



Phyletic Evolution of *Ophiocordyceps sinensis* through Interkingdom Host Colonization between Plants and Insects

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Authors' contributions

This work was carried out in collaboration between all authors. Authors XL and GZ designed the study. Author WL performed the statistical analysis. Authors WL and XS wrote the protocol. Author WL wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Ophiocordyceps sinensis, an entomogenous fungus parasitic in the larvae of ghost moths (Lepidoptera), has always been used as one of the most valued Traditional Chinese Medicines throughout the Orient World. However, *O. sinensis* fails to be artificially cultivated so far, because its occurrence and developmental mechanisms are crucial but almost completely unknown. In this study, the genetic marker mitochondrial small subunit (mtSSU) gene was cloned and identified by PCR and bioinformatic tools, and then the phylogenetic relationship between *O. sinensis* and related species was established based on mtSSU sequences with Maximum-Parsimony method. This phylogenetic project was reconstructed to show the evolution of host specificity and the process of interkingdom host jumping of *O. sinensis*, which revealed a natural outcome of

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co-evolution among this fungus, plants and *Thitarodes* insects. Therefore, this study will contribute to promote our understanding for life history and occurrence mechanism of *O. sinensis*.

Keywords: *Ophiocordyceps sinensis*; *Thitarodes* insect; plant; mitochondrial small subunit; evolution.

1. INTRODUCTION

Ophiocordyceps sinensis (syn. *Cordyceps sinensis*), a well-known medicinal mushroom, is entomogenous fungus naturally distributed in mountain shrubbery and meadows on the Tibetan Plateau. Its anamorph *Hirsutella sinensis* parasitizes the larvae of the genus *Thitarodes*, and then the fruit-bodies may produce to be used for medicine under certain condition. However, the development mechanism of *O. sinensis* is largely unknown, especially the anamorph infection and parasitic processes, which greatly restricted the sustainable exploration and utilization of *O. sinensis* [1,2]. A widely accepted theory is that *Thitarodes* insects are the only hosts of this fungus [3], but based on the research of interkingdom parasitism of *Cordyceps* fungi [4,5], the potential host and new parasitic pattern are considered to exist in the life cycle of *O. sinensis*. Moreover, in our previous study, a considerable amount of *O. sinensis* was detected in plant tissues, suggesting that plants may be new hosts of this fungus [6], and these plants may build key linkage between *Thitarodes* larvae and *O. sinensis* [6-8]. Therefore, it is of great importance to carry out further investigation on the interkingdom parasitism of *O. sinensis*, which will modify our understanding for its occurrence and development.

In order to interpret the systematic evolution properties of *O. sinensis* host specificity and diversity given its interkingdom colonization between plants and insects, in this study, we construct the phylogenetic tree of *O. sinensis* and other related fungi based on the molecular marker mitochondria small subunit gene (mtSSU) from population genetics, species enrolled contain 6 fungi parasites and 18 insect parasites.

2. MATERIALS AND METHODS

The *O. sinensis* samples were collected from Mount Sejila in Tibet, and their mycelia were isolated from sclerotia of *O. sinensis* and cultured artificially on potato dextrose agar (PDA) media at 18°C.

2.1 Cloning of mtSSU Gene

2.1.1 DNA preparation

Genomic DNA was isolated from pure culture of *O. sinensis* mycelia using the AxyPrep Multisource Genomic DNA Miniprep Kit (Axygen Scientific Inc, California, USA), and the quantity and quality were detected by ultramicrospectrophotometer NanoDrop 2000 and polyacrylamide gel electrophoresis. Total DNA was then kept at -80°C ultra-low temperature freezer.

DNA was extracted following the user's manuscript of AxyPrep Genomic DNA extraction kit, described as 2.1.2.

2.1.2 Molecular cloning

mtSSU rDNA fragment of *O. sinensis* was amplified by universal primer MSA0 (5'-TRCTTGACACATGCTAATCGAA-' and MSX3 (5'-AYTACACGAACNTATTTTCGACTT-3') [4].

The 25 µl reaction mix contained 1.0 µl genomic DNA of *O. sinensis*, 2.5 µl 10× PCR Buffer, 2.0 µl dNTP, 0.5 µl MSA0-primer, 0.5 µl MSX3-primer, 0.2 µl LA *Taq* polymerase and 18.9 µl ddH₂O. The amplification reactions were performed for 30 cycles consisting of 94°C for 30s, 55°C for 30s, 72°C for 4min, and then followed by 10min of amplification at 72°C. PCR product was subject to agarose gel electrophoresis, and the amplified DNA fragment was cloned to PMD19-T easy vector before being sequenced.

