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Comparative Phylogenetic Relationships and Genetic Structure of the Caterpillar Fungus *Ophiocordyceps sinensis* and Its Host Insects Inferred from Multiple Gene Sequences[§]

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Ophiocordyceps sinensis **(Ascomycota: Ophiocordycipitaceae) is a native fungal parasite of Hepialidae caterpillars and one of the most economically important medicinal caterpillar fungi in China. However, little is known about the phylogenetic and evolutionary relationships between *O. sinensis* and its host insects. In this study, nuclear ITS and β-tubulin sequences from *O. sinensis* and mitochondrial COI, COII, and Cyt*b* sequences from its hosts were analyzed across 33 populations sampled from five regions in China. Phylogenetically, both *O. sinensis* and its hosts were divided into three geographically correlated clades, and their phylogenies were congruent. Analysis of molecular variance and calculated coefficients of genetic differentiation revealed significant genetic divergence among the clades within both *O. sinensis* (F_{ST}= 0.878, N_{ST}=0.842) and its hosts (F_{ST}=0.861, N_{ST}=0.816). Estimated gene flow was very low for *O. sinensis* (Nm=0.04) and the host insects (Nm=0.04) among these three clades. Mantel tests demonstrated a significant correlation (*P*<0.01) between the genetic distances for *O. sinensis* and its hosts, as well as a significant association (*P*<0.05) between geographic and genetic distances in both. The similar phylogenetic relationships, geographic distributions, and genetic structure and differentiation between *O. sinensis* and its hosts imply that they have coevolved.**

Keywords: *Ophiocordyceps sinensis*, host insect, phylogenetic relationship, genetic structure, coevolution

Introduction

Ophiocordyceps sinensis (Ascomycota: Ophiocordycipitaceae) (Berk.) Sung, Sung, Hywel-Jones & Spatafora, a fungal parasite of Hepialidae caterpillars, has been routinely used in traditional Chinese medicine to treat asthma and respiratory, kidney, and other diseases for centuries (Zhu *et al.*, 1998; Sung *et al.*, 2007). This species is only distributed in alpine meadows regions of the Qinghai-Tibet plateau of western China. Overexploitation driven by its high commercial value and habitat degradation has endangered the species in recent years. The number of natural *O. sinensis* populations is extremely limited, and its specific host insects are also potentially threatened (Zhu and Mou, 2006). Therefore, policies to protect *O. sinensis* and its host are critically needed.

When *O. sinensis* infects its host, it forms a complex of stroma and host cadaver in three stages: infection, parasitism, and saprophytism (Li and Tsim, 2004). Molecular studies suggest that *Hirsutella sinensis* is the sole anamorph of *O. sinensis* (Chen *et al.*, 2001, 2004; Xiao *et al.*, 2009), while alternative hypotheses hold that *O. sinensis* is a "cryptic" species or has multiple genotypes (Stensrud *et al.*, 2007; Zhu *et al.*, 2010). The host insects of *O. sinensis* are taxonomically diverse based on traditional morphological classifications (Yang *et al.*, 1996; Liu *et al.*, 2005; Wang and Yao, 2011).

Recently, individual cultivation of *O. sinensis* and its hepialid hosts has been successful, however, stromata formation following artificial inoculation of *O. sinensis* on hepialid larvae remains difficult and unreliable. In addition, whether *O. sinensis* from different geographic regions parasitize specific hepialid hosts remains unknown. Thus, the genetic relationships, genetic structure, and geographic distribution of *O. sinensis* and its hosts, as well as their interaction, should be clarified for the sustainable development and use of *O. sinensis*.

