuica* | FCNU1041 | Shandong, China | MNS13712 | MNS13639 | MNS13855 | MNS13938 | MNS13446 | MNS13776 |
| *M. yishuica* | FCNU1042 | Shandong, China | MNS13712 | MNS13639 | MNS13855 | MNS13938 | MNS13446 | MNS13776 |
| *M. yishuica* | FCNU1043 | Shandong, China | MNS13712 | MNS13639 | MNS13855 | MNS13938 | MNS13446 | MNS13776 |
| *M. yishuica* | FCNU1044 | Shandong, China | MNS13712 | MNS13639 | MNS13855 | MNS13938 | MNS13446 | MNS13776 |
| *M. yishuica* | FCNU1045 | Shandong, China | MNS13712 | MNS13639 | MNS13855 | MNS13938 | MNS13446 | MNS13776 |

* Du et al. IMA Fungus (2020) 11:4 Page 3 of 19
Table 1 Fungal names, specimen voucher, locations and GenBank accession numbers (Continued)

| Taxon | Specimen voucher | Location | GenBank accession number | Pi of MAT1–1/1 | Pi of MAT1–2/1 |
|-------|------------------|----------|-------------------------|----------------|----------------|
| Mes 9 | FCNU1046         | Shandong, China | MNS13749 MNS13676 MNS13895 MNS13978 MNS13979 MNS13812 |
| Mes 10 | FCNU1048        | Yunnan, China   | MNS13692 MNS13619 MNS13833 MNS13916 MNS13427 × | 0.0031/0.0000 |
| Mes 10 | FCNU1049        | Henan, China    | MNS13711 MNS13638 MNS13854 MNS13937 × | MNS13775 |
| Mes 15 | FCNU1056        | Yunnan, China   | MNS13752 MNS13679 MNS13899 MNS13982 MNS13482 MNS13816 | 0.0000/0.0005 |
| Mes 15 | FCNU1057        | Yunnan, China   | MNS13752 MNS13683 MNS13899 MNS13982 MNS13482 MNS13816 |
| Mes 15 | FCNU1058        | Sichuan, China  | MNS13739 MNS13666 MNS13885 MNS13968 MNS13471 MNS13802 |
| Mes 15 | FCNU1059        | Yunnan, China   | MNS13694 MNS13621 MNS13836 MNS13919 MNS13429 × |
| Mes 15 | FCNU1060        | Guizhou, China  | MNS13718 MNS13645 MNS13862 MNS13945 MNS13452 MNS13783 |
| Mes 19 | FCNU1069        | Hubei, China    | MNS13716 MNS13643 MNS13859 MNS13942 MNS13450 MNS13780 | 0.0008/0.0004 |
| Mes 19 | FCNU1070        | Henan, China    | MNS13760 MNS13687 MNS13910 MNS13993 MNS13490 MNS13827 |
| Mes 19 | FCNU1071        | Chongqing, China | MNS13726 MNS13653 MNS13870 MNS13953 MNS13459 MNS13789 |
| Mes 19 | FCNU1072        | Chongqing, China | MNS13725 MNS13652 MNS13869 MNS13952 MNS13458 MNS13788 |
| Mes 19 | FCNU1073        | Henan, China    | MNS13724 MNS13651 MNS13868 MNS13951 MNS13457 MNS13787 |
| Mes 19 | FCNU1074        | Yunnan, China   | MNS13753 MNS13680 MNS13900 MNS13983 MNS13483 MNS13817 |
| Mes 20 | FCNU1075        | Henan, China    | MNS13761 MNS13688 MNS13911 MNS13994 MNS13491 MNS13828 | 0.0006/0.0010 |
| Mes 20 | FCNU1076        | Henan, China    | MNS13729 MNS13656 MNS13873 MNS13956 MNS13462 MNS13792 |
| Mes 20 | FCNU1077        | Henan, China    | MNS13762 MNS13689 MNS13913 MNS13996 MNS13493 MNS13830 |
| Mes 20 | FCNU1078        | Henan, China    | MNS13763 MNS13690 MNS13914 MNS13997 MNS13494 MNS13831 |
| Mes 20 | FCNU1079        | Shandong, China | MNS13746 MNS13673 MNS13892 MNS13975 MNS13476 MNS13809 |
| Mes 21 | FCNU1080        | Henan, China    | MNS13732 MNS13659 MNS13876 MNS13959 × | MNS13795 0.0006/0.0007 |
| Mes 21 | FCNU1081        | Henan, China    | MNS13734 MNS13661 MNS13879 MNS13962 MNS13467 MNS13798 |
| Mes 21 | FCNU1082        | Gansu, China    | MNS13707 MNS13634 MNS13850 MNS13933 MNS13443 × |
| Mes 21 | FCNU1083        | Henan, China    | MNS13703 MNS13630 MNS13845 MNS13928 MNS13438 MNS13770 |
| Mes 21 | FCNU1084        | Henan, China    | MNS13702 MNS13629 MNS13844 MNS13927 MNS13437 MNS13769 |
| Mes 22 | FCNU1085        | Yunnan, China   | MNS13754 MNS13681 MNS13901 MNS13984 × | MNS13818 0.0000/0.0069 |
| Mes 22 | FCNU1086        | Yunnan, China   | MNS13755 MNS13682 MNS13902 MNS13985 MNS13484 MNS13819 |
| Mes 22 | FCNU1088        | Hubei, China    | MNS13715 MNS13642 MNS13858 MNS13941 MNS13449 MNS13779 |
| Mes 22 | FCNU1089        | Hubei, China    | MNS13717 MNS13644 MNS13860 MNS13943 × | MNS13781 |
| Mes 23 | FCNU1087        | Guangdong, China | MNS13701 MNS13628 MNS13843 MNS13926 MNS13436 MNS13768 | 0.0000/0.0000 |
| Mes 23 | FCNU1090        | Sichuan, China  | MNS13740 MNS13667 MNS13886 MNS13969 MNS13472 MNS13803 |
| Mes 23 | FCNU1091        | Sichuan, China  | MNS13741 MNS13668 MNS13887 MNS13970 MNS13473 MNS13804 |
| Mes 23 | FCNU1092        | Hubei, China    | MNS13722 MNS13649 MNS13866 MNS13949 × | MNS13785 |
| Mes 23 | FCNU1093        | Zhejiang, China | MNS13728 MNS13655 MNS13872 MNS13955 MNS13461 MNS13791 |
| Mes 23 | FCNU1094        | Hubei, China    | MNS13723 MNS13650 MNS13867 MNS13950 MNS13456 MNS13786 |
| Mes 24 | HMAS96865       | Beijing, China  | JQ322043 JQ322002 MNS13834 MNS13917 × × N |
| Mes 25 | FCNU1096        | Henan, China    | MNS13730 MNS13657 MNS13874 MNS13957 MNS13463 MNS13793 | 0.0000/0.0025 |
| Mes 25 | FCNU1097        | Sichuan, China  | MNS13691 MNS13618 MNS13832 MNS13915 MNS13426 × |
| Mes 26 | FCNU1098        | Shandong, China | MNS13742 MNS13669 MNS13888 MNS13971 MNS13474 MNS13805 | 0.0000/0.0000 |
| Mes 26 | FCNU1099        | Shandong, China | MNS13670 MNS13743 MNS13889 MNS13972 × | MNS13806 |
| Mes 27 | FCNU1101        | Zhejiang, China | MNS13720 MNS13647 MNS13864 MNS13947 MNS13454 × | 0.0000/0.0000 |
| Mes 27 | FCNU1108        | Zhejiang, China | MNS13721 MNS13648 MNS13865 MNS13948 MNS13455 MNS13784 |

* a: This sample failed to generate amplicons of this gene
spacer region (ITS) and translation elongation factor 1-α (EF1-a) gene were used to identify these eight-three samples in this study (Table 1). Additionally, 28S–18S ribosomal intergenic spacer region (IGS) and partial sequences of histone acetyltransferase ELP3 (F1) with assumed functions independent of sexual reproduction in *Morchella* were selected for comparative purposes. IGS and F1 are referred to as non-reproductive genes in this paper.

