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Tutorial

Live cell confocal laser imaging studies on the nuclear behavior during meiosis and ascosporogenesis in *Morchella importuna* under artificial cultivation

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ABSTRACT

The commercial production of *Morchella* mushrooms, especially *M. importuna*, has been realized since 2012 in China, which facilitates the fundamental studies of *Morchella* spp. In this paper, the cytological characteristics at three stages of ascocarpic development and the nuclear behavior during meiosis and ascosporogenesis in cultivated strain *M. importuna* 1# was visualized by confocal laser scanning microscopy. The results suggested that the strain sporulated at sporulation stage of ascocarpic development. A total of six nuclear divisions typically took place during ascosporogenesis. The first and second divisions were meiotic in which the single diploid nucleus divided into four haploid nuclei. The subsequent mitosis gave rise to eight nuclei, and eight incipient ascospores with one nucleus in each spore were formed after spore delimitation in the clavate ascus. Then, the nucleus in most of the young ascospore underwent three successive mitoses producing 6–8 haploid nuclei in each mature spore, and thus the multinucleate ascospores in each ascus were all homokaryons. To the best of our knowledge, this is the first dynamic tracing study of nuclear behavior during meiosis and ascosporogenesis in cultivated morels, and the spore delimitation time is also the first report. The study will be beneficial for the genetics study and strain breeding of *Morchella* mushrooms.

1. Introduction

True morels (*Morchella*, Order Pezizales) are valuable ascomycetous mushrooms mainly occurring in temperate regions of the Northern Hemisphere and highly prized for their edibility and appearance (Pagliaccia et al., 2011). They have always attracted mycologists due to their commercial value, medicinal properties, culinary qualities and good taste (Ferreira et al., 2009; He et al., 2012; Nitha et al., 2007). Despite obvious commercial applications and the popularity of morels, many aspects of their genetics and biology remain poorly understood, presumably due to the general scarcity of the ascocarps both in nature and in the laboratory. The nuclear behavior during ascosporogenesis in other ascomycetes was extensively studied (Gibson and Kimbrough, 1988; Goh and Hanlin, 1997; Lu, 1967; Raju, 2002; Raju, 2008). However, the cytological study on *Morchella* development was not enough. Maire (1905) described the developmental cytology of the ascus, including the nuclear divisions in the ascus mother cell and the ascus. Greis (1940) traced the path of nuclei from vegetative hyphae into the hymenium of the wild ascocarp of *M. conica*, *M. esculenta*, and *M. elata*. Volk and Leonard (1990) examined the progression of cytological events in the morel life-cycle followed the development of ascoma fruiting in association with tuberous begonias in semi-controlled conditions and the indoor maturation of wild fruiting bodies of *M. esculenta*. These studies provided the static state of nuclear divisions in the ascus and ascospores of wild morels. Furthermore, some detailed events, e.g. the time of sporulation and especially the spore delimitation were ambiguous.

Ower (1982) innovatively achieved the morel cultivation with *M. esculenta*, now thought to be *M. rufobrunnea* (Kuo, 2008) being the first indoor artificially cultivated *Morchella* species. On the basis of Ower's pioneering study (Ower, 1982), the commercial production of *Morchella* mushrooms by technology of field soil cultivation has been realized since 2012 in China. In production seasons from 2016 to2017, the total cultivation area exceeded 1560 hm^2 in more than 15 provinces, municipalities and autonomous regions of China (Liu et al., 2017b). The cultivated species of field soil cultivation were those belong to Elata Clade, including *M. importuna*, a landscape specialist that occurring in gardens, planters, woodchip beds and urban landscaping settings (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.*

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importuna, e.g. $1^{\#}$, $2^{\#}$ and $4^{\#}$, were widely applied and accounted for about 95% of the total production (He et al., 2015; Liu et al., 2017b). The commercial cultivation facilitates the fundamental study of *Morchella* spp.

In this paper, the time of sporulation in morel development was tracked and the nuclear behavior during ascosporogenesis in cultivated strain of *M. importuna* $1^{\#}$ was monitored by confocal laser scanning microscopy. The results suggested that the strain sporulated at sporulation stage of ascocarpic development under artificial cultivation, spore delimitation took place after post-meiotic mitosis, and the multinucleate ascospores in each ascus were all homokaryons. These findings could effectively facilitate the application of single-ascospore strains as materials in fundamental genetic research and strain breeding of cultivated *Morchella* mushrooms.

2. Material and methods

2.1. Sampling

In production seasons from 2015 to 2016, artificial cultivation with *M. importuna* $1^{\#}$ was carried out by technology of field soil cultivation in Sangzhe Town, Miao-Tujia Autonomous County of Pengshui, Chongqing Municipality, China, and Niuzhuang Town, Tujia Autonomous County of Wufeng, Yichang City, Hubei Province, China. A total of 74 samples were collected, including 27 primordia, 34 young ascomata of different sizes after differentiation of pileus and stipe and 13 mature ascocarps characterized by halt of ascocarpic enlargement, lightening of the pileus (especially the ridges), obvious separation of pits with ridges, and tendency for reduction of dry weight (Table 1; Supplementary Fig. S1). The samples could represent the successive ascocarpic development during the stage of initiation, maturation and sporulation (Manachere, 1974). The samples were fixed in 4.0% formaldehyde, and preserved at 4 °C for further use. The cultivated strain of *M. importuna* $1^{\#}$ used in this study is available from Peixin He and Wei Liu in Zhengzhou University of Light Industry, China.