2.2 Molecular Evolution Analysis

mtSSU sequences from *O. sinensis* and representative sequences of other 24 *Cordyceps* species, 6 fungi colonization species and 18 insect colonization species (Table 1), were subject to multiple alignment using ClustalX software [9]. Subsequently, the evolutionary tree was constructed by MEGA4.1 procedure based on Maximum-Parsimony (MP) method with 100 repeats and the genetic distance was calculated.

Table 1. mtSSU rDNA sequences and their accession numbers

Host	Species	GenBank accession no.
Fungi	<i>Elaphocordyceps capitata</i>	AB027340
	<i>Elaphocordyceps jezoensis</i>	AB027341
	<i>Elaphocordyceps japonica</i>	AB027342
	<i>Elaphocordyceps ophioglossoides</i>	AB027343
	<i>Elaphocordyceps inegoensis</i>	AB027344
	<i>Elaphocordyceps paradoxa</i>	AB027345
Insects	<i>Cordyceps prolifica</i>	AB027346
	<i>Cordyceps kanzashiana</i>	AB027347
	<i>Cordyceps ramosopulvinata</i>	AB027348
	<i>Ophiocordyceps heteropoda</i>	AB027349
	<i>Ophiocordyceps sobolifera</i>	AB027350
	<i>Cordyceps</i> sp. 97003	AB027352
	<i>Ophiocordyceps tricentri</i>	AB027353
	<i>Ophiocordyceps cochliidiicola</i>	AB027355
	<i>Cordyceps</i> sp. 97009	AB027356
	<i>Cordyceps militaris</i>	AB027357
	<i>Ophiocordyceps coccidiicola</i>	AB031197
	<i>Ophiocordyceps konnoana</i>	AB031194
	<i>Ophiocordyceps sinensis</i>	JN255253
	<i>Isaria tenuipes</i>	AB027358
	<i>Beauveria brongniartii</i>	AB027359
<i>Beauveria bassiana</i>	AB027360	
<i>Metarhizium anisopliae</i>	AB027361	
Outgroup	<i>Hypocrea lutea</i>	AB027362
	<i>Hypomyces chrysospermus</i>	AB027363

3. RESULTS

3.1 mtSSU rDNA Sequence

The rDNA sequence of *O. sinensis* mtSSU was successfully cloned and identified by bioinformatic methods (Figs. 1, 2), and the sequence was submitted to GenBank Database and acquired the accession number: JN255253. The sequence was analyzed using primers MSA0 and MSX3 to have a length of 1.4 k bp, but we amplified a much longer sequence via PCR. The variation was attributed to the inserted group I and group II introns, which should be excluded in further sequence analyses.