**Single spore isolates and cultural conditions**

Twenty single ascospores were randomly isolated from each species, except for *M. steppicola*, *Mes-24* and *Mes-27*, because samples from the three species were immature. Ascospores were washed, suspended in sterilized water, and 200 μL of a solution adjusted to a concentration of 200–300 ascospores mL⁻¹ was spread on potato dextrose agar (PDA) (Solarbio, China) and incubated at 23–25 °C for 1–2 days. Single germinated ascospores were picked using a dissecting needle under a dissecting microscope (Zeiss 455,094), and transferred to a new PDA petri dish, and incubated at 23–25 °C for 2 weeks. Then, the mycelium were harvested by scraping the surface of PDA using a clean surgical blade and used for DNA extraction.

**DNA extraction**

Samples (mycelium/ascomata) were ground to a fine powder in a 1.5-mL microcentrifuge tube using a Kontes pellet pestle (Kaimu, China). Once pulverized, the samples were suspended in 700 μL CTAB extraction buffer (100 mM Tris–Cl pH 8.4, 1.4 M NaCl, 25 mM EDTA, 2% CTAB), and incubated for 1.5–2.0 h at 65 °C during which time they were gently inverted 3–5 times. After the samples were cooled to room temperature, 700 μL chloroform–isoamyl alcohol (24:1) was added to each tube. The mixture was vortexed briefly, centrifuged at 12,000 g for 10 min, and 500 μL of the upper phase was carefully transferred to a new 1.5-mL microcentrifuge tube. After a second chloroform–isoamyl alcohol (24:1) extraction was performed, the supernatant was transferred to a new 1.5-mL microcentrifuge tube and an equal volume of 100% isopropanol at 20 °C was added to each tube. The tube contents were mixed briefly by inversion to obtain a homogeneous solution and then they were stored overnight at 20 °C to precipitate total genomic DNA. After the tubes were warmed to room temperature, they were centrifuged at 12,000 g for 10 min and the supernatant was discarded. The DNA pellet was washed consecutively with 70 and 100% ethanol, air-dried and resuspended in 100 μL of sterile double-distilled H₂O. All genomic DNA samples were stored frozen at –20 °C.

**PCR amplification and DNA sequencing**

The primer sequences of *MAT1–1-1, MAT1–2-1*, ITS, *EF1-a*, IGS and F1 genes are reported in Table 2 and used for the following PCR and DNA sequencing. PCR was carried out on an ABI 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) using the following program: pre-denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s and elongation at 72 °C for 30 s. Afterwards, a final elongation at 72 °C for 8 min was performed. PCR products were sent to Kunming Shuoqing Biotech Ltd. to carry out DNA sequencing. Raw sequence data of *MAT1–1-1, MAT1–2-1*, ITS, *EF1-a*, IGS and F1 were edited and aligned with SeqMan implemented in Lasergene v7.1 (DNASTAR Inc., USA) and automatically aligned with MAFFT v6.853 using the E-INS-i strategy (Katoh et al. 2002). Aligned sequences were visually inspected and manually adjusted using BioEdit v7.0.9. Sequences generated in the present study have been deposited in GenBank under accession numbers MN513618-MN513997 and MN513426-MN513494.

**Identification of species and phylogenetic analysis of all the loci**

Considering the difficulty of accurately distinguishing all the morel species according to morphology, species identification of all the samples used in this study was previously performed based on molecular phylogenetic analysis of the ITS rDNA and EF1-a datasets, which were previously used for species identification in the genus *Morchella* (O’Donnell et al. 2011, Du et al. 2012). For the concatenated and two individual matrices (ITS-EF1-a, MAT1–1-1 and MAT1–2-1), the taxon *M. steppicola* was used as the outgroup in this study as inferred by O’Donnell et al. (2011) and Du et al. (2012). Because of our inability to sequence locus F1 and IGS for *M. steppicola*, *M. americana* was used as the outgroup in the F1 datasets as inferred by O’Donnell et al. (2011) and Du et al. (2012); whereas mid-rooted trees were generated for the IGS datasets as no suitable taxon could serve as the outgroup. All eighty-three samples from twenty-two species successfully generated amplicons from ITS, *EF1a, MAT1–1-1* and *MAT1–2-1*. However, some samples failed to generate amplicons of IGS and F1 and were shown in Table 1.

Using these generated sequences, phylogenetic analyses were performed on the following datasets: (i) for the combined ITS and *EF1-a* dataset, 83 sequences, 22 species; (ii) for the *MAT1–1-1* dataset, 83 sequences, 22 species; (iii) for the *MAT1–2-1* dataset, 83 sequences, 22 species; (iv) for the IGS dataset, 68 sequences, 20 species; and (v) for the F1 dataset, 69 sequences, 20 species. The combined nucleotide sequences of ITS and *EF1-a* were used to construct the species tree for all samples in Table 1.
Phylogenetic analyses using a Maximum parsimony (MP) analysis and Maximum Likelihood (ML) analysis were subsequently conducted on PAUP V 4.0b10 (Swofford 2002) and RAxML v7.2.6 (Stamatakis 2006), respectively. For the concatenated two-gene matrices, single-gene analyses were conducted to assess incongruence among individual genes using the MP method (results not shown). Because no well-supported (BS > 70%) conflict was detected among the topologies of the two genes, their sequences were then concatenated together for further analyses. For the two-gene datasets, a partitioned model, with the model (GTR + I + G) for both of genes, was used by defining the sequences of ITS and EF1-a as two partitions. For the MP analysis, heuristic searches were conducted with tree bisection–reconnection branch swapping algorithm (TBR) after exclusion of uninformative characters, random sequence additions, and with Multitrees option on. Bootstrap values (BP) of the most parsimonious trees were obtained from 1000 replications. Gaps were defined as a fifth character in all analyses. For the ML analyses, all of the parameters were kept at their default settings, except that the model was set as GTRGAMMA1 (Stamatakis 2006) which was applied for the concatenated and four individual matrices (ITS-EF1-a, MAT1–1-1, MAT1–2-1, F1 and IGS), while statistical support was obtained using nonparametric bootstrapping with 1000 replicates. Trees generated by the two analyses were viewed and exported in FigTree v. 1.4.2 (Rambaut 2009).