Previous studies of *O. sinensis* have focused mainly on morphology, ecology, and life history (Yang *et al.*, 1996; Liu *et al.*, 2005), and genetic diversity estimates have been made from a limited number of individuals or populations using molecular markers such as RAPD, ISSR, rDNA-ITS, and MAT1-2-1 (Chen *et al.*, 1999; Liang *et al.*, 2008; Hao *et al.*, 2009; Zhang *et al.*, 2009). However, few reports have also included phylogenetic analyses of their hepialid hosts (Chen *et al.*, 1998; Cheng *et al.*, 2007). Even less common are simultaneous phylogenetic and population genetic analyses of both parasitism partners. Such studies could advance efforts to conserve, cultivate, and use this resource.

We previously developed an effective approach for amplifying the mitochondrial Cyt*b* gene of the hepialid hosts and the nrITS gene of *O. sinensis* from the same complex of host cadaver and stroma (Cheng *et al.*, 2007; Hao *et al.*, 2009). This method allows us to simultaneously evaluate the phylogenetic relationships and genetic structures of both *O. si-*

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nensis and its host at the population level, as well as the evolutionary history of their interaction.

In this study, nrITS and β-tubulin sequences of *O. sinensis* and mitochondrial COI, COII, and Cyt*b* sequences of host insects were analyzed for 33 *O. sinensis* populations in five regions of China. The phylogenetic relationships and genetic structure/differentiation were clarified for both *O. sinensis* and its host insects and were also simultaneously compared between the two at the population level. The results will provide important information on the phylogenetic and evolutionary relationships between an entomogenous fungus and its insect host, which will be useful for the artificial cultivation, protection, and sustainable use of this system.

Materials and Methods

Sampling

Thirty-three *O. sinensis* populations were sampled across the species' main distribution in China (Qinghai, Tibet, Sichuan, Gansu, and Yunnan provinces). The populations were distributed widely from 27°47′N–38°01′N to 89°25′E–102°30′E, at altitudes of 2238–4500 m. Locations and abbreviations for the populations are given in Supplementary data Table S1. See Fig. 1 for detailed locations. Each population included about 10–20 individuals that comprised a complex of *O. sinensis* stroma and host cadaver. Specimens were preserved in the Herbarium of the Institute of Bioresources and Applied Technology, Tongji University, China.

Genomic DNA extraction and electrophoresis

After collection, each sample was subdivided into stroma and host cadaver components to extract the DNA of *O. sinensis* and its host individually. DNA was extracted for each component using a modified CTAB method (Liang *et al.*, 2005). The extracted DNA was electrophoresed on a 1.5% agarose gel and viewed under UV light after staining with ethidium bromide.

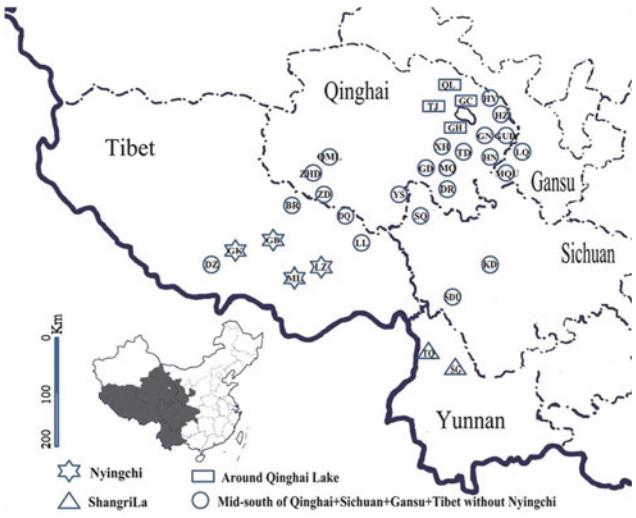

Fig. 1. Distribution map of 33 *O. sinensis* populations collected across the major distributing regions in China. Population codes are defined in Supplementary data Table S1. Symbols show populations belong to different geographic areas and correspond to the phylogenetic clades of both *O. sinensis* and host insects in Fig. 2.

