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ORIGINAL PAPER

Interspecific hybridization between cultivated morels *Morchella importuna* and *Morchella sextelata* by PEG-induced double inactivated protoplast fusion

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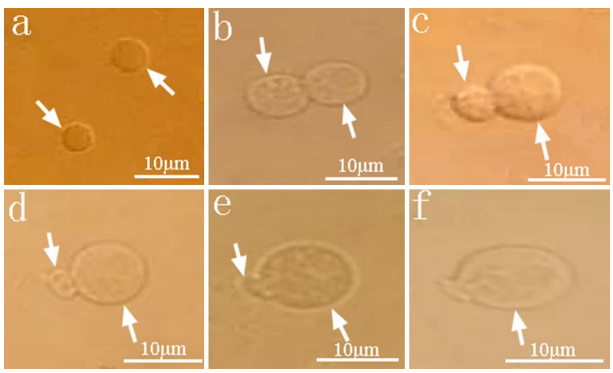
Abstract

The commercial production of *Morchella* mushrooms calls for urgent breeding of excellent varieties or strains with appropriate tools, such as protoplast fusion. However, the protoplast fusion in morels has not been studied. In this paper, interspecific hybridization between cultivated morels of *M. importuna* and *M. sextelata* by PEG-induced protoplast fusion was conducted. Apart from functional complementation of double inactivated protoplasts, the fusants were characterized by cultural and cultivated characters and molecular markers of random amplified polymorphic DNA (RAPD). The results suggested that the hybrids and their parents showed significant difference in their inoculum recovery time, mycelial growth rate, yield of cultivation and total amino acid content of ascocarps. Moreover, positive barrage reactions were observed between parental strains as well as between each parent and a hybrid line. A dendrogram created on the basis of RAPD fingerprints exhibited three major clusters, in which morel hybrids showed intra-cluster variations, *M. sextelata* #6 formed an out group, while *M. importuna* #4 was phylogenetically closer to morel hybrids. All the results demonstrated that real fusants were obtained in our study. Protoplast fusion may provide an ideal alternative for new strain selection, and thus will promote the healthy development of morel industry.

Graphic Abstract



Keywords Characterization · *Morchella importuna* · *Morchella sextelata* · Protoplast fusion · RAPD markers

Abbreviations

ANOVA	Analysis of variance
CYM	Complete yeast extracts medium
GB	Guo Biao (Chinese National Standard)
PEG	Polyethylene glycol
RAPD	Random amplified polymorphic DNA
RCYM	CYM supplemented with 0.6 M mannitol
SD	Standard deviation
UPGMA	Unweighted pair-group method with mathematic averages

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Introduction

True morels (*Morchella* spp., Pezizales, Ascomycota) are edible and medicinal mushrooms, highly appreciated for their commercial value, profound bioactivities, culinary qualities and good taste (Liu et al. 2018a, b, c; Tietel and Masaphy 2018). The outdoor cultivation of morel mushrooms has been rapidly developed in China (Liu et al. 2018a, b, c). In production season of 2018–2019, the total cultivation area exceeded 9000 ha in more than 20 provinces, municipalities and autonomous regions of China. The cultivated morels are only confined to several black species, including the landscape species *M. importuna* (Kuo et al. 2012) and two fire-associated species, *M. sextelata* (Kuo et al. 2012) and *M. exmia* (Richard et al. 2015). Some strains of *M. importuna*, e.g. #2, #4 and #8, and *M. sextelata*, e.g. #6 and #13, were widely cultivated under field condition and accounted for about 95% of the total production (He et al. 2015; He et al. 2017; He et al. 2018a; Liu et al. 2017). Despite the rapid commercial cultivation of *Morchella* mushrooms, "uncertainty" has been being the prominent problem of morel outdoor cultivation. Statistics showed that the cultivation of about 70% farmers (mostly dispersible cultivation with field area less than 0.5 ha) is of poor profitability every year with average yield less than 1500 kg fresh morels per ha. The yields may be more for experienced farmers with reliable spawn sources. Occasionally, the production may exceed 6000 kg per ha in small-scale cultivation. However, the good harvest was difficult to achieve the repeatability. The key to uncertainty was lack of reliable breeding technology and thus scarcity of excellent cultivated varieties or strains. Moreover, as ascomycetous mushrooms, morels are liable to strain aging and degeneration in comparison to Basidiomycetes, which places greater demand on strain breeding (He et al. 2015). In China, almost all the commercial cultivated strains of *Morchella* are domesticated from naturally grown morels (Liu et al. 2017). The wide popularization of commercial morel cultivation calls for urgent breeding programs to develop excellent varieties or strains.