### RESULTS

**Identification of MAT1–1-1 and MAT1–2-1 genes in the genome of yellow morels**

Through the BLAST analysis of the alpha-box- and HMG-containing sequences of _M. importuna_ (KY508074 and KY508167) and _M. sextelata_ (KY508145 and KY508228) belonging to the Elata Clade of _Morchella_, we revealed one MAT1–1-1 and one MAT1–2-1 genes in _Morchella_ sp. _Mes-21_ genome. This was confirmed by BLAST analysis in Genbank and was best matching with _M. importuna_ (APF29253 and APF29258), _Tuber indicum_ (AHE80940, AHE80941) and _Penicillium kewense_ (CBY44653), respectively. The primer pairs EMAT11L/EMAT11R and EMAT12L/EMAT12R were designed on the most conserved regions of the alpha-box domains of MAT1–1-1 and HMG-box of MAT1–2-1 genes, and then were used to amplify orthologs of the MAT1–1-1 and MAT1–2-1 genes from the eighty-three samples of twenty-two species.

### Table 2 PCR and sequencing primers used in this study

| Locus   | Primer          | References       | Sequence (5’-3’)a | Tm   |
|---------|-----------------|------------------|------------------|------|
| MAT1–1-1 | EMAT1–1L        | This study       | TGAGTCCGTTATGATTCTGG | 50°C |
|         | EMAT1–1R        | This study       | GGACCATTCGCTTTCTCATA  | 50°C |
| MAT1–2-1 | EMAT1–2L        | This study       | GATATGCTCACAACCCTGTA  | 50°C |
|         | EMAT1–2R        | This study       | TACGATCGAATAATGGCTCC  | 50°C |
| EF-1a   | EF-595F         | Kauerud and Schumacher (2001) | CTTACTCCTGCAATCGTAGT  | 49°C |
|         | EF-1R           | Du et al. (2012) | GGARGAAYCATCTTGACGA  | 50°C |
| ITS     | ITS4            | White et al. (1990) | TCTCCGCTTTAGTATGC  | 50°C |
|         | ITS5            | White et al. (1990) | GGAAGTAAAAGTCGTAACAAGG  | 50°C |
| F1      | F1F             | Du et al. (2016) | GGCTAAGATACGAGCTACGAGA  | 49°C |
|         | F1R             | Du et al. (2016) | ACATCAATGAGAGCCATTGA  | 50°C |
| IGS     | IGSYL           | This study       | CTTACTCCTGAATCGTAGT  | 49°C/50°C |
|         | IGSYR           | This study       | TGGTACCCTGCCTCATC     | 50°C |

**Ratio assessment of mating types and karyological analysis**

The ratios of both mating types were assessed for intra- and interspecies single-spore isolates. The mating-type ratios within and among species were compared. Ascospores were stained with DAPI (4’-6-diamidino-2-phenilindole) to visualize nuclei. Pools of purified ascospores were incubated for 15 min in the staining reagent (4 mM DAPI, 100 mM Tris-HCl pH 7.5, and 20% glycerol) directly on the microscope slide. Ascospore nuclei were observed under fluorescent microscopy and laser scanning microscopy with an Olympus Fluoview FV1000 laser scanning microscope.

**Mating-type detection and screening for single ascospore culture**

Considering that the sequence lengths of MAT1–1-1 and MAT1–2-1 are 708 bp and 869–880 bp, respectively, mating-type gene detection of the 380 single ascospores could be performed by observing amplicon length over an ultraviolet transilluminator after electrophoresis for first screening. This method greatly reduced the sequencing cost and accelerated mating-type detection.
Genetic diversity of MAT idiomorphs

The most conserved regions of MAT1–1-1 and MAT1–2-1 were successfully amplified from the twenty-two species. MAT1–1-1 was 708 bp and contained two exons and one intron while MAT1–2-1 varied between 869 and 880 bp and included three exons and four introns. As the basal taxa of the Esculenta Clade, the length of MAT1–1-1 of M. steppicola was similar to the ones of other species in Esculenta Clade without deletion or insertion; however, the length of MAT1–2-1 for this species was much longer than the other species since it contained an insertion of fourteen bases. The nucleotide sequences alignment of MAT1–1-1 included a total of 654 sites after trimming and contained 53 parsimony-informative sites and 67 singleton variable sites. Its estimated nucleotide diversity (pi) was 0.0177 with 120/654 (18.35%) variable nucleotide sites. The sequences alignment of MAT1–2-1 included a total of 827 sites after trimming and contained 74 parsimony-informative sites and 84 singleton variable sites. It had an estimated nucleotide diversity (pi) of 0.0237 with 158/827 (19.10%) variable sites. Therefore, the two mating types occurred equally among ascospores of a single ascomata and the reproductive modes of nineteen species were assumed to be heterothallic.

The results of fluorescence microscopy suggest that ascospores of these species were multinuclear. The number of nuclei in each ascospores roughly divided these species into three groups (Fig. 1 and Table 3): (1) having two, four, six, eight, or more than ten nuclei, including Mes-6, Mes-9, Mes-19, Mes-20, Mes-22, Mes-23, M. esculenta, M. dunensis, and M. yangii; (2) having more than ten nuclei, including Mes-10, Mes-15, Mes-21, Mes-25, Mes-26, M. americana, M. palazonnii, M. yishuica, and M. clinovela; and (3) having more than twenty nuclei, including M. galilaea.

Notably, when observed under the laser scanning confocal microscopy, not only multi-nuclei, but also many mitochondrial nucleoids were observed in ascospores of each species (Fig. 2). Due to the many mitochondria nucleoids in ascospores, we could not further count their nuclei under the laser scanning confocal microscopy. The results from both the fluorescence microscopy and the laser scanning confocal microscopy suggest that ascospores in these species were haploid homokaryotic multinuclear that harbored many mitochondria nucleoids.

Genealogies from reproductive gene (MAT1–1-1 and MAT1–2-1) datasets and species phylogeny

A species phylogeny framework for the twenty-two species (Fig. 3) was constructed using the ITS and EF1-a combined dataset with 2019 bp using the maximum likelihood and parsimony phylogenetic method which was also used for inferring the genealogies of both MAT1–1-1 and MAT1–2-1 datasets, respectively with 654 bp and 828 bp (Figs. 4 and 5). The phylogenetic trees of MAT1–1-1 and MAT1–2-1 were used to represent the reproductive genes phylogeny of these species (Figs. 4 and 5). Both the MP and ML trees of MAT1–1-1 and MAT1–2-1 had the similar topological phylogenies, but their backbones were weakly supported. The species trees supported that each of the twenty-two species formed a monophyletic group; however, the phylogenetic trees for MAT1–1-1 and MAT1–2-1 displayed several topological conflicts with the species tree and between each other (Figs. 3, 4 and 5).

First, the topological positions of the species differed between the MAT1–1-1 genealogy and the species tree (Figs. 3 and 4). In the species tree, M. clinovela, M. yishuica, M. yangii and Mes-20, respectively formed well-supported monophyletic groups, in contrast to the MAT1–1-1 genealogy, in which samples from these
species did not form monophyletic groups. Samples from Mes-22 and Mes-23 could be well divided into two monophyletic groups with high support (ML and MP bootstrap > 80%) in the species tree where the relationship between them was indicated to be very close; however, the MAT1–1-1 genealogy supported samples from Mes-22 and Mes-23 belonging to one species with 100% ML and MP bootstrap value.