Polymerase chain reaction (PCR) amplification and DNA sequence

Primer sequences for nrITS and β-tubulin sequences of *O. sinensis* and the mitochondrial COI, COII, and Cyt*b* sequences of host insects are listed in Supplementary data Table S2. To clarify accurately any phylogenetic correlation among these genes and to avoid an excessive amount of confusing data, one individual was randomly selected to represent each population. All loci were amplified by PCR in a total volume of 50 μl containing 2 mM $MgCl_2$, 0.25 mM dNTPs, 0.2 μM primers, 50 ng of genomic DNA, 1× buffer, and 2 U ExTaq DNA polymerase (TaKaRa, Japan). PCR was conducted in a Mastercycler Gradient PCR (Eppendorf, Germany) as follows: ITS: 94°C for 5 min; 30 cycles of 94°C for 1 min, 55°C for 30 sec, 72°C for 1 min; and a final extension of 72°C for 10 min. β-Tubulin: 94°C for 5 min; 35 cycles of 94°C for 35 sec, 57°C for 55 sec, 72°C for 90 sec; and a final extension of 72°C for 10 min. COI and COII: 94°C for 1 min; 6 cycles of 94°C for 1 min, 45°C for 90 sec, 72°C for 75 sec; then 36 cycles of 94°C for 1 min, 51°C for 90 sec, 72°C for 75 sec; and an extension of 72°C for 5 min. Cyt*b*: 94°C for 5 min; 40 cycles of 94°C for 45 sec, 46°C for 1 min, 72°C for 1 min; and a final extension at 72°C for 10 min. All amplified products were detected by electrophoresis on 1.5% agarose gels in 1× TAE buffer (100 V for 35 min) then purified and sequenced at Shanghai GeneCore Biotechnologies, Shanghai, China. The GenBank accession numbers of all sequences were shown in Supplementary data Table S1.

Data analysis

Nucleotide sequences were aligned using ClustalW (Thompson *et al.*, 1994). The number of variable sites, A+T and G+C contents, and the transition/transversion ratio were calculated. Genetic distances among populations were determined based on the Kimura's two-parameter model using MEGA5.2 (Kimura, 1980; Tamura *et al.*, 2011). Geographical distances of the 33 populations were estimated based on their latitudes and longitudes using GenAlEx6 software (Peakall and Smouse, 2006). Correlation among the genetic distances of the five genes was determined with Mantel tests (Mantel, 1967). Correlation between geographic and genetic distances of the populations was also examined. All Mantel tests were conducted using GenAlEx6 with 9,999 randomizations.

Neighbor-joining phylogenetic trees were constructed based on genetic distances for each of the five genes using MEGA5.2 software (Kimura, 1980; Tamura *et al.*, 2011). Bootstrap support values were obtained from 1,000 replications for each dataset. Genetic divergences between clades (F_{ST}) were calculated by analyses of molecular variance (Excoffier *et al.*, 1992) using Arlequin v. 3.11 (Excoffier *et al.*, 2005). The numbers of haplotypes, differentiation coefficients (N_{ST}), and average gene flow (Nm) among clades were calculated using DNASP4.0 (Rozas *et al.*, 2003).

Table 1. Variation parameters and base compositions of *O. sinensis* and host insects based on five different gene sequences, respectively

Gene	Fragment (bp)	Variable sites (V)	Parsimony informative sites (Pi)	Singleton sites (S)	Rate of A+T (%)	Rate of variation (%)	Rate of transition/ transversion (%)
ITS	546	14	11	3	37.40	2.60	3.0
β-Tubulin	1201	11	9	2	42.30	0.92	2.8
COI	686	108	86	22	69.40	15.70	2.6
COII	602	63	58	5	77.10	10.40	4.6
Cyt*b*	485	68	53	15	74.80	14.00	2.2

Results

Analysis of DNA sequence

The alignment of *O. sinensis* ITS sequences contained no indels and was 546 bp in length with an average pairwise divergence of 2.6% (Table 1). Similarly, nine parsimony informative sites were detected in the 1,201 bp β-tubulin alignment, which had an average divergence of 0.92%. The A+T content was significantly lower than that of G+C in both datasets. From the insect hosts, alignments of 686, 602, and 485 bp were obtained for the COI, COII, and Cyt*b* sequences, respectively. A+T contents of the three mitochondrial loci were 69.4%, 77.1%, and 74.8%, respectively. High divergence rates from 10.4% to 15.7% were detected in the three sequences, with COI having the highest.