Protoplast fusion is efficient at overcoming the natural barriers of vegetative incompatibility to establish heterokaryosis in fungi (Kim et al. 1997; Yoo et al. 2002). Protoplast fusion can be performed intraspecifically (Gupta and Gautam 1995; Linardi et al. 1993; Zhao and Chang 1995), interspecifically (Eyini et al. 2006; Kirimura et al. 1997; Selvakumar et al. 2015; Singh et al. 2007; Zhao and Chang 1997), intergenerically (Chakraborty and Sikdar 2008, 2010; Zhao and Chang 1996) and interhetero generically (He et al. 2018a, b; Kim et al. 1997; Okamura et al. 2000; Zhao et al. 2009), and fusions can be induced by polyethylene glycol (PEG) or electric field and electric pulse. Protoplast fusion is potentially important to develop fungal hybrids with enhanced potential for the production of antibiotics, enzymes, useful metabolites, and high productivity, especially when conventional methods cannot achieve these results (Eyini et al. 2006; Selvakumar et al. 2015; Singh et al. 2007). In addition, protoplast fusion has been mainly conducted in many Basidiomycetes, while seldom in Ascomycetes (Gupta and Gautam 1995; Kirimura et al. 1997; Linardi et al. 1993). Although protoplast preparation and regeneration were well studied, the technique of protoplast fusion has not been engaged in *Morchella* spp. (Chen et al. 2003, 2007; Cui and Luo 2003; Liu et al. 2008; Moriguchi and Kotegawa 1985; Zhang et al. 1989).

Clamp connection has been commonly used to characterize the somatic hybrids and their recombinant progenies in Basidiomycetes (Eyini et al. 2006; Dhitaphichit and Pornsuriya 2005; Djajanegara and Masduki 2010; Selvakumar et al. 2015; Yoo and Lee 1994; Zhao and Chang 1995). However, the morphological marker is absent in Ascomycetes. Besides, the induced markers like auxotrophic and drug resistant mutants were often unstable and generally undesirable for breeding because physical or chemical mutagens always cause some non-specific mutation (Eyini et al. 2006; Peberdy 1989; Yoo et al. 1987; Zhao and Chang 1995). Consequently, the nongenetic method based on inactivation of protoplasts before fusion and reactivation in the fusion products by their fusion partner which is either not inactivated or inactivated using a different treatment, proved to be a good alternative of selective methods (Chakraborty and Sikdar 2008; He et al. 2018a, b; Menczel 1984; Zhao and Chang 1995, 1997). In addition, random amplified polymorphic DNA (RAPD) is a useful tool for identification of somatic hybrids following protoplast fusion (Chakraborty and Sikdar 2008, 2010; Yoo et al. 2002; Zhao and Chang 1996, 1997; Zhao et al. 2009).

In this paper, interspecific hybridization between cultivated *M. importuna* and *M. sextelata* was conducted by PEG-induced double inactivated protoplast fusion. Morphologic and genetic characterization of the somatic hybrids and their production potentials were determined. The results demonstrated that protoplast fusion protocol can give an alternative method for development of new hybrids of *Morchella* mushrooms.

Materials and methods

Strains and culture media

The parental strains, *M. importuna* #4 and *M. sextelata* #6, were commercially grown morels domesticated from wild populations in Sichuan Province, China (Liu et al. 2017).

They are available from Peixin He in Zhengzhou University of Light Industry and Wei Liu in Huazhong Agricultural University, China. The stocks were maintained on Complete Yeast extracts Medium (CYM) (glucose 20 g/L, yeast extracts 2 g/L, peptone 2 g/L, K_2HPO_4 1 g/L, $MgSO_4$ 0.5 g/L, KH_2PO_4 0.46 g/L and agar 20 g/L) and preserved at 4 °C. Before protoplast isolation, the strains were cultured in liquid CYM medium under stationary condition for 3 days at 25 ± 1 °C. CYM supplemented with 0.6 M mannitol (RCYM) was used for protoplast reversion and fusants selection.

Protoplast preparation and reversion

The fresh young mycelia cultured in liquid CYM medium were washed three times with sterile distilled water and 0.6 M mannitol, respectively. For protoplast isolation, the combination of 2.0% (w/v) Lywallzyme (Guangdong Academy of Agricultural Science) and 1.0% of snailase (Beijing Solarbio Science & Technology Co., Ltd.) in 0.6 M mannitol was used as lysing enzymes. The filter sterilized enzymatic solution was added to harvested fresh mycelia in the ratio of 1:5 (w/v). The mixtures were incubated at 30 °C with gently shaking (50 rev/min) for 3 h. The protoplasts were purified by filtration with cotton syringe (Yan et al. 2004; Zhao and Chang 1993), washed with 0.6 M mannitol solution and recovered by centrifugation. The final protoplast pellet was suspended in 200 µl 0.6 M mannitol and the density of protoplasts was determined with a haemocytometer. Protoplast regeneration and reversion was performed by sandwich method (Chang et al. 1985). The regeneration plates were incubated at 25 °C.

Inactivation of protoplasts

The protoplasts of *M. importuna* and *M. sextelata* were inactivated with heating (45 °C, 20 min) and UV irradiation (30 W, 30 cm, 5 min), respectively.

Protoplast fusion

The fusogen for protoplast fusion was 25% (v/v) PEG 4000 (Sigma-Aldrich), 0.05 M $CaCl_2$, pH 8.0. PEG-induced fusion of double inactivated protoplasts was carried out as Zhao and Chang (1995) with some modifications. The incubation temperature of fusion solution was 25 oC and the fused protoplasts were regenerated in RCYM by sandwich method (Chang et al. 1985). The inactivated protoplasts of both parental strains were fused with themselves and used as controls. The fusion frequency was expressed as the number of regenerated colonies on RCYM as a fraction of the number of regenerated protoplasts.