2.2. Frozen section, freehand section and confocal laser scanning microscopy

The fixed samples were rinsed with 0.01 M phosphate buffer solution (pH 7.0) (PBS) and embedded with Epon-812 as embedding agent. Frozen sections of 5–8 μm were prepared by freezing microtome (Thermo CryoStar NX50). In addition, the freehand sections of about 10 μm were also prepared from fixed and rinsed samples, by directly cutting with sharp scalpel. The frozen and freehand sections were stained with 5 μg/mL of DAPI (Sigma-aldrich) solution for DNA staining at ambient temperature for 10–15 mins away from light. After rinsing with PBS three times, the sections were mounted in glycerol on a microscope slide, a cover slip was placed on the mounted material, and then the coverslip was tapped lightly with a rubber eraser to disperse the asci, and then examined with a confocal laser scanning microscope (Carl Zeiss LSM 710, German) using the 40 × water objective (numerical aperture of 1.1) and the 63 × oil immersion (numerical aperture of 1.4). DNA localizations were visualized with an excitation wavelength of two-photon laser 780 nm and with a 400–550 nm filter. To enhance the bright field contrast, differential interference contrast (DIC) was used when observed and recorded under 40 × and 63 × objectives. Three-dimensional (3-D) imaging was realized with a Z-step interval of 1.5 or 2.0 μm. The 3-D images were reconstructed with the original 3-D interactive visualization software of zen 2010, and exported as video files, which could be played by Windows Media Player (Microsoft). Each sample was repetitively prepared and observed for five times and more than five different fields of the nuclear behaviors in each observation were recorded. Moreover, the nuclear numbers of 160 different mature ascospores were counted under the 3-D condition by zen 2010 software package.

3. Results

3.1. The cytological characteristics of different stage of ascocarpic development

Ascospores were not formed at stage of initiation and maturation (Fig. 1a and 1b). In sections of primordia, the densely-packed parallel paraphysal cells derived from branched hyphae were observed on the bottom of the pre-apothecia (pits) (Fig. 1a). In phases of hymenial layers of developing ascocarps, young asci can be occasionally observed interspersed among the tightly ordered sterile paraphyses (Fig. 1b). There were no signs of antheridia or trichogynes. Young asci arose through the swelling of the terminal segments of the basal ascocarp hyphae (Fig. 1b). Large amounts of ascospores were formed at sporulation stage of ascocarpic development (more than 20 days after primordial formation closely related to the environmental conditions such as temperature and humidity) (Table 1). Ascus and ascospores at different phases of ascosporogenesis were observed in sections of pits of mature ascomata (Fig. 1c). The paraphyses were clavate, somewhat apically inflated, straight, septate, branched at base, 6.5–12 × 66–94 μm (Fig. 1a–c) and multinucleated (Fig. 1a'–c'). The young asci were clavate, 13.7–20.2 × 174–284 μm (Fig. 1b), and contained a larger diploid nucleus of early meiosis (see Section 3.2.1)

Table 1
States, sites, sizes and numbers of samples and the environmental conditions of ascocarpic development of *M. importuna* $1^{\#}$ under artificial cultivation.

State (stage)	Time of sampling (2016)	Soil temperature (min./max.,°C)[1]	Air temperature (min./max.,°C)[2]	Soil moisture (%)[3]	Air relative humidity (%)	Site[4]	Size of sample (length × diameter, pileus/stipe, mm)	Num. of samples
Primordia (initiation)	February 27th	8–11/4.5–7	8–20/3–8	25–28	85–95	A	5–8 × 2–3(length × diameter)	15
	March 5th	7–10.5/6–8	11–16/4–10	23–25	85–95	B	5–8 × 2–3(length × diameter)	12
Young ascocarps (maturation)	March 15th	9–13/6–8.5	8–21/3–14	22–26	80–95	A	10–15 × 5–8/6–12 × 4–6	5
						A	15–25 × 8–15/7–13 × 5–8	5
						A	25–45 × 15–20/14–25 × 10–18	7
	March 18th	8.5–11/5–7.5	10–22/4–10	20–22	80–95	B	10–15 × 5–8/6–12 × 4–6	4
						B	15–25 × 8–15/7–13 × 5–8	6
						B	25–45 × 15–20/14–25 × 10–18	7
Matured ascocarps (sporulation)	March 23rd	11–15/7–10	9–23/5–14	20–24	80–90	A	45–90 × 20–45/20–45 × 12–25	7
	March 31st	11–16.5/5.5–9.5	12–25/6–12	18–22	80–90	B	45–90 × 20–45/20–45 × 12–25	6

[1] The minimum and maximum temperature of soil 5–6 cm underground was determined at 6:00 AM and 1:00 PM, respectively.
[2] The minimum and maximum air temperature 1.5 m from the ground was determined at 6:00 AM and 1:00 PM, respectively.
[3] The difference of soil moisture in two bases was depended on their soil sediment concentration.
[4] A: Sangzhe Town, Miao-Tujia Autonomous County of Pengshui, Chongqing Municipality, China; B: Niuzhuang Town, Tujia Autonomous County of Wufeng, Yichang City, Hubei Province, China.