Correlation analysis of multiple gene sequences

Significant correlation was detected between ITS and β-tubulin sequences from *O. sinensis* based on genetic distance matrices, but the correlation value (r=0.168, P<0.05) was low. Higher correlations, in the range of 0.923–0.966, that were highly significant (P<0.01) were detected among the genetic distances for the COI, COII, and Cyt*b* sequences of the host insects. This finding suggested that these three mitochondrial sequences would reveal the same genetic relationships for the host insects. The highest correlation coefficient (r=0.993, P<0.01) was detected between the genetic distances of COI and the combined mitochondrial dataset (COI+COII+Cyt*b*), indicating that the COI sequence was an appropriate representative of the combined data. Mantel tests showed that the genetic distances of *O. sinensis* based on the ITS sequence were most strongly correlated (r=0.735, P<0.01) in genetic distance with the COI sequence of the host insect, while the correlation coefficient between β-tubulin and COI sequence was low (r=0.165, P<0.01).

Phylogenetic analysis

The phylogenetic tree constructed with ITS sequences of *O. sinensis* divided the 33 populations into three clades that matched their geographic distributions (Fig. 2). The largest clade (I) included 22 populations with almost identical sequences that were mainly from the mid-south of Qinghai,

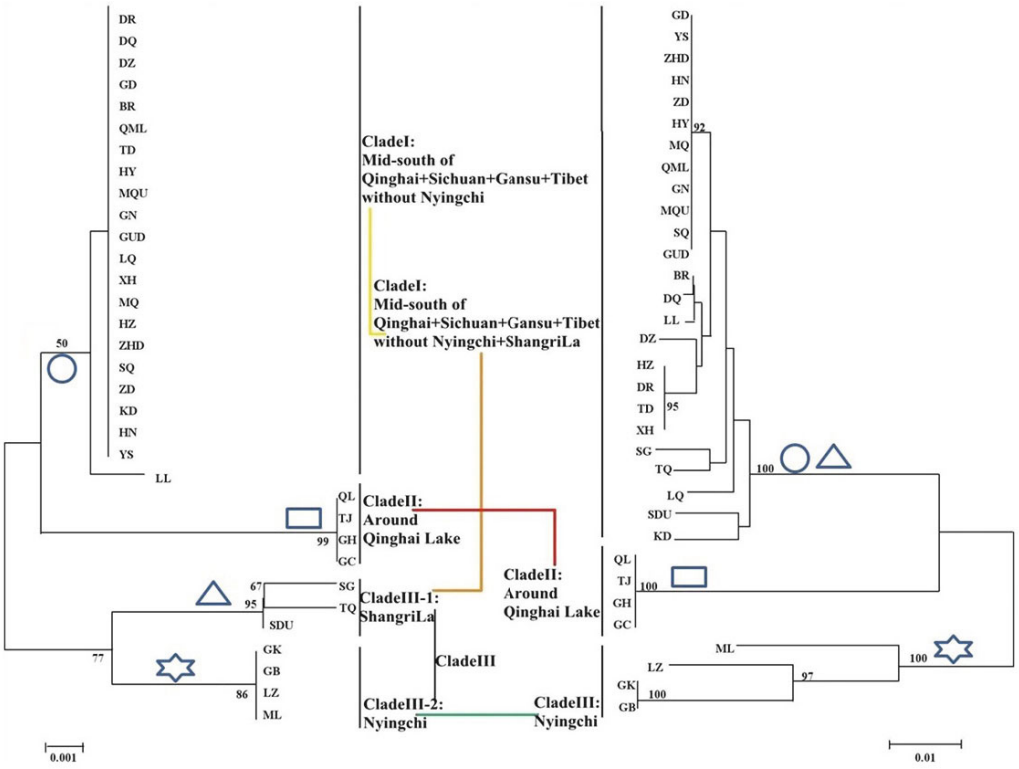

Fig. 2. Phylogenetic clades match between *O. sinensis* (left) and host insects (right) inferred from ITS and COI sequences, respectively. Bootstrap support values exceeding 50% are indicated above branches. Symbols represent different geographic areas of *O. sinensis* populations as shown in Fig. 1. Population codes are defined in Supplementary data Table S1.