Analysis of fusion hybrids

The presumptive somatic hybrid colonies were picked up cautiously and transferred to fresh CYM slants for further culture and then for future use. For assay of cultural characteristics of parental strains and potential somatic hybrids, mycelial plugs (4 mm diameter) from respective slant culture were placed in the center of CYM plates. The inoculated plates were incubated at 25 °C in dark place for 14 days. The mycelial growth rate was calculated when the mycelia near the side of a Petri dish. The morphological characteristics including sclerotial development were recorded (He et al. 2015). Each culture was repeated three times with inocula from the same slant. The barrage reactions of parental strains and presumptive somatic hybrids were assayed on CYM plates. The plates were incubated at 25 °C for 14 days, after which the macro-morphology of contact zone was observed (Chakraborty and Sikdar 2010).

DNA extraction and RAPD reactions

Genomic DNA of actively growing mycelia of the parental strains and their presumptive hybrids cultures from CYM plates covered with autoclaved cellophane membranes were isolated using Ezup Column Fungi Genomic DNA Purification Kit (Shanghai Sangon Biotech) according to the manufacturer's instructions (He et al. 2018a, b). Genomic DNA was quantitated with NanoDrop8000 spectrophotometer (Thermo Scientific) by measuring the OD at 260 nm and 280 nm. RAPD amplifications were performed as Liu et al. (2018b).

Outdoor planting experiment

The parental and several hybrid strains were artificially cultivated by technology of outdoor cultivation (Liu et al. 2017). The outdoor planting experiments were conducted in production season of 2018–2019 in western suburb of Zhengzhou City, Henan Province, China. The cultivation of each strain was repeated for three times in different plastic canopies. The area of each small plot was about 15 m^2. The whole process of morel artificial cultivation can be roughly divided into six major stages: spawn production, land preparation, spawning, exogenous nutrition supplying, fruiting management and harvesting (Liu et al. 2017). The spawning time was on November 14th, 2018. The dosage of seeding (0.5 kg/m^2) and exogenous nutrition (1.2 kg/m^2) and other management measures were the same. The mature ascocarps were harvested and weighed until the end of production season (before April 20th, 2019).

Determination of morel total amino acid content

After drying at 50 °C, the harvested morels were ground to a particle size of 74 µm. Total amino acid contents of the artificially cultivated ascocarps of the parental and hybrid strains were determined with amino acid analyzer according to the protocol of Chinese National Standard (Guo Biao) GB 5009.124–2016.

Statistical analysis

The data were expressed as mean ± standard deviation (SD). Data were subjected to one-way ANOVA followed by the Duncan's multiple range test ($\alpha = 0.05$). RAPD products were scored as present (1) or absent (0) for each primer genotype combinations. Data generated from 5 elaborately selected decamer primers for 18 mushroom individuals were entered into a binomial matrix. The bivariate 0–1 data were analyzed using the software NTSYS-pc for Windows version 2.10e. Jaccard's proximity matrix was then used to construct a dendrogram to show the genetic relationship between the parents and their presumptive hybrids employing the Unweighted Pair-Group method with Mathematic Averages (UPGMA) algorithm.

Results

Protoplast isolation, protoplast inactivation, protoplast fusion and generation of hybrid lines.

Combination of Lywallzyme and snailase gave high yield of protoplasts both for *M. importuna* #4 and *M. sextelata* #6 (Table 1), which was enough to be used in protoplast fusion. Protoplasts of both morels regenerated in 3–4 days of incubation. The regeneration rate in RCYM with 0.6 M mannitol as osmotic stabilizer was 1.82 ± 0.26% and 1.53 ± 0.31% in *M. importuna* and *M. sextelata*, respectively (Table 1).

The self-fused inactivated protoplasts of both parental strains did not regenerate, which suggested that the inactivation of protoplasts before fusion using a different treatment was a good alternative of selective methods and thus can be used as marker of somatic hybridization. The fusion frequency of double inactivated protoplasts was 0.82 ± 0.06%, which was lower than that of intraspecific hybridization, corresponded with interspecific hybridizations, however, higher than some distant somatic hybridizations of some basidiomycetous mushrooms. In addition, the approach, contact and fusion of morel protoplasts induced by PEG, the cell agglutinator and membrane modifier were observed under the microscope.

Macro-colonies of 16 putative hybrid lines were obtained and analyzed with RAPD markers. Furthermore, the parental strains (designated as P1 for *M. importuna* #4 and P2 for *M. sextelata* #6) and six hybrid lines (designated as R1, R2, R3, R4, R5 and R8) were subjected to further morphological and cultivated characterizations.