Second, the topological positions of the species differed between the MAT1–2-1 genealogy and the species tree. In the species tree, M. dunensis, Mes-6 and Mes-21 respectively formed well-supported monophyletic groups (ML and MP bootstrap > 85%) and were closely related sister groups; however, in the MAT1–2-1 genealogy, all samples from these three species formed a well-supported monophyletic group (ML and MP bootstrap = 98%). In the species tree, Mes-9 and Mes-20 could each form a well-supported monophyletic group, in contrast to the MAT1–2-1 genealogy, in which samples from two species did not form monophyletic groups. Samples from Mes-22 and Mes-23 could be well divided into two monophyletic groups with high support (ML and MP bootstrap > 80%) in the species tree where the relationship between them was indicated to be very close.

Table 3 Approximate numbers of nuclei in ascospores of each species

| Numbers of nuclei in ascospores | N = 2/4 /6 /8 /> 10 | N > 10 | N > 20 |
|---------------------------------|----------------------|-------|-------|
| Species                         | Mes-6, Mes-9, Mes-19, Mes-20, Mes-22, Mes-23, M. dunensis, M. esculenta, M. yangii | M. american, M. clivicola, M. palazonii, M. yishuica | M. galilaeae | M. palazonii | M. yangii | M. yishuica |
bootstrap > 80%) in the species tree; however, the MAT1–2-1 genealogy clustered most of the samples from both Mes-22 and Mes-23 into one monophyletic group (ML and MP bootstrap = 100%), except for FCNU1085, which was grouped together with M. dunensis, Mes-6 and Mes-21 and presumed to be a hybrid individual.

Third, the placement of these species also differed between the MAT1–1-1 and MAT1–2-1 genealogy (Figs. 4 and 5). Samples from Mes-9 and Mes-20 did not form monophyletic groups in either the MAT1–1-1 genealogy or the MAT1–2-1 genealogy, both of which is in conflict with the species phylogeny, while they showed different topological positions between each other as well. Samples from Mes-10 had no variation on MAT1–2-1 sequences while they highly varied on MAT1–1-1 sequences. The same phenomenon was found in M. palazonii and M. dunensis. Samples from Mes-25 had no variation on MAT1–1-1 sequences while they highly varied on MAT1–2-1 sequences. The same phenomenon was found in M. americana and Mes-6.

**Genealogies from non-reproductive (F1 and IGS) datasets and species phylogeny**

The primers of F1 and IGS developed in this study did not result in an amplification product for fourteen and fifteen of the samples used in the present study, respectively, although we redesigned new primers and extracted DNA several times to amplify these samples, we always failed. The final aligned lengths of F1 and IGS datasets used for generating phylogenetic trees were, respectively, 755 bp and 909 bp. The MP and ML trees of F1 and IGS datasets had different topological structures. Though the same samples were always clustered together in both MP and
ML trees of F1, the placements of branches always changed and their backbones were weakly supported. For the MP and ML trees of IGS, it’s the similar case as the ones of F1. Conflicts were found among the genealogies of F1 (Fig. 6) and IGS (Fig. 7) and the species phylogeny (Fig. 3).

In the F1 genealogy, samples from *M. steppicola* and *Mes-24* failed to generate amplicons, and only one sample from each of the *Mes-10*, *Mes-26*, and *M. palazonii* obtained F1 amplicons, so when conflicts between the F1 phylogeny and the species phylogeny were considered, these five species were excluded. Samples from *M. americana*, *M. clivicola*, *M. yishuica*, *Mes-15*, and *Mes-27* each formed a monophyletic group in the F1 genealogy as well as in the species phylogeny, although the placements of the five species differed between the F1 genealogy (Fig. 6) and the species tree (Fig. 3). Samples of *Mes-6* did not form a monophyletic group in the F1 genealogy and the sample FCNU1035 from this species formed a monophyletic group together with FCNU1097 of *Mes-25* with high support (ML and MP bootstrap > 95%). This indicated that some hybridization events might have happened between *Mes-6* and *Mes-25*, and that FCNU1035 might be a hybrid. Samples from *M. esculenta*, *Mes-9* and *Mes-20* did not form monophyletic groups in the F1 genealogy. Samples of *M. galilaea* also did not form a monophyletic group as well as in the F1 genealogy, whereas one sample of this species, FCNU1064, from this species formed a monophyletic group as well as in the F1 genealogy, whereas one sample of this species, FCNU1064, from this species formed a monophyletic group as well as in the F1 genealogy, whereas one sample of this species, FCNU1064, from this species formed a monophyletic group as well as in the F1 genealogy, whereas one sample of this species, FCNU1064, from this species formed a monophyletic group as well as in the F1 genealogy, whereas one sample of this species, FCNU1064, from this species formed a monophyletic group as well as in the F1 genealogy, whereas one sample of this species, FCNU1064, from this species formed a monophyletic group as well as in the F1 genealogy, whereas one sample of this species, FCNU1064, from this species formed a monophyletic group as well as in the
Mes-22 and Mes-23 formed a monophyletic group in the F1 genealogy. Samples from M. steppicola and Mes-24 failed to generate IGS amplicons and only one of the samples of M. americana, Mes-10, Mes-25, and Mes-27 had amplicons from IGS, thus, these five species were excluded. Samples from M. clivicola, M. yishuiica and Mes-15 each formed a monophyletic group in the IGS genealogy as well as in the species phylogeny. Samples from M. esculenta, M. palazonii, M. dunensis, M. yangii, Mes-6, Mes-9, Mes-19, Mes-20, Mes-21, Mes-22, Mes-23 and Mes-26 did not form monophyletic groups in the IGS genealogy. One sample from M. dunensis, one sample from M. esculenta, two samples from Mes-20 and five samples from Mes-23 formed a monophyletic group with high support (ML and MP bootstrap > 95%). Three different IGS sequences were obtained from the four samples of Mes-21 (FCNU1080, FCNU1081, FCNU1083, and FCNU1084), and the IGS sequence of FCNU1080 and those of the three samples of M. yangii were clustered into a monophyletic group together. Three different IGS sequences from three samples of M. esculenta (FCNU1038, FCNU1039 and FCNU1041) and two from two samples of Mes-26 (FCNU1098 and FCNU1099) were obtained. The IGS sequences of FCNU1088 and FCNU1089 of Mes-22 and that of FCNU1099 of Mes-26 were grouped into a monophyletic group with high support (ML and MP bootstrap > 95%), suggesting that some hybridization events might have happened between Mes-22 and Mes-26.

Genealogical conflicts among MAT1–1-1, MAT1–2-1, F1, IGS, and species phylogeny

Conflicts were identified among the MAT1–1-1, MAT1–2-1, F1, IGS, and species phylogenies. The percentages of species

![Fig. 4](image-url) The phylogenetic tree was inferred from RaxML and MP analysis based on 83 MAT1–1-1 sequences. Bootstrap values over 70% are shown at the nodes of the tree.
in MAT1–1-1, MAT1–2-1, F1, and IGS genealogies which were in conflict with species phylogeny were 30, 35, 66.7 and 81.3%, respectively (Table 4). The IGS genealogy had the most conflicts with species phylogeny, followed by F1, MAT1–2-1, and MAT1–1-1. Herein, the conflicts mainly referred to samples forming a monophyletic group in species phylogeny but not in the four other genealogies. It is interesting that M. yishuica and Mes-15 did not have conflicts among the four genes genealogies and with the species phylogeny. For M. americana and Mes-27, except IGS with only one sample having amplicons in both species, no conflicts were found in the MAT1–1-1, MAT1–2-1 and F1 genealogies. Among the four genes, the success rate of amplification in IGS was the lowest.