Table 2. **Correlations between geographic distances and genetic distances of *O. sinensis* and host insects based on five different gene sequences, respectively**

Species	Gene	R value
O. sinensis	ITS	0.373**
	β-tubulin	0.111*
	ITS+β-tubulin	0.302**
Host insect	COI	0.267**
	COII	0.266**
	Cyt*b*	0.213*
	COI+ COII+ Cyt*b*	0.255**

*p<0.05; ** p<0.01

Sichuan, Gansu, and Tibet, but not the Nyingchi area. Four populations (GH, GC, TJ, and QL) from the area around Qinghai Lake formed clade II, which was separated from other clades at a genetic distance of 0.009–0.017. Those populations from the ShangriLa and Nyingchi areas comprised clade III, which was subdivided into two sub-clades; sub-clade III-1 included the three populations (SG, TQ, and SDU) from the ShangriLa area and had small genetic distances (0.002–0.004), while sub-clade III-2 contained four populations (LZ, ML, GK, and GB) from the Nyingchi area with identical sequences. The Mantel test showed that genetic distances of ITS were extremely significantly correlated with the geographical distance among populations (r=0.373, P< 0.01) (Table 2).

The phylogenetic tree obtained from the β-tubulin sequences of *O. sinensis* was obviously different from that constructed using ITS sequences. Three major clades were also distinguished, but they did not match the population geographic distribution (Supplementary data Fig. S1). The Mantel test revealed that the geographical and genetic distances of the populations were still significantly correlated, but with a very small r-value (r=0.111, P<0.05) (Table 2). Most of the populations that were geographically near one another clustered into different clades, with the exception of four populations from the area around Qinghai Lake, which formed a separate clade. Those populations from the mid-south of Qinghai and northern Tibet, which had relatively close genetic relationships in the ITS tree, were separated into different clades in the β-tubulin tree, perhaps because of the lower divergence rate (0.92%) of the β-tubulin sequences.

The phylogenetic trees constructed using the COI, COII, and Cyt*b* sequences of the host insects of *O. sinensis* had similar topological structures with three clades (Fig. 2, Supplementary data Figs. S2 and S3). Clade I was composed of 25 populations, mainly from the mid-south of Qinghai, Sichuan, Gansu, and Tibet but not the Nyingchi area. Four populations (GH, GC, TJ, and QL) from the area around Qinghai Lake were clustered together in clade II. Four populations (LZ, ML, GK, and GB) from the Nyingchi area of Tibet formed clade III. The components of three clades in the host mitochondrial trees were similar to those in the ITS tree of *O. sinensis*.

Genetic distances based on the three mitochondrial sequences were significantly correlated to the geographical distances among the 33 populations (Table 2), indicating that the host insect populations had a distinct geographic distribution pattern. The correlation between genetic and geographical distances was highest (r=0.267, P<0.01) for COI, suggesting that the geographic distribution of these insects matched the best with the COI phylogenetic tree.

Genetic differentiation and gene flow

Our phylogenetic analyses showed that both *O. sinensis* and its host insects from 33 populations could be divided into three corresponding clades. Seven and six haplotypes for the ITS and β-tubulin sequences, respectively, were identified for *O. sinensis*. The highest haplotypic diversity was detected within the clade of the ShangriLa area in the ITS tree. The genetic differentiation coefficients F_{ST} and N_{ST} revealed that significant genetic differentiation occurred among the three clades of *O. sinensis* in both the ITS and β-tubulin datasets (Table 3). Estimated gene flow among the clades was very low based on both ITS (Nm=0.04) and β-tubulin (Nm=0.01) sequences.