Table 1 Protoplast yield and regeneration rate of *Morchella importuna* and *M. sextelata*

Strain	Proptoplast yield (protoplasts/g of young mycelia)	Regeneration rate
M. importuna #4	5.6×10^6	1.82 ± 0.26%
M. sextelata #6	3.3×10^6	1.53 ± 0.31%

Morphological characterizations

The six putative hybrid lines and their parents were characterized for their inoculum recovery time, mycelial growth rate, colony morphology, barrage reaction, and yield of artificial cultivation and total amino acid content of ascocarps. The mycelium grew rapidly on CYM plates with the mycelia covered the plate (9 cm in diameter) in 5–6 days, and then formed sclerotial initials and thereafter secreted yellow to light brown pigments. Colony morphology of the tested strains was similar, however, the number of sclerotia formed on CYM plate of parent strain P1 (average 426 per plate) were more than those of parental strain P2 and hybrid lines (average 101–200 each plate) (Fig. 1 a–e). Moreover, statistically the hybrids and their parents showed significant difference ($P < 0.05$) in their inoculum recovery time, mycelial growth rate, yield of artificial cultivation and total amino acid content of ascocarps (Table 2). The inoculum recovery time of the tested strains was in the range of 11.26 ± 0.25 h and 14.08 ± 0.42 h. Faster recovery after inoculation was observed in P1, R5 and R4, then was R1, R3 and R2, while P2 and R8 recovered slowly (Table 2). The mycelial growth rates of the parental strains and hybrid lines were close, ranged from 0.568 ± 0.026 cm/days (R8) to a maximum of 0.635 ± 0.025 cm/days (R3) (Table 2). Moreover, the fusion products were also distinguished from their parents on the basis of barrage reaction. A distinct characteristic demarcation line indicating somatic incompatibility was observed between parental strains as well as between each parent and a hybrid line (Fig. 1 f–h).

Cultivated characterizations

Outdoor planting experiments were conducted in 2018–2019 (Fig. 1 i). No significant difference was observed in cultivated characteristics of time and quantity of mitospore appearance, time of fruiting, size of ascocarps and so on. However, parental strain P2 and putative hybrid R8 gained the same low yield of 20.00 ± 0.44 g/m^2 in fresh weight, while the yield of the parental strain P1 and other

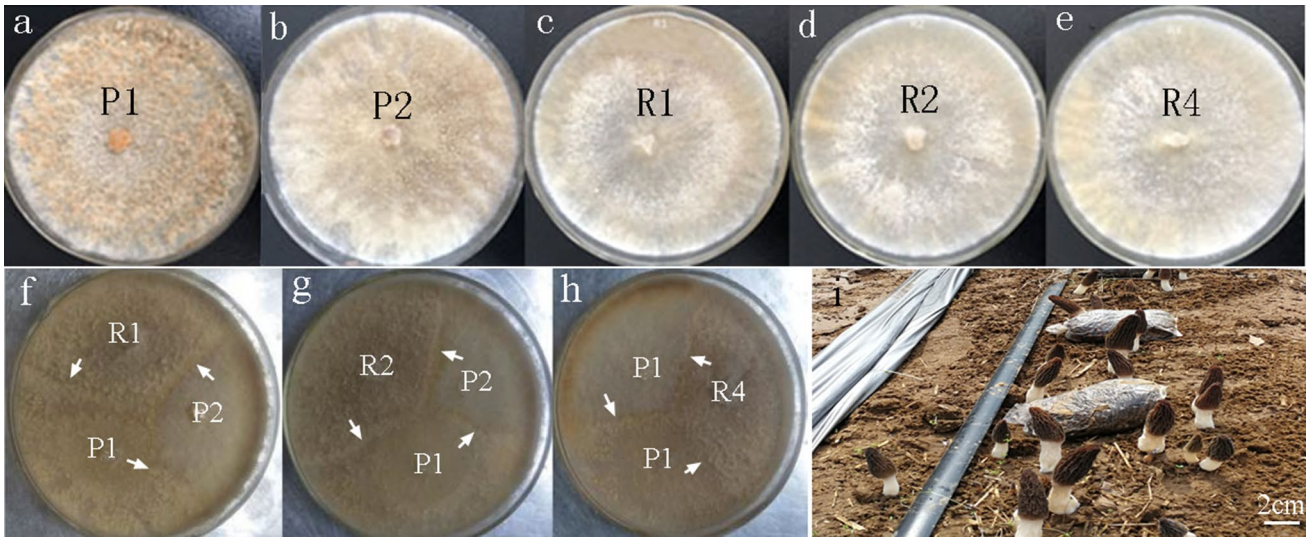

Fig. 1 Morphological and cultivated characterizations of the hybrid lines. Colony morphology of parental strains of P1 (**a**), P2 (**b**) and hybrid lines of R1 (**c**), R2 (**d**) and R4 (**e**) on CYM plates cultured at 25 °C for 10 days. Barrage reactions (arrows) between hybrid strains of R1 (**f**), R2 (**g**) and R4 (**h**) and their parental strains of P1 and P2 after incubation at 25 °C for 14 days. Fructification of hybrid strain R1 in artificial cultivation (**i**)

Table 2 Cultural and cultivated characteristics of the somatic hybrids and their parents