The phylogenies of reproductive genes (MAT1–1-1 and MAT1–2-1) showed more resolution in these yellow morel species than the non-reproductive genes (F1 and IGS) (Figs. 4, 5, 6 and 7). In the MAT1–1-1 and MAT1–2-1 genealogies, fourteen and fifteen well-supported branches consistent with species phylogeny were formed, respectively (Figs. 4 and 5). In the F1 and IGS genealogies, only five or three well-supported branches and consistent with the species phylogeny were formed, respectively (Figs. 4 and 5).

**TAXONOMY**
Not applicable.

**DISCUSSION**
The availability of the genome sequence of Mes-21 created the opportunity to unveil MAT loci and mating strategies of the Esculenta Clade in the genus *Morchella*. 
In this study, primers were developed to amplify and sequence \textit{MAT1–1-1} and \textit{MAT1–2-1} genes from twenty-two species of the Esculenta Clade. Phylogenies resulting from these \textit{MAT} sequences were compared with those resulting from the analyses of \textit{F1} (Du et al. 2016), IGS and a species phylogeny framework of \textit{Morchella} and revealed multiple conflicts among them.

Comparison of \textit{MAT} loci provides insights into the evolution of the genus \textit{Morchella}

In the Elata Clade, the length of \textit{MAT1–1-1} (729–736 bp) is longer than \textit{MAT1–2-1} (398–408 bp) (Du et al. 2017). In the Esculenta Clade, the current results indicate that \textit{MAT1–1-1} (651–654 bp) is shorter than \textit{MAT1–2-1} (813–827 bp). This difference indicated that the mating-type genes in the Elata and Esculenta Clades appear to have a differential evolutionary history. Analyses of the \textit{MAT} sequence showed higher levels of nucleotide diversity ($\pi$) in both \textit{MAT1–1-1} (0.0178) and \textit{MAT1–2-1} (0.0236) within these species than intraspecies nucleotide diversity ($\pi$). This indicated that \textit{MAT} genes evolved rapidly among morel species. Strong purifying selection against deleterious mutations were suggested in \textit{MAT} genes (Turgeon 1998; Heitman 2015), which probably resulted in the low intraspecific polymorphism observed in \textit{MAT} genes of morels. Additionally, \textit{MAT1–2-1} was found to be more variable than \textit{MAT1–1-1} among the twenty-two species, which is consistent with the results in the Elata Clade (Du et al. 2017). Du et al. (2017) suggested that different sexual competences of \textit{MAT1–1-1} and \textit{MAT1–2-1} might exist in black morels which deserved to be investigated in yellow morels.

Dominant heterothallic reproductive modes in the genus \textit{Morchella}

The \textit{MAT1–1-1} and \textit{MAT1–2-1} genes are scarcely conserved through different classes of fungi which is the
main obstacle to identify sexual genes and decipher their reproductive modes in morels. Recently, the sequence of the *M. eximia* genome allowed the first set of MAT genes, namely *MAT1–1-1* and *MAT1–2-1*, to be mined from fourteen black morel species in the genus *Morchella* and proved that the reproductive modes of these species are mainly heterothallic. However, at that time, *MAT1–1-1* and *MAT1–2-1* genes were not obtained from yellow morel species after multiple attempts due to the conservation of MAT genes (Du et al. 2017). To this end, based on the genome of *Mes–21*, we employed PCR primers designed for the most conserved domains, namely the alpha and HMG domains, of *Mes–21* MAT genes, to amplify the MAT genes from twenty-two yellow morel species. Although no single ascospores were obtained from *M. steppicola*, *Mes–24*, and *Mes–27*, these three species are suggested to be heterothallic based on the fact that all species in the genus *Morchella* investigated thus far are heterothallic and that both MAT genes were obtained from their strains.

Heterothallic reproductive modes of the twenty-two species of the Esculenta Clade were supported by the evidence that amplicons specific to both MAT genes were evenly obtained by PCR from he single spores collected. Combined with the reported heterothallic reproductive modes of fourteen black morel species in the Elata Clade (Du et al. 2017), species in the genus *Morchella* might mainly be heterothallic. The prevalence of heterothallism as mating strategy in fungi is an important feature with implications on the genetic variability and evolutionary potential of a species (Lopes et al. 2018).

Similar to the results reported in black morel species (Du et al. 2017), ascospores of yellow morel species were shown to be haploid homokaryotic multinuclear by DAPI-staining methods under both the fluorescence microscopy and laser scanning confocal microscopy. According to the number of nuclei in the ascospores, these species could be divided into three kinds. *Morchella galilaeae*, the widest distributed species of the Esculenta Clade had the most nuclei in ascospores among...
these species. It is possible that the number of ascospore nuclei might be positively correlated with the distribution range of the species, as the number of nuclei in ascospores might contribute to their vitality and spreading ability resulting in their wide distribution. However, more samples of *M. galilaea* and other morel species should be collected for further study and comparison.

In addition to nuclei, many mitochondria nucleoids were observed in ascospores of these yellow morel species by laser scanning confocal microscopy, similar to what was observed in other fungi by DAPI-staining (Oakley and Rinehart 1985; Chen and Butow 2005; Kucej and Butow 2007). The presence of many mitochondria nucleoids in black morels ascospores was previously shown. In fungi, mitochondrial–nuclear interactions are involved in the control of aging processes and the age-related changes in the mitochondrial DNA are proven to be part of the process leading to organismal degenerations (Osiewacz and Kimpel 1999; Osiewacz 2002). Whether the number of mitochondria nucleoids in morel ascospores would be related to morel strain degradation is not known yet and should be studied in the future.

Conflicts among the genealogies of four targeted genes and species phylogeny

In this study, we assessed the capability of gene trees from reproductive genes (*MAT1–1–1* and *MAT1–2–1*) and non-reproductive genes (*F1* and *IGS*) to resolve species relationships by comparing genes trees with a species phylogeny framework of the twenty-two species inferred from ITS and *EF1-a* combined datasets which were derived from multi-locus analyses and were previously used in studies of the genus *Morchella* (Taşkun et al. 2010; O’Donnell et al., 2011; Du et al. 2012, 2019b; Loizides et al. 2016; Baroni et al. 2018). One of the findings in this study is the topologies for the reproductive gene trees (mating-type) and non-reproductive genes (*F1* and *IGS*) were in conflict with the species phylogeny framework of the Esculenta Clade. Conflicts between the gene tree genealogies and the species phylogeny could be signatures of evolutionary processes such as hybridization, introgression, gene transfer and incomplete lineage sorting (Degnan and Rosenberg 2009).

