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## Isolation of the MAT1-1 mating type idiomorph and evidence for selfing in the Chinese medicinal fungus *Ophiocordyceps sinensis*

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### ABSTRACT

*Ophiocordyceps sinensis* is one of the most valued medicinal fungi in China. Research on the mating system and sexual development is vitally important to this endangered species. Previous efforts devoted to investigate the mating type (MAT) locus of *O. sinensis*, however, resulted in an incomplete understanding. In this study, the MAT1-1 locus of *O. sinensis* was investigated. The conserved  $\alpha$ -box and HMG-box regions of the MAT1-1-1 and MAT1-1-3 genes, respectively, and a conserved region of the DNA lyase gene were successfully amplified using degenerate PCR. A combination of TAIL-PCR and long-range PCR were used to connect these genes and obtain the sequence of the MAT1-1 locus. Screening of 22 single spore isolates by PCR demonstrated that both the MAT1-1-1 and MAT1-2-1 genes co-occurred within the same isolate. Additionally, both MAT1-1-1 and MAT1-2-1 are expressed in vegetative mycelia, providing evidence that *O. sinensis* is likely capable of selfing. DAPI (4,6-diamidino-2-phenylindole) staining of ascospores and hyphae showed that a majority of hyphal compartments are binucleate, suggesting that *O. sinensis* may be pseudohomothallic. Analyses of sequence diversity showed lower levels of genetic diversity in MAT1-1-1 compared to MAT1-2-1, indicating the possibility that different selective pressures act on the two MAT idiomorphs. The MAT1-1-1 sequences of *O. sinensis* and *Tolypocladium inflatum* cluster as a monophyletic group consistent with phylogenetic classification of Ophiocordycipitaceae. Comparison of the structure of the MAT1-1 locus across hypocrealean taxa showed that *O. sinensis* contains all three mating type genes (MAT1-1-1, MAT1-1-2, and MAT1-1-3) and supported previous observations that of the four families in Hypocreales, MAT1-1-3 has undergone a lineage specific loss only in some members of the Cordycipitaceae.

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## Introduction

*Ophiocordyceps sinensis* (Berk.) G.H. Sung, J.M. Sung, Hywel-Jones, & Spatafora, previously known as *Cordyceps sinensis* (Berk.) Sacc., is an economically and medicinally valuable fungus that parasitizes the Himalayan ghost moth (Hepialidae) to form the combination of insect and fungus known as *Dong Chong Xia Cao* (winter worm, summer grass) in Chinese. This valued herbal medicine has been used in traditional Chinese medicine for centuries (Pegler et al. 1994). Due to a dramatic increase in the market price of this medicinal fungus over the past several decades, *O. sinensis* has been overharvested to the point of becoming an endangered species in China (State Forestry Administration and Ministry of Agriculture 1999) and the long term effects of overharvesting on population viability, genetic variation, and survival of the species remain unknown. Efforts to cultivate the fungus have also been hampered due to the lack of knowledge of the mating biology and conditions required for fruiting. Even mating crosses in the laboratory have not been successful to date. Thus, understanding the mating biology of this important fungus is critical both to conservation of natural populations as well as to development of artificial cultivation systems.

Sexual reproduction in ascomycetous fungi is governed by a single locus called the mating type (MAT1) locus (Turgeon 1998; Turgeon & Yoder 2000). The allelic variants of this locus, MAT1-1 and MAT1-2, are highly divergent and nonhomologous, and are thus termed idiomorphs rather than alleles (Metzenberg & Glass 1990). The MAT1-1 idiomorph contains the MAT1-1-1 protein with a motif called the  $\alpha$ 1-domain, while the MAT1-2 idiomorph is characterized by the MAT1-2-1 gene that encodes a protein with an HMG domain (Turgeon & Yoder 2000). Although sordariomycetous fungi generally contain three genes in the MAT1-1 idiomorph including MAT1-1-1, MAT1-1-2, and MAT1-1-3, other ascomycetes may have one to three of these genes (Turgeon 1998; Turgeon & Yoder 2000). The MAT1-2 idiomorph of ascomycetes generally contains only the single MAT1-2-1 gene (Turgeon & Yoder 2000).