Strains	Inoculum recovery time (h)	Mycelial growth rate (cm/d)	Yield (g/m^2) in fresh weight	Total amino acid content of ascocarps (%)
P1	11.26 ± 0.25a	0.596 ± 0.014abc	61.13 ± 3.06a	21.83 ± 0.03b
P2	13.82 ± 0.47e	0.582 ± 0.020a	20.00 ± 0.44g	21.59 ± 0.04ab
R1	12.28 ± 0.26bc	0.573 ± 0.017ab	55.20 ± 3.21b	21.29 ± 0.03a
R2	13.04 ± 0.44d	0.584 ± 0.023ab	14.03 ± 0.59h	23.44 ± 0.04d
R3	12.65 ± 0.27cd	0.635 ± 0.025c	27.50 ± 0.62f	23.41 ± 0.05d
R4	11.64 ± 0.18ab	0.617 ± 0.018bc	42.73 ± 1.36c	22.71 ± 0.05c
R5	11.47 ± 0.35a	0.576 ± 0.015ab	37.70 ± 2.55d	22.40 ± 0.04c
R8	14.08 ± 0.42e	0.568 ± 0.026a	20.00 ± 0.44g	21.77 ± 0.03b

All the values are means ± SD of three repeated experiments. Means within a column labeled with the same letter are not significantly different according to Duncan's multiple range tests at a 5% probability level

hybrid lines was significantly different. The highest yield (61.13 ± 3.06 g/m^2 in fresh weight) was observed in parental strain P1, then was R1, R4, R5 and R3, and the lowest yield (14.03 ± 0.59 g/m^2 in fresh weight) was seen in hybrid line of R2 (Table 2). The overall yields of tested strains were lower than those of commercially cultivated strains. For example, the yield of cultivated strain of *M. sextelata* #13 exceeded 300 g/ m^2 in fresh weight at the same site under the same managements. The reason may be strain ageing occurred in parental strain (P1 and P2) and their somatic lines during long-time of preservation at 4 °C, while the cultivated strains are preserved at ultralow temperatures (at least − 80 oC) under the protection of protectants, which may effectively shelter from ageing. Moreover, the results demonstrated that no hybrid line with the yield higher than parental strain of P1 (*M. importuna* #4) was obtained. The cause may be the degeneration of parental strain P2 (*M. sextelata* #6). Some properties of strain degeneration e.g. increase of mushroom malformation, decrease of yield and poor stress resistance etc. had been observed in cultivation of *M. sextelata* #6, and thus the strain had been gradually replaced by *M. sextelata* #13 in outdoor cultivation over the past three years. The results also indicated the necessity of selection of parental strains without obvious drawbacks in breeding by protoplast fusion. The total amino acid content of ascocarps of the tested strains was in the range of 21.29 ± 0.03% and 23.44 ± 0.04%. High percentage of total amino acid content was found in ascocarps of R2 and R3, then was R4 and R5, and low figures were observed in ascocarps of P1, P2, R1 and R8 (Table 2).

Although both pitted and ridged, *M. importuna* can be distinguished from *M. sextelata* in morphology of ascomata.

The hymenophore of *M. importuna* shows a laddered appearance created by 12–20 primary vertical ridges and numerous transecting horizontal ridges, while the arrangements of primary vertical ridges and numerous shorter, secondary vertical ridges and sunken transecting horizontal ridges were irregular in *M. sextelata*. Furthermore, the swelling of bottom stipe in *M. sextelata* #6 is obviously in comparison to *M. importuna* #4. The morphology of ascomata of six putative hybrid lines can be divided into three types: parental P1 type (R5), parental P2 types (R1, R2 and R4) and intermediate parental morphology types of R3 with cap similar to P2 while stipe similar to P1, and R8 with cap similar to P1 while stipe similar to P2 (Fig. 2). Two non-parental fusants obtained in this study were not sufficient to infer the occurrence of nuclear fusion. The exact mechanism of occurrence of intermediate parental types needs further study.

Characterization with RAPD markers

Genetic variations among parental strains and 16 morel hybrids were determined using RAPD markers. PCR amplification of genomic DNA from the parents and their hybrid lines were conducted using fiv selected primers. Specific DNA polymorphisms were observed in somatic hybrids (Fig. 3). RAPD-PCR band profiles of the hybrid lines were compared with the parental strains. The results suggested that all or part of the bands of parental strains were amplified