In the 33 populations of host insects, 16, 16, and 17 haplotypes were identified for the COI, COII, and Cyt*b* genes, respectively (Table 3). The mitochondrial haplotypic diversity ranged from 0.84 to 0.89, and both haplotypic number and diversity were greater than in *O. sinensis*. The F_{ST} (F_{STCytb}= 0.829) and N_{ST} (N_{STCytb}=0.770) values for the Cyt*b* sequences were lower than those of other two genes (F_{STCOI}=0.861, N_{STCOI}=0.816; F_{STCOII}=0.871, N_{STCOII}=0.845). Based on the combined mitochondrial dataset (COI + COII + Cyt*b*), the genetic differentiation coefficients (F_{ST}=0.865, N_{ST}=0.812) of the host insects were similar to those of *O. sinensis*. Gene flow in the host insects was also low (Nm=0.04–0.07) among the clades (Table 3), as in *O. sinensis*.

Discussion

Low genetic diversity was found among the 33 populations

Table 3. **Statistic summary of genetic diversity and differentiation for *O. sinensis* and host insects**

Species	Gene	nh	(Hd)	N_{ST} (Nm)	AMOVA F_{ST} (Nm)
O. sinensis	ITS	7	0.58	0.842 (0.05)	0.878 (0.04)
	β-tubulin	6	0.74	0.948 (0.01)	0.959 (0.01)
host insect	COI	16	0.87	0.816 (0.06)	0.861 (0.04)
	COII	16	0.84	0.845 (0.05)	0.871 (0.04)
	Cyt*b*	17	0.89	0.770 (0.07)	0.829 (0.05)
	COI+COII+Cyt*b*	25	0.95	0.812 (0.06)	0.865 (0.04)

The parameters include number of haplotypes (nh), haplotype diversity (Hd), coefficient of genetic differentiation (N_{ST}), and analyses of molecular variance (AMOVA).

of *O. sinensis* sampled from across the main parts of its distribution in China. The highest genetic distances were 0.017 for ITS and 0.007 for β-tubulin. *Ophiocordyceps sinensis* isolates are considered same species if their ITS distance values are lower than 0.03 (Chen *et al.*, 2004). Our results based on specimens from an extensive geographic region also supported the hypothesis that *O. sinensis* is a single species (Xiao *et al.*, 2009), rather than two species or a species complex (Stensrud *et al.*, 2007).

In this study, both phylogenetic analysis and the Mantel test suggested that COI is a reliable marker to reveal the phylogenetic relationships and geographic distribution patterns of the host insects, although COI, COII, and Cyt*b* are all effective for analyzing the phylogenetic relationships of Lepidoptera (Hebert *et al.*, 2003; Hajibabaei *et al.*, 2007; Silva-Brandão *et al.*, 2009). Similarly, for *O. sinensis*, the ITS sequences showed the highest genetic distance correlation with the insect COI sequences. The ITS and COI sequences could be used alone to clarify the phylogenetic correlations between these two interacting species.

Phylogenetic analysis indicated that both *O. sinensis* and its host insects form three clades that match well with their geographic distribution along a latitudinal gradient. This paper provides the first population-level analysis of the geographic distributions of these insects, although we previously reported on that of *O. sinensis* (Liang *et al.*, 2005, 2008).