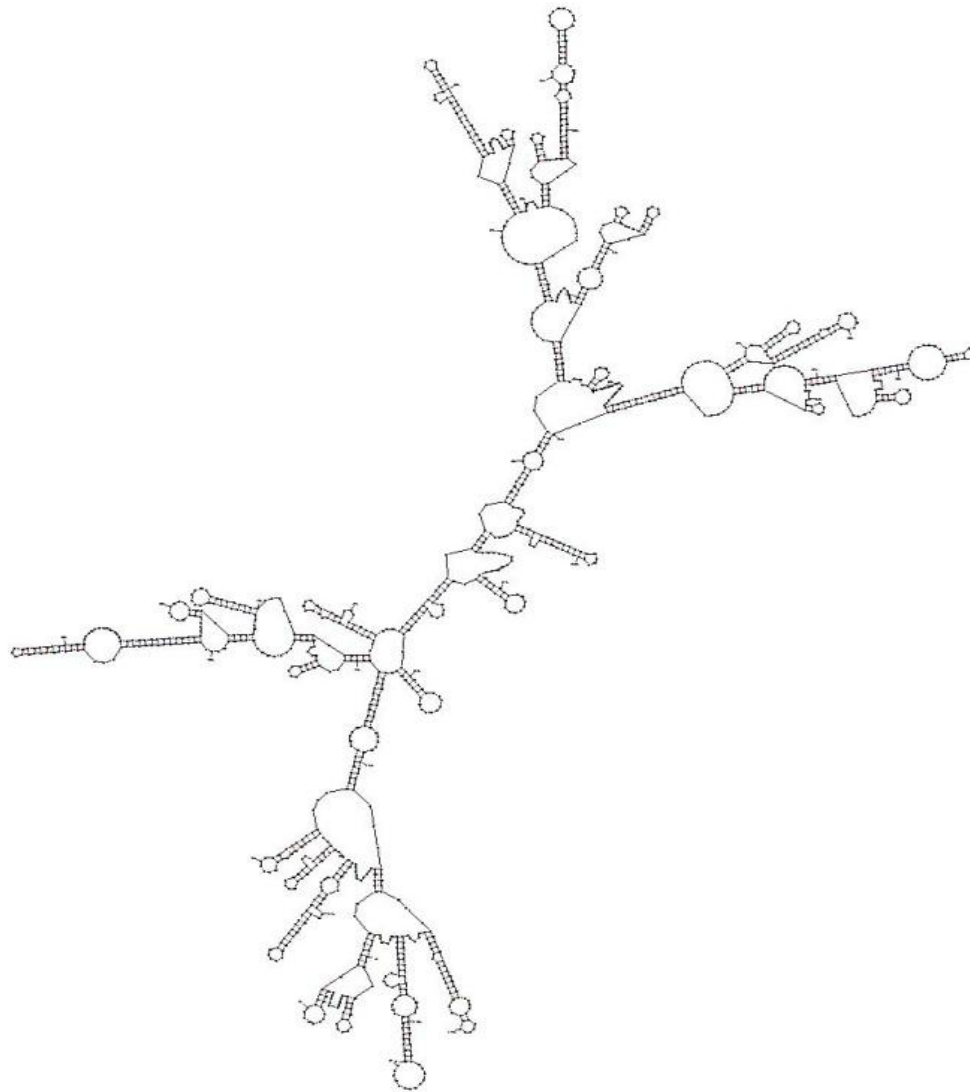
3.2 Construction and Analysis of Phylogenetic Tree

MP phylogenetic tree of *O. sinensis* and the related fungi was constructed before being appropriately adjusted and classified (Fig. 3). Except the outgroup *Hypomyces*, 4 fungi colonization formed a cluster, which was called fungi host group, and the rest insects colonization formed another cluster, called

insects host group. More importantly, *O. heteropoda* had been proved to parasitize both insects and plants [5]. In the phylogenetic tree, *O. heteropoda* clustered in the same group with *O. sinensis*. They shared a similarity of 73%, and located in the overlap region of fungi host group and insects host group, suggesting that *O. sinensis* might also parasitize both insects and plants.

4. DISCUSSION

In this study, phylogenetic tree of *O. sinensis* and the related fungi was constructed, and host specificity and diversity switch phenomenon of the *O. sinensis* anamorph was analyzed, which proved phylogenetic tree to be an effective way for interpreting phylogenetic differentiation of fungi from different colonizing host [10]. These fungal species were clustered into two topological groups, including fungi host group and insects host group, which were coincidence with the previous findings [10,11]. Phylogenetically, *O. sinensis* may parasitize both plants and insects, and plants were the main hosts. This interkingdom colonization is the result of *O. sinensis* natural evolution.



dG = -289.08 [Initially -342.90] aaa

Fig. 1. The secondary structure of mtSSU region

The former results strongly substantiated that it's necessary to add a "plant host switch" session in the life cycle of *O. sinensis*, which was a significant supplement for *O. sinensis* developmental process. Based on origin and evolution of entomogenous fungus, the anamorph of *O. sinensis* may parasitize plants firstly and then insects, and finally return fungi host [12]. Because the *Thitarodes* larvae and the nearby plants share the same environment, and get frequent contact via the soil media, which

accelerate the host switch of *O. sinensis* anamorph from one to another [13]. Moreover, the anamorph of *O. sinensis* colonizes its food plants, and the *Thitarodes* larvae may get infected through food intake. When the larvae eat the roots carrying *O. sinensis*, this fungus then enters the insect intestinals from inside and meanwhile infects the hosts. Under appropriate situation, the infected larvae turn to muscardine cadaver and grow to teleomorph of *O. sinensis* with fruit body.