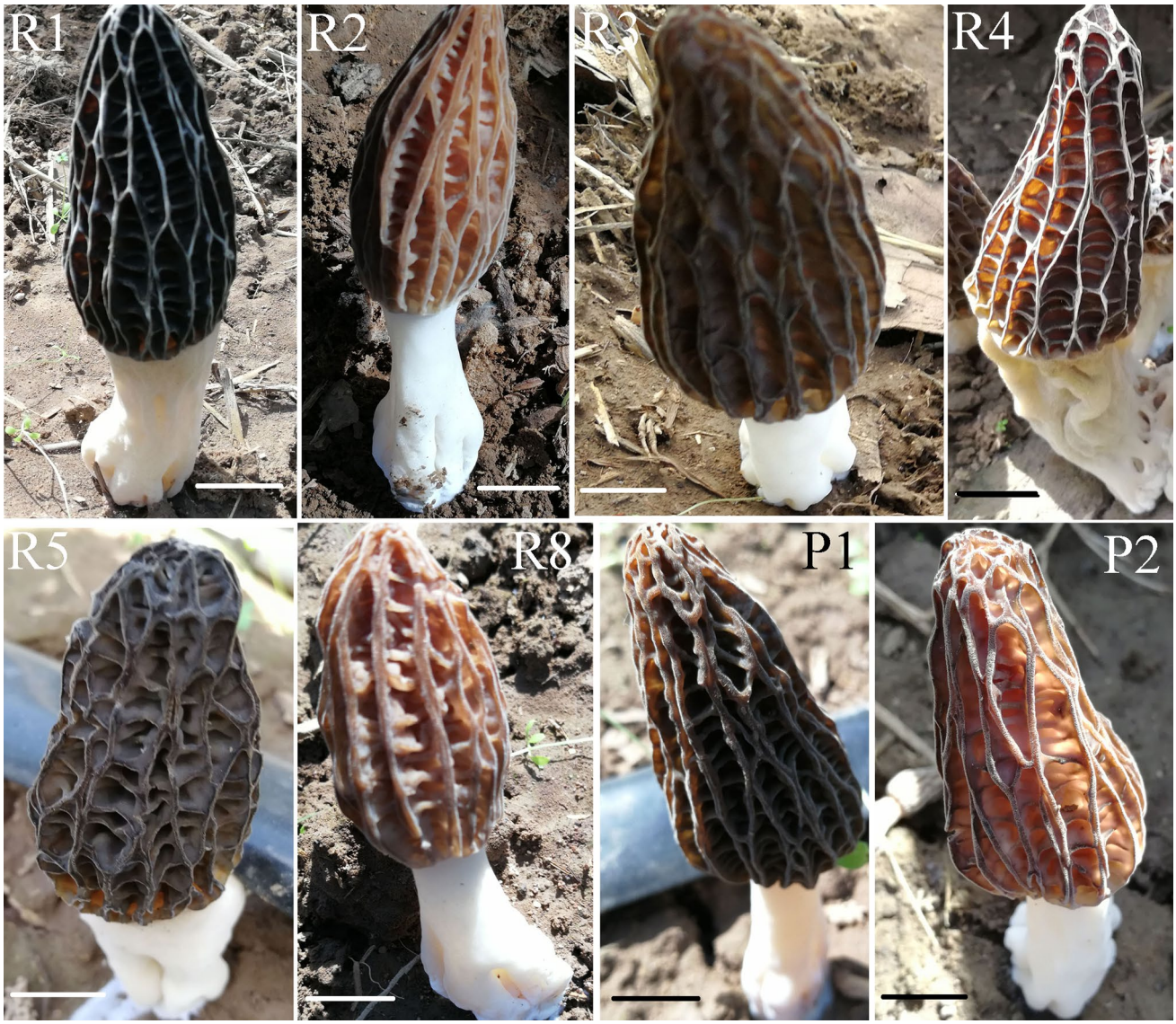

Fig. 2 Ascocarpic morphology of parents (P1 and P2) and hybrid lines (R1, R2, R3, R4, R5 and R8) in artificial cultivation. Size bars correspond to 1 cm