1. Conflicts between reproductive genes and species trees

Individual *MAT1–1–1* and *MAT1–2–1* gene genealogies resolved most of the species proving their potential usefulness as phylogenetic markers for species delimitation in the Esculenta Clade (Figs. 4 and 5). Although *MAT1–2–1* has previously been considered to be a better phylogenetic marker than *MAT1–1–1* (Martin et al. 2010), here we found both MAT genes to be equally efficient in resolving species in the Esculenta Clade, with six species conflicting with the species phylogeny in the *MAT1–1–1* genealogy and seven species in the *MAT1–2–1* genealogy.

Taylor et al. (2000) proposed that conflicts among gene trees are possibly the result of recombination among individuals and that the transition from concordance to conflict determines the limits of the species. This is the principle of phylogenetic species recognition widely which is applied to fungi and specifically to *Morchella*. O’Donnell (2000) suggested that gene genealogy concordance is well suitable to identify hybrid individuals because hybrids should be grouped with different species in different single-gene genealogies. Several studies revealed that phylogenies of mating-type genes are in general consistent with those of other genes, such as ITS, *EF1-a*, *Beta-tubulin* and *RPB2* (Turgeon 1998; Waalwijk et al. 2002; O’Donnell et al. 2004; Inderbitzin et al. 2005; Yokoyama et al. 2006; Duong et al. 2013; Du et al. 2017; Lopes et al. 2017, 2018), while other studies

![Table 4 Phylogenetic conflicts present in each species according to these gene genealogies](image)

| Species     | Species phylogeny | MAT1–1–1 | MAT1–2–1 | F1 | IGS |
|-------------|-------------------|----------|----------|----|-----|
| *M. americana* | ×                  | ×        | ×        | ×  | O   |
| *M. clivicola*  | ×                  | ×        | ×        | ×  |     |
| *M. dunensis*   | ×                  | ×        | ×        |    |     |
| *M. esculenta*  | ×                  | ×        | ×        |    |     |
| *M. galilaea*   | ×                  | ×        | ×        |    |     |
| *M. palazonii*  | ×                  | ×        | ×        |    |     |
| *M. steppicola* | O                  | O        | O        | N  | N   |
| *M. yangii*     | ×                  | ×        | ×        |    |     |
| *M. yishuica*   | ×                  | ×        | ×        |    |     |
| *Mes–6*        | ×                  | ×        | ×        |    |     |
| *Mes–9*        | ×                  | ×        | ×        |    |     |
| *Mes–10*       | ×                  | ×        | ×        |    |     |
| *Mes–14*       | ×                  | ×        | ×        |    |     |
| *Mes–20*       | ×                  | ×        | ×        |    |     |
| *Mes–21*       | ×                  | ×        | ×        |    |     |
| *Mes–22*       | ×                  | ×        | ×        |    |     |
| *Mes–23*       | ×                  | ×        | ×        |    |     |
| *Mes–24*       | ×                  | ×        | ×        |    |     |
| *Mes–25*       | ×                  | ×        | ×        |    |     |
| *Mes–26*       | ×                  | ×        | ×        |    |     |
| *Mes–27*       | ×                  | ×        | ×        |    |     |

**Total conflicts** / 30% 35% 66.7% 81.3%

| &nbsp; | &nbsp; | &nbsp; | &nbsp; | &nbsp; | &nbsp; |
|---|---|---|---|---|---|
| &nbsp; | &nbsp; | &nbsp; | &nbsp; | &nbsp; | &nbsp; |
| &nbsp; | &nbsp; | &nbsp; | &nbsp; | &nbsp; | &nbsp; |
| &nbsp; | &nbsp; | &nbsp; | &nbsp; | &nbsp; | &nbsp; |
| &nbsp; | &nbsp; | &nbsp; | &nbsp; | &nbsp; | &nbsp; |
| &nbsp; | &nbsp; | &nbsp; | &nbsp; | &nbsp; | &nbsp; |

* No conflicts were detected in this species according to the current genealogy
* Only one sample of this species obtained amplicons from this gene
* No sample of this species obtained amplicons from this gene

* ITS, *EF1-a*, *Beta-tubulin* and *RPB2* (Turgeon 1998; Waalwijk et al. 2002; O’Donnell et al. 2004; Inderbitzin et al. 2005; Yokoyama et al. 2006; Duong et al. 2013; Du et al. 2017; Lopes et al. 2017, 2018), while other studies...
found they are conflicting (Wik et al. 2008; Strandberg et al. 2010).

Conflicts among genealogies of \textit{MAT1}–1–1 and \textit{MAT1}–2–1 and the species phylogeny indicated that some recombination or hybridization events probably occurred between \textit{Mes}-20 and \textit{Mes}-9. As sister species with a similar distribution area, \textit{Mes}-20 and \textit{Mes}-9 have the geographical advantage and potential incomplete reproductive isolation for hybridization to occur. The fact that both \textit{MAT1}–1–1 and \textit{MAT1}–2–1 genealogies showed the same conflicts with the species tree for \textit{Mes}-20 and \textit{Mes}-9 is an important finding, indicating that the two mating types likely introgressed from a single ancestral source rather than independently from different ancestors. This pattern suggested a non-random process of acquisition of the MAT alleles in the evolutionary history of the two species.

\textit{MAT} genes are divergent and known to evolve quickly (Turgeon 1998). However, the \textit{MAT1}–1–1 and \textit{MAT1}–2–1 sequences of \textit{Mes}-22 and \textit{Mes}-23 samples had no differences between the two lineages, suggesting that \textit{Mes}-22 and \textit{Mes}-23 retained the ancestral character of interbreeding due to recent divergence. Thus, in this case, by having identical \textit{MAT} gene sequences as a proxy for mating, they would in fact represent distinct phylogenetic species. Additionally, there was the possibility that incomplete lineage sorting caused the current patterns of \textit{Mes}-22 and \textit{Mes}-23 in the \textit{MAT1}–1–1 and \textit{MAT1}–2–1 genealogies. In the genealogy of \textit{MAT1}–2–1, \textit{Mes}-6, \textit{Mes}-21 and \textit{M. dunensis} formed a monophyletic group with almost identical \textit{MAT1}–2–1 gene sequences. Having identical \textit{MAT} gene sequences as a proxy for mating was suggested to be the ancestral character of interbreeding. \textit{Morchella clivicola}, \textit{M. palazonii}, and \textit{M. yangii} did not form monophyletic groups in the genealogy of \textit{MAT1}–1–1. In addition, either of the \textit{MAT} genealogies, such as \textit{MAT1}–2–1 for \textit{Mes}-6, \textit{Mes}-21, and \textit{M. dunensis}, and \textit{MAT1}–1–1 for \textit{M. clivicola}, \textit{M. palazonii}, and \textit{M. yangii} had conflicts with the species tree indicating that the two mating types independently evolved and one introgressed from different ancestors. This pattern suggested a random process of acquisition of the \textit{MAT} alleles in the evolutionary history of these six species.

Unlike the phylogenies of heterothallic \textit{MAT} loci shown in the Elata Clade (Du et al. 2016), the phylogeny of \textit{MAT} loci in the Esculenta Clade presented here contained a few well-supported deviations from the species tree. This could be taken as evidence that lateral gene-flow existed in Esculenta Clade. Furthermore, the existence of conflicts between the phylogenies of \textit{MAT1}–1–1 and \textit{MAT1}–2–1 provided insights into the evolution of the Esculenta Clade and indicated that both loci evolved independently.