Strains of heterothallic (self-sterile) fungi contain only a single idiomorph and require mating with a strain of the opposite mating type for successful fruiting (Yun et al. 2000). Homothallic (self-fertile) fungi contain both idiomorphs (MAT1-1 and MAT1-2) within the same strain and nucleus. A second type of self-fertile mating system, intermediate between strict heterothallism and homothallism, termed pseudohomothallism, appears to have evolved multiple times in fungal evolution and has been characterized in both ascomycetes (Dodge 1927; Raju & Perkins 1994) and basidiomycetes (Langton & Elliott 1980). In pseudohomothallic species, some ascospores formed during meiosis receive two nuclei of opposite mating type to form stable heterokaryons capable of self-reproduction, while other ascospores receive a single nucleus containing a single mating type and are thus able to outcross (Dodge 1927; Raju & Perkins 1994).

The structure of the mating type locus in most heterothallic ascomycetes is relatively conserved and flanked by a set of conserved genes: SLA2 (cytoskeleton assembly control) and APN1 (DNA lyase) (Ramirez-Prado et al. 2008). The organization of the mating type loci in homothallic fungi is more varied. In

some ascomycetes, such as *Cochliobolus* and *Fusarium*, homothallism is generally thought to have independently evolved multiple times through crossover events from heterothallic ancestors (Yun et al. 1999; O'Donnell et al. 2004). However, in *Aspergillus* spp., some have hypothesized that heterothallic species having evolved from ancestral homothallic ones (Poggeler 2001). It is likely that transitions have occurred multiple times in both directions over the course of fungal evolution (Lee et al. 2010). Thus, the molecular organization of the mating type locus in different homothallic species is often unique (Yun et al. 1999). The two idiomorphs can exist within the same fungal strain either as a single fused MAT1-1/MAT1-2 locus or at separate loci located in dispersed locations within the genome (Yun et al. 1999, 2000; Turgeon & Yoder 2000; Poggeler 2001).

The mating type loci of a wide range of filamentous ascomycetes have been cloned (Arie et al. 1997; Bennett et al. 2003; McGuire et al. 2004; Yokoyama et al. 2005). However, mating type loci in the order Hypocreales, especially within the family Ophiocordycipitaceae to which *O. sinensis* belongs, remain poorly characterized. Fungi previously assigned to Clavicipitaceae *sensu lato* have recently been reclassified into three major families within Hypocreales: Cordycipitaceae (represented by *Cordyceps militaris*), Clavicipitaceae (represented by *Metarhizium anisopliae*, *Epichloë festucae*), and Ophiocordycipitaceae (represented by *O. sinensis*, *Tolyposcladium inflatum*) (Sung et al. 2007a). Previous studies (Yokoyama et al. 2004, 2006) utilized different sets of degenerate primers designed to the conserved  $\alpha$ 1 and HMG domains of the MAT1-1-1 and MAT1-2-1 genes to amplify short (approximately 200 bp) fragments of the mating type genes from 41 species across all three families. The majority of these species contained only a single mating type (either MAT1-1 or MAT1-2) and thus were presumed to be heterothallic. Both *Cordyceps takaomontana* (Yokoyama et al. 2003) and *C. militaris* (Shrestha et al. 2004) (Cordycipitaceae) were also shown to be heterothallic, and the latter was confirmed by the recent genome sequence of the species (Zheng et al. 2011). The genomes of *M. anisopliae* and *Metarhizium acridum* (Clavicipitaceae) also contain only a single idiomorph in each strain, indicating that these taxa are heterothallic (Gao et al. 2011).

While the majority of fungi closely related to *O. sinensis* are presumed heterothallic, evidence for homothallism within Hypocreales clearly exists and several heterothallic species show evidence of having evolved from a homothallic ancestor. The genus *Fusarium*, for example, which belongs to the family Nectriaceae of Hypocreales, clearly shows evidence of both heterothallic and homothallic species (Yun et al. 2000). The MAT1-2 idiomorph sequenced from one isolate of *C. takaomontana* contains a degenerate MAT1-1-1 pseudogene adjacent to the MAT1-2-1 gene, suggesting that the ancestor of *C. takaomontana* may have been homothallic with a fused MAT1-1/MAT1-2 locus (Yokoyama et al. 2005). Similarly, the MAT1-2 idiomorph of a heterothallic *Trichoderma reesei* strain also contains a region of the 3' end of the MAT1-1-1 gene next to the MAT1-2-1 gene (Seidl et al. 2009).

Interestingly, some heterothallic species (e.g. *C. militaris*) display unusual mating behaviour that does not conform to strict heterothallism or homothallism. While being primarily

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