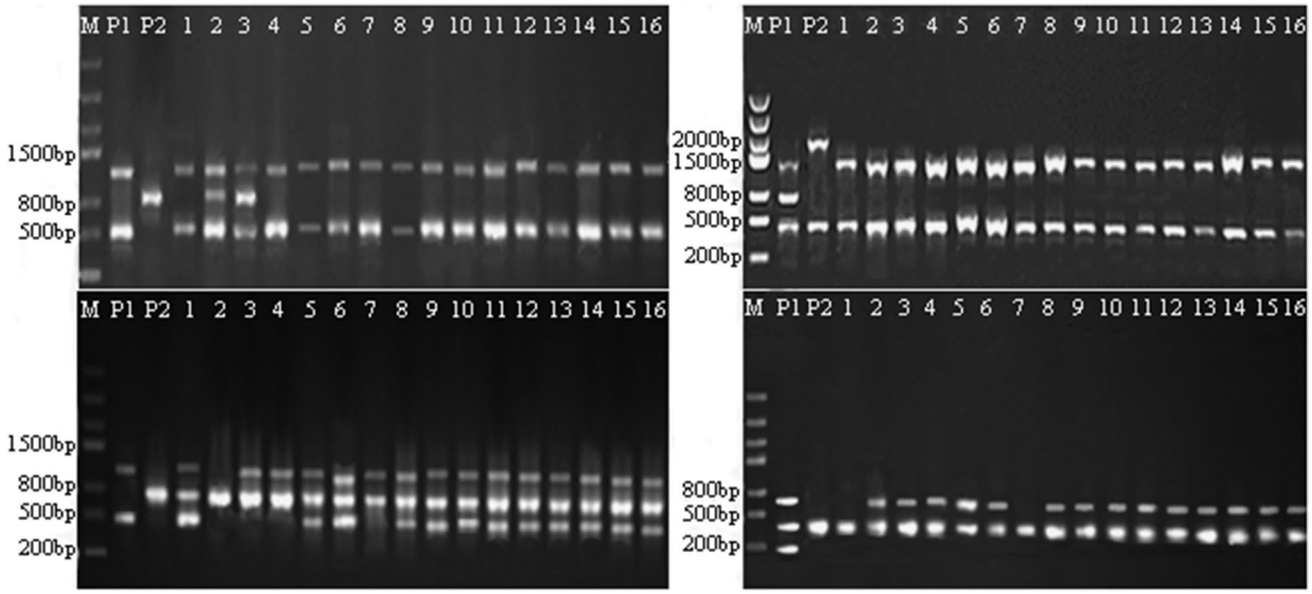

Fig. 3 RAPD profiles of *M. importuna* #4 (P1), *M. sextelata* #6 (P2) and their 16 hybrid lines (1–16). **a** Primer OPA25, **b** Primer OPA27, **c** Primer OPA28, **d** Primer OPA33. M represents DNA marker

Table 3 Sequences of RAPD primers and characters of DNA fingerprinting of the parents and their somatic hybrids

Primer	Sequence (5′-3′)	No. of RAPD bands	Size range (approx. base pairs)	PIC value
OPA03	AGTCAGCCAC	5	300–1000	1.040
OPA25	TGCCCGTCGT	3	500–1000	1.224
OPA27	GACCGACCCA	4	450–2000	1.079
OPA28	ACTGAACGCC	3	500–1000	1.235
OPA33	CCTTGACGCA	3	200–800	1.128

PIC Polymorphism information content

in 16 hybrid lines (Fig. 3). No new band was amplified in hybrid lines. All the five primers amplified easily recognizable and consistent DNA bands ranged from 0.2–2.0 kb in length. Among which, primer OPA03 showed highest polymorphism in hybrids which amplified five marker bands, then was OPA27 (4 bands), followed by OPA25, OPA28 and OPA33 (3 bands) (Table 3). To determine genetic relationships among the parental strains and the somatic hybrids, a dendrogram was constructed from similarity matrix of RAPD amplified fragments employing the UPGMA algorithm. The dendrogram exhibited three major clusters: morel hybrids that showed intra-cluster variations, P1 and P2. *M. sextelata* #6 (P2) seemed to be genetically distant from their hybrid progeny thereby forming an out group, while *M. importuna* 4# (P1) was phylogenetically closer to morel hybrids, also evident from the RAPD profiles. Among the hybrid lines, R2 and R3, R4 and R7, R1 and other fusants shared the closest similarity (Fig. 4).

Results of morphological, cultivated and molecular characterizations suggested that real fusants were obtained in our PEG-induced double inactivated protoplast fusion between two cultivated morels. The hybrid lines may be heterokaryons with each nucleus undergoing many times of subsequent mitosis. Not all bands of the parental strains were amplified in some fusants in the RAPD experiments may attribute to the disturbance of cytoplasmic genetic factors and the relatively poor stability of the technique. Positive barrage reactions may be interpreted as leading-in of new nucleus into hyphal cells of the hybrid lines, possible occurrence of mutation accumulation during iterative mitosis and nuclear-cytoplasmic interaction in fusants.

Discussion

Compound enzymes were used in protoplast isolation of *Morchella* spp.

Our study showed that combination of Lywallzyme and snailase gave high yield of protoplasts both for *M. importuna* and *M. sextelata* (Table 1). Glucans and chitin have been shown to be essential components in cell walls of Basidiomycetes, and thus the enzymatic mixtures of α-and/or β-glucanases and chitinase have been used for their protoplast production (Mendoza 1992). Lywallzyme has been widely applied in protoplast isolation of basidiomycetous mushrooms. The enzyme was the crude preparation of *Trichoderma longibrachiatum* 958–11 under the induction of cell wall of stipe of *Agaricus bisporus*

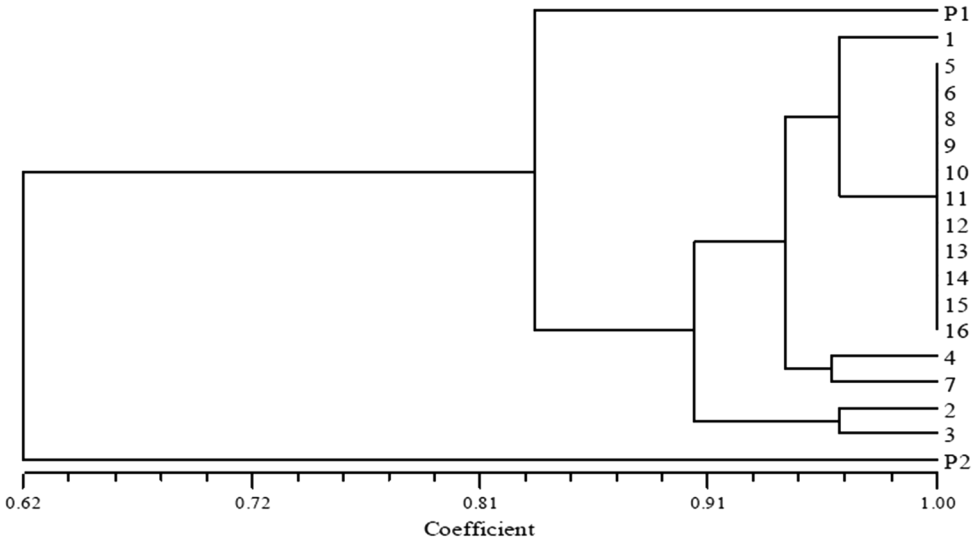

Fig. 4 Dendrogram showing the relationship between morel somatic hybrid lines and their parental strains using UPGMA method of clustering