2. Conflicts between non-reproductive genes (\textit{F1} and IGS) and the species phylogeny reveal potential hybridization

The conflicts between the genealogies of \textit{MAT} genes and species phylogenies called for a further analysis of species relationship and the evolution of mating-type loci in \textit{Morchella}. One way to improve the resolution of the species tree is to increase the number of loci used in the study. The \textit{F1} fragment was firstly used in the Elata Clade of the genus \textit{Morchella} for population studies and some potential hybridization or gene transfer events were revealed in several species (Du et al. 2016). The phylogeny of intergenic spacer region (IGS) was reported to conflict with the phylogeny of \textit{EF1-a} in the genus \textit{Fusarium} (Mbofung et al. 2006; Silva et al. 2014). By comparing the reproductive (\textit{MAT1}–1–1 and \textit{MAT1}–2–1) and the non-reproductive (\textit{F1} and IGS) datasets, we tested whether the conflicts are inherent only to the reproductive genes or they also exist in other parts of the genome in species of the Esculenta Clade. According to the above results, the phylogenies of both \textit{F1} and IGS were shown to conflict with each other and with the species phylogeny (Figs. 6 and 7).

In \textit{F1}, only five species were in consistent with the species phylogeny. Strong conflicts were detected between the genealogy of \textit{F1} and the species phylogeny in the Esculenta Clade, which were more serious than those reported in the Elata Clade (Du et al. 2016). Three samples (FCNU1062, FCNU1063, and FCNU0164) from \textit{M. galilaea} generated three kinds of \textit{F1} sequences and the phylogenetic position of FCNU1064 inferred from the \textit{F1} phylogeny was clustered with samples of \textit{Mes}-19. This indicated that some hybridization events might have happened between \textit{Mes}-19 and \textit{M. galilaea}. Hybridization processes in nature occur between pairs of taxa within a species complex (Garbelotto and Gonthier 2013). \textit{Morchella galilaea} and \textit{Mes}-19 are related species and both are widely distributed in China, which gives them the chance to meet in sympatry or allopatry. It is interesting to note that interspecific mating and hybridization has been reported for fungi such as \textit{Ophiostoma} spp., \textit{Heterobasidion} spp., \textit{Melampsora} spp. and \textit{Microbotryum} spp. (Chase and Ullrich 1990; Brasier et al. 1998; Newcombe et al. 2000; Gonthier et al. 2007; Gladieux et al. 2011). Hybridization occurred through secondary contact following initial divergence in allopatry for some fungi (e.g., \textit{Microbotryum}) (Gladieux et al. 2011). The sample FCNU1035 from \textit{Mes}-6 had the same \textit{F1} sequence as the sample FCNU 1097 from \textit{Mes}-25 and they were grouped into a monophyletic group. Thus, we assume that horizontal gene transfer or hybridization events might have happened between \textit{Mes}-6 and \textit{Mes}-25.

In IGS, only three species were in consistent with the species phylogeny. A high degree of conflicts were shown between the genealogy of IGS and the species phylogeny. According to the species phylogeny, different kinds of IGS sequences were obtained from samples
belonging to the same species. Frequent potential hybridization or horizontal gene transfer events of IGS were suggested to exist in the Esculenta Clade. When they detected conflicts between phylogenies of IGS and EF1-α in the genus Fusarium, Mbofung et al. (2006) proposed that unequal rates of evolution between loci and incomplete concerted evolution within loci could be among the factors responsible for the observed discrepancies. Most likely, these factors also contributed to the conflicts shown here.

With the increasing availability of molecular data, phylogenetic trees generated from different genes are often recognized to have conflicting branching patterns (Maddison 1997; Nichols 2001; Pamilo and Nei 1988). Davin et al. (2018) suggested that different genetic markers could yield conflicting estimates of the species phylogeny. In recent years, genome-scale sequence data have become increasingly available in phylogenetic studies for understanding the evolutionary histories of species (Liu et al. 2015). Phylogenetic analyses of genomic data revealed that different genes within a genome can have different evolutionary histories, i.e. phylogenetic conflicts (Spatafora et al. 2017; Shen et al. 2017). However, research on the sources of these conflicts, including incomplete lineage sorting, hybridization and horizontal gene transfer, as well as on the detection and characterization of these conflicts according to phylogenetic inference, is still in the early stage (Mirarab et al. 2014; Spatafora et al. 2017).

We presumed that the conflicts among the phylogenies of F1, IGS, and the species phylogeny were likely due to gene transfer and hybridization. Gene transfer is often suggested as an explanation for incongruencies between gene trees and species trees; however, although it is widely assumed to be more common in prokaryotes, it’s considered to be a common phenomenon in eukaryotes (Dujon 2005; Syvanen 1994; Gogarten 2003; Galtier and Daubin 2008). Hybridization is now widely recognized as an important evolutionary process which might play a crucial role in speciation (Gross and Rieseberg 2005; Mallet 2007; Schumer et al. 2014; Taylor and Larson 2019). Hybridization followed by reproductive isolation has been reported to contribute to rapid speciation of yeast (Leducq et al. 2016) and the same has been supposed to occur in some filamentous fungi (Kohn 2005; Gladieux et al. 2014; Sillo et al. 2019). Du et al. (2012) reported the 85.2% of the species in the Esculenta Clade (including almost 90% of the Chinese species lineages) diversified and went through rapid speciation in East Asia since the middle Miocene, which might have contributed to the potential hybridization and gene transfer events detected in the Esculenta Clade. Of note, we could not exclude the possibility that conflicts arising from gene trees and species phylogeny might be due to weak phylogenetic signal for F1 and IGS.

CONCLUSIONS

The results from our study showed that heterothallism is the reproductive mode in the Esculenta Clade and in the genus Morchella. The primers designed for MAT loci in the Esculenta Clade supplemented the primers previously developed for the Elata Clade (Du et al. 2017). MAT genes of the Elata Clade had similar topologies to the species phylogeny in the study of Du et al. (2017). However, the present study indicated that MAT genes of the Esculenta Clade were in conflict with species tree. Divergent evolutionary patterns were suggested for MAT genes between the Esculenta and the Elata Clades. Complex evolutionary trajectories of MAT1–1–1, MAT1–2–1, F1 and IGS in the Esculenta Clade were highlighted. Our findings contribute to a better understanding of the importance of hybridization and gene transfer in Morchella and especially for shaping reproductive modes during its evolutionary process. The genus Morchella will be useful for studying the complexities and evolution of mating types and genomes in the Ascomycota.

Abbreviations

EF1-α: Translation elongation factor 1-α; F1: Partial sequences of histone acetyltransferase ELP3.; IGS: 28S–18S ribosomal intergenic spacer region.; ITS: Nuc-rDNA internal transcribed spacer region.; MAT: mating type.