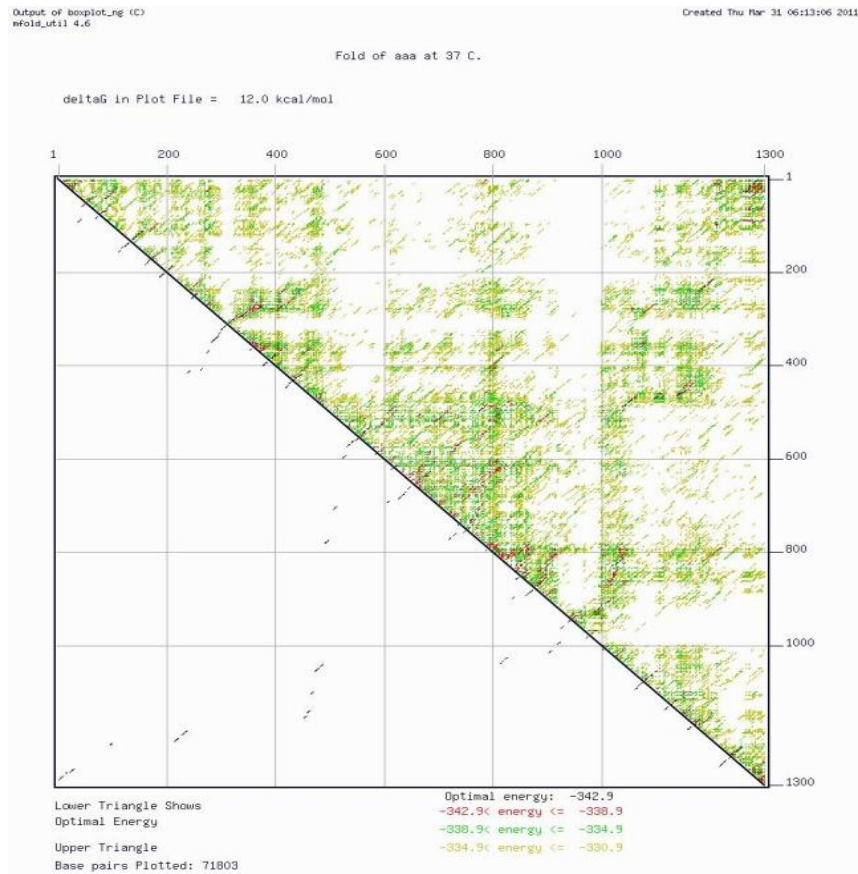


Fig. 2. Refolding free energy of mtSSU secondary structure

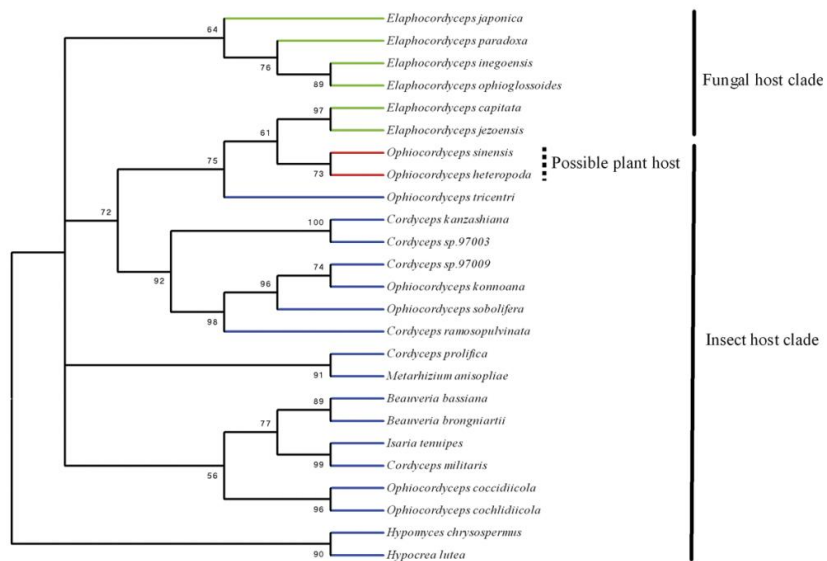


Fig. 3. Phylogenetic relationship of the *O. sinensis* and its related species based on mtSSU rDNA sequences. Bootstrap values obtained with 1000 replications are shown at the nodes. The clade names are shown on the right

5. CONCLUSION

mtSSU gene of *O. sinensis* was cloned and identified for the first time, and then the phylogenetic relationship between *O. sinensis* and related species was established to show the evolution of host specificity and the process of interkingdom host jumping of *O. sinensis*. As a result, it revealed a natural outcome of co-evolution among this fungus, plants and *Thitarodes* insects. Therefore, this study will contribute to promote our understanding for the life history and occurrence mechanism of *O. sinensis*.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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