Both the fungi and host insects from four populations (GH, GC, TJ, and QL) around Qinghai Lake were distinctly clustered into single clades with just one haplotype each. The host species in this area was different from that of other populations in Qinghai Province based on the criterion of COI sequence variation (Hebert *et al.*, 2003). This unique clade of *O. sinensis* was also recovered using ISSR markers and morphological characters (Liang *et al.*, 2005, 2008). Qinghai Lake, surrounded by Datong, Riyue, and Amuni mountains, is the largest extant closed-basin lake in China. It was formed by tensional geological movement and violent decline (Yuan *et al.*, 1990; Madsen *et al.*, 2008). The regional climate, rainfall, and plant distributions in this area were shaped by the lake and the topography of the basin (Qi and Guo, 2007). Thus, the genetic differentiation of both *O. sinensis* and its host insects in this area may be due to its long-term geographical isolation and distinct ecological environment. Consequently, the complete one-to-one corresponding relationship between *O. sinensis* and its host insects in this area was shaped by their interaction.

Both *O. sine*nsis and its host insects from the populations of the Nyingchi area also clustered into independent clades. There was only one haplotype of *O. sinensis* in this region, but the host insects had multiple haplotypes, perhaps because of different modes of transmission between *O. sinensis* and its hosts. The host insects cannot easily fly long distances and disperse, and the adults live only 3–8 days (Yang *et al.*, 1996). The unique landform, forest vegetation, and microclimate of Namjagbarwa Mountain area have resulted in a rich lepidopteran fauna, unlike in other regions of China and the world, and the similarity coefficients for Lepidoptera from this area are also very low (Lu and Tang, 2006). However, the *O. sinensis* fungal spores are dispersed by wind and can travel long distances (Li and Tsim, 2004). Therefore, in this area, several distinct and geographically separated insect populations could be parasitized by a single haplotype of *O. sinensis*. These results conflict with a previous report that *O. sinensis* from the Nyingchi area had numerous haplotypes (Zhang *et al.*, 2009). More samples will be necessary to clarify further the relationships between *O. sinensis* and host insects in this area.

Three populations from the ShangriLa area showed that both *O. sinensis* and their host insects had substantial genetic differentiation. This area, in the southeast Qinghai-Tibetan plateau, belongs to the Hengduan Mountains, which have a unique geographical location, complex geomorphology, and diverse climatic conditions. No extensive ice cap is known to have developed during the Quaternary stages in the Qinghai-Tibetan Plateau, especially in its southeast region (Li, 1995; Shi *et al.*, 1998; Wang *et al.*, 2008), which thus comprises the major component of the south-central "biodiversity hotspot" (Myers *et al.*, 2000; Wang *et al.*, 2008). Our results also implied that the ShangriLa area may be a diversity center of *O. sinensis* and its host insects, as indicated by their significant genetic differentiation.

The comparative genetic structure of hosts and their parasites is critical in understanding how hosts adapt to parasites (Dybdahl and Lively, 1996; Jarne and Théron, 2001; Prugnolle *et al.*, 2005; Keeney *et al.*, 2009). Our study demonstrated that similar genetic structures and gene flow patterns between *O. sinensis* and its host insects are also important for parasites to adapt to hosts, possibly because the life cycle of *O. sinensis* depends on its hosts (Li and Tsim, 2004). The fungus must parasitize its host to reproduce, indicating that the host insect played an important role in the genetic differentiation of the fungus. Yang *et al.* (1996) reported that the fungus usually used different species of hosts in different mountain ranges and on different sides or at different altitudes on the same mountain. Genetic isolation of the host often results in isolation of the parasite population and in the parasite's subsequent speciation (Paterson and Banks, 2001). The significant correlations between the genetic and geographic distances of both *O. sinensis* and its host populations indicate that geographical isolation and environmental factors may be important influences on the evolution of both species.

Coevolution between host and parasite species is defined as the extent to which their phylogenies are congruent (Legendre *et al.*, 2002). In this study, a significant correlation was detected between the genetic distances of *O. sinensis* and their host insects, and their phylogenies were similar at the population level. This finding was consistent with Fahrenholz's Rule that parasite phylogeny mirrors host phylogeny (Klassen, 1992; Paterson and Banks, 2001). Therefore, similar phylogenetic trees and genetic structures and a significant correlation of genetic distances between *O. sinensis* and its host insects indicated that these two species have coevolved.

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