Acknowledgments

The authors thank Zhu L. Yang and Hong Luo (Kunming Institute of Botany, Chinese Academy of Science), Jianping Xu (Department of Biology, McMaster University), Kerry O’Donnell (Bacterial Foodborne Pathogens and Mycology Research Unit, National Center for Agricultural Utilization), Hanma Zhang and Bo Li (Chongqing Normal University), Franck Richard and Jean-Michel Bellanger (Centre d’Écologie Fonctionnelle et Évolytive, CNRS), for kind help during the study. Yu Liu and Jianrui Wang (Ludong University), Xinsheng He (Southwest University of Science and Technology), Hao Hu, Bei Xiao, Xue-Feng Jia (Biotechnology Research Institute, Xinxang Agricultural Reclamation Academy of Sciences), and many other kind people are acknowledged for their help during collecting samples and the authors’ field trip. Nan Xu, Chao Zhou, Dan Kuang and Lu Liu (Chongqing Normal University) are acknowledged for excellent technical assistance. Many thanks also are due to the anonymous reviewers for constructive comments and suggestions.

Adherence to national and international regulations

Not applicable.

Authors’ contributions

Xi-Hui Du designed the study. Xi-Hui Du, Dongmei Wu, Heng Kang, Hanchen Wang, Nan Xu, Tingting Li and Keliang Chen collected the samples and conducted the laboratory work. Xi-Hui Du analyzed the data and wrote the manuscript. All authors reviewed the manuscript. All authors read and approved the final manuscript.

Funding

This work was supported by Scientific and Technological Research Program of Chongqing Municipal Education Commission (No. KJ1703052), Chongqing basic science and frontier research project (No. cstc2017jcyjAX1079) and Chongqing Natural Science Foundation (No. cstc2018jcyjA3693). The funders had no role in study design, interpretation of data or the preparation of the present manuscript.

Availability of data and materials

Sequences generated in the present study have been deposited in GenBank under accession numbers MN513618-MN513997 and MN513426-MN513494. The full alignments of these datasets were submitted to the TreeBASE (25132).
The genome data of Mes-21 generated in this project has been deposited at DDBJ/ENA/GenBank and the accession WSNQ00000000. The version described in this paper is version WSNQ00000000.

Ethics approval and consent to participate
This work conforms with all regulations pertaining to ethics approval and the consent to participate. In general, this is not applicable to our study, as there were no human subjects subject to research.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
1College of Life Sciences, Chongqing Normal University, Chongqing 401331, China. 2Biotechnology Research Institute, Xinjiang Academy of Agricultural Reclamation of Sciences, Shihezi 832000, China. 3Institute of Applied Mycology, Huazhong Agricultural University, Wuhan 430070, Hubei, China.

Received: 10 July 2019 Accepted: 6 January 2020
Published online: 12 February 2020

References
Baroni TJ, Breug MW, Cantrell SA, Clements TA, Iturria T et al (2018) Four new species of *Morchella* from the Americas. Mycologia 110(6):1205–1221
Brasier CM, Kirk SA, Pipe ND, Buck KW (1998) Rare interspecific hybrids in natural populations of the Dutch elm disease pathogens *Ophiostoma ulmi* and *O. novo-ulmi*. Mycological Research 102:45–57
Burt A (2000) Perspective: sex, recombination, and the efficacy of selection. Annual Review of Phytopathology 51:39–59
Gioti A, Muhsegian AA, Strandberg R, Stajich JE, Johannesson H (2012) Unidirectional evolutionary transitions in fungal mating systems and the role of transposable elements. Molecular Biology and Evolution 29:325–336
Gladeux P, Vercken E, Fontaine MC, Hood ME, Jonot O et al (2011) Maintenance of fungal pathogen species that are specialized to different hosts: allopatric divergence and introgression through secondary contact. Molecular Biology and Evolution 28:459–471
Gogarten JP (2003) Gene transfer: gene swapping craze reaches eukaryotes. Current Biology 13:53–54
Gonthier P, Niccotti G, Linzer R, Guglielmo F, Garbelotto M (2007) Invasion of European pine stands by a north American forest pathogen and its hybridization with a native interfertile taxon. Molecular Ecology 16:1389–1400
Greenevald M, Barnes I, Bradshaw RE, Brown AV, Dlae A et al (2007) Characterization and distribution of mating type genes in the *Dothistroma* needle blight pathogens. Mycology 97(7):825–834
Greenevald M, Greenevald JZ, Harrington TC, Abelin ECA, Crous PW (2006) Mating type gene analysis in apparently asexual *Cercospora* species is suggestive of cryptic sex. Fungal Genetics and Biology 43:813e825
Gross BL, Reeseberg LH (2005) The ecological genetics of homoploid hybrid speciation. Journal of Heredity 96:241–252
Heitman J (2015) Evolution of sexual reproduction: a view from the fungal kingdom supports an evolutionary epoch with sex before sexes. Fungal Biology Reviews 30:108–117
Indenbitzen P, Harkness J, Turgeon BG, Berbee ML (2005) Lateral transfer of mating system in *Stemphylium*. Proceedings of the National Academy of Sciences of the United States of America 102:1390–1395
Kato K, Misawa K, Kuma K, Miyata T (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Research 30:3959–3966
Kauerud H, Schumacher T (2001) Outcrossing or inbreeding: DNA markers provide evidence for type of reproductive mode in *Phellinus nigrolineatus* (*Basidiomycota*). Mycological Research 105:676–683
Kohn LM (2005) Mechanisms of fungal speciation. Ann Rev Phytopathology 43:279–308
Kronstad JW, Staben C (1997) Mating types in filamentous fungi. Annual Review of Genetics 31:245–276
Kuczy M, Butow RA (2007) Evolutionary tinkering with mitochondrial nucleoids. Trends in Cell Biology 17:586–592
Kuo M, Dewsbury DR, O’Donnell K, Carter MC, Rehner SA et al (2012) Taxonomic revision of true morels (*Morchella*) in Canada and the United States. Mycologia 104:1159–1177
Leducq JB, Nielly-Thibault L, Charron G, Eberlein C, Verta JP et al (2016) Speciation driven by hybridization and chromosomal plasticity in a wild yeast. Nature Microbiology 1:503
Lee SC, Ni M, Li W, Shertz C, Heitman J (2010) The evolution of sex: a perspective from the fungal kingdom. Microbiology and Molecular Biology Reviews 74:329–340
Liu L, Wu SY, Yu LL (2015) Coalescent methods for estimating species trees from phylogenomic data. Journal of Systematics and Evolution 53(5): 380–390
Loizides M, Bellanger JM, Clopez P, Richard F, Moreau PA (2016) Combined phylogenetic and morphological studies of true morels (*Pezizes*, *Ascomycota*) in Cyprus reveal significant diversity, including *Morchella arbutiphilus* and *M. dispersus* spp. nov. mycological Progress 15:39
Lopes A, Linaldeed BU, Phillips AJ, Alves A (2018) Mating type gene analyses in the genus *Diplodia* from cryptic sex to cryptic species. Fungal Biology 122: 629–638
Lopes A, Phillips AJL, Alves A (2017) Mating type genes in the genus *Norfusisocoma*: mating strategies and usefulness in species delimitation. Fungal Biology 121:394e404
Maddison WP (1997) Gene trees in species trees. Systematic Biology 46:523e536
Mallet J (2007) Hybrid speciation. Nature 446:279
Martin SH, Steenkamp ET, Wingfield MJ, Wingfield BD (2013) Mate-recognition and species boundaries in the ascomycetes. Fungal Diversity 58:1e12