or *Volvariella volvacea* (Qiu et al. 1982). Unlike Basidiomycetes, protoplasts were difficult to be released from the mycelia of *Morchella* spp. when cells were treated singly with Lywallzyme (Cui and Luo 2003). The components and structure of the cell wall of *Morchella* spp. are different from Basidiomycetes. The main compounds in cell walls of morels were protein (33%), non-nitrogenous polysaccharides (17%) and chitin (16%), with glucose being the major neutral monosaccharide. Moreover, the cell wall of *Morchella* did not contain chitosan and cellulose (Ruíz-Herrera and Osorio 1974). Therefore, compound enzymes should be used in protoplast isolation of *Morchella* spp., e.g. chitinase and zymolyase (Moriguchi and Kotegawa 1985), combination of snailase with lywallzyme (Chen et al. 2007; Liu et al. 2008) and with driselase (Zhang et al. 1989). Additionally, addition of cellulase to the mixture of lysing enzymes in morel protoplast production may be not helpful (Moriguchi and Kotegawa 1985), because there was no cellulose in morel cell wall (Ruíz-Herrera and Osorio 1974), although several reports used cellulase in protoplast isolation of *Morchella* mushrooms (Chen et al. 2007; Liu et al. 2008; Zhang et al. 1989). Snailase (snail enzyme) was extracted from the digestive juice of *Helix pomatia*. It was the mixture of many enzymes, including chitinase, pectinase, amylase, glucanase, mannase, protease, laminaribiase, β-acetylaminodeoxyglucosidase and phosphomonoesterase, and thus has been used in protoplast isolation of yeasts (Anderson and Millband 1966). In this study, the combination of Lywallzyme and snailase were used in protoplast isolation from hyphae of two *Morchella* mushrooms, and resulted in ideal protoplast yields. In addition, the protoplast regeneration rates achieved in this study (Table 1) were lower than some Basidiomycetes (Chakraborty and Sikdar 2008, 2010; Eyini et al. 2006) and ascomycetous *Aspergillus* spp. (Kirimura et al. 1997), while higher than *Pleurotus eryngii* (He et al. 2018a, b), *Lentinula edodes* and *Ganoderma lucidum* (Bok et al. 1994).

Protoplast fusion may reinforce the genetic potential in morels and provide an ideal alternative for morel breeding

The cultivated morels of *M. importuna* and *M. sextelata* are both heterothallic (Chai et al. 2017; Du et al. 2017; Liu et al. 2018a). Thus the normal development of ascocarp needs spermatization between ascogonium and spermatium with different mating type in the subhymenial layer of fruiting body (He et al. 2017; Volk and Leonard 1990). Nevertheless, the occurrence of monosporic fruiting in *Morchella* mushrooms was common (He et al. 2019; Liu et al. 2019), which demonstrated that the nuclei representing opposite mating type were introduced possibly through spermatization during cultivation (Liu et al. 2019). The mitospores (microconidia) were commonly occurred in both wild and cultivated morels, and may function as spermatia (Carris et al., 2015; He et al. 2017; Liu et al. 2016). Meanwhile, the mitospores are direct products of hyphal differentiation, and hence the genetic diversity in morel mycelial system will in turn determine the genetic potential of the microconidia. Morels may employ mechanisms such as hyphal anastomoses and heterokaryogamy to maintain genetic diversity in their mycelial colonies (Pagliaccia et al. 2011; Rayner 1991; Volk and Leonard 1989). The mechanisms may allow colonies to adapt to changing environmental conditions and mask the expression of deleterious mutations (Pilz et al. 2007). Furthermore, the genetic diversity of mitospores shaped by heterokaryogamy along with the ability of outcrossing may be the main causes of high levels of genetic polymorphism among morels (Dolgleish and Jacobson 2005; Gessner et al. 1987; Pagliaccia

et al. 2011), although such vegetative heterokaryons do not occur frequently (He et al. 2019; Volk and Leonard 1989).

Fortunately, heterokaryons can be reliably realized by technology of protoplast fusion, which may reinforce the genetic potential and enhance the genetic diversity in mycelial colonies of *Morchella* spp. Moreover, our study demonstrated the feasibility of PEG-induced double inactivated protoplast fusion in morel breeding. The hybrid lines with ideal characteristics of inoculum recovery, mycelial growth, yield of production and quality of ascocarps were obtained through protoplast fusion. The fusion of elaborately selected parents with respective advantages and without obvious drawbacks may result in excellent hybrid lines. Protoplast fusion may provide an ideal alternative for new strain selection of *Morchella* mushrooms, and thus will promote the healthy development of morel industry.

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