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A Real-Time qPCR Assay to Quantify *Ophiocordyceps sinensis* Biomass in *Thitarodes* Larvae

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Ophiocordyceps sinensis*, an entomogenous fungus parasitic in the larvae of moths (Lepidoptera), is one of the most valuable medicinal fungi, and it only distributed naturally on the Tibetan Plateau. The parasitical amount of *O. sinensis* in various tissues of the host *Thitarode*s larvae has an important role in study the occurrence and developmental mechanisms of *O. sinensis*, but there no an effective method to detect the fungal anamorph. A real-time quantitative PCR (qPCR) system, including a pair of species-specific ITS primers and its related program, was developed for *O. sinensis* assay with high reliability and efficiency. A calibration curve was established and exhibited a very good linear correlation between the fungal biomass and the C_T values (R^2=0.999419) by the qPCR system. Based on this method, *O. sinensis* was detected rapidly in four tissues of its host caterpillars, and the results were shown as following: the maximum content of *O. sinensis* parasitized in the fat-body, and next came body-wall; both of them were much larger than that observed in the haemolymph and intestinal-wall. Taken together, these results show that qPCR assays may become useful tools for study on developmental mechanism of *O. sinensis*.

***Keywords*:** *O. sinensis*, real-time quantitative PCR, caterpillars, internal transcribed spacer, DNA

Introduction

Ophiocordyceps sinensis (syn. *Cordyceps sinensis*), a traditional and precious medicinal ascomycetes from the Tibetan Plateau, has been used throughout the Orient world for centuries (Sung *et al*., 2007; Winkler, 2008). Its recent progress has focused on the sanitarian function such as immune activation, cancer prevention, radiation mitigation and kidney nourishment, etc., while this fungus, called commonly as one of the most valuable fungi on earth, sells at the price of U.S. $ 60000/kg for the top quality product in 2011 (Kuo *et al*., 2007; Yoon *et al*., 2008; Tsai *et al*., 2010; Bi *et al*., 2011). *O. sinensis* parasitize and grow inside the body of its host *Thitarodes* larvae (caterpillars) (Li *et al*., 2011). Under a certain condition, these endoparasites break out and eventually kill the caterpillar (Zhang *et al*., 2008; Shrestha *et al*., 2011). At last, the fungal stromata (teleomorphs) start to form and then emerge from the ground. In this case, parasitism of the endoparasitic *O. sinensis* is considered as the crucial factor impacting occurrence and development of *O. sinensis*, but it can not be isolated from the host caterpillar to obtain the pure culture (Li *et al*., 2000; Dong and Yao, 2010). Despite of some reports about the fungal qualitative detection, there is hardly any method studying quantitatively the population level and parasitical dynamics of *O. sinensis* in the host caterpillars (Kaarakainen *et al*., 2009; Şakalar and Abasıyanık, 2012).

Polymerase chain reaction (PCR) has been currently used to detect *O. sinensis* but it just provides the preliminary information related to presence or absence (Zhao *et al*., 1999; Kuo *et al*., 2005; Sung *et al*., 2007; Zhong *et al*., 2010). Real-Time quantitative PCR (qPCR) technology, the advanced version of the conventional PCR assay, represents another powerful leap forward in the field of DNA analysis (Heather *et al*., 2008). In recent years, qPCR enables more sensitive and reliable detection of numerous fungal pathogens within animal, plants, and mushrooms tissues (Laube *et al*., 2010; Babu *et al*., 2011; Lee *et al*., 2011; Mujico *et al*., 2011). qPCR's attributes, such as rapid detection, multiple test, and accurate quantification will enable practical detection of *O. sinensis* so that study projects can be established to reveal the occurrence and development mechanism of *O. sinensis*. A key consideration is the relationship between pathogen genomics DNA and its biomass (Bjornsdottir-Butler *et al*., 2011; Hwang *et al*., 2011). A proposed strategy is to create calibration curve based on the liner correlation between amounts of fungal mycelia and qPCR test value (Huang and Kang, 2010; Lee *et al*., 2011). However, it is difficult to prepare adequate mycelia for biomass determination by weight. Even though there were some published studies involving detection of pathogenic fungi, their accuracy needed to be improved further. In addition, both primer specificity and optimal amplification condition play an important role in endogenetic fungi assay. Furthermore, it is well-known that the life cycle of *O. sinensis* includes asexual and sexual generations, which show the distinct morphological characters and tissue structures (Zeng *et al*., 2006). In particular, it is not available directly from the host and environment so far.

Due to relative stability of ITS gene in fungal cell, there is an absolute positive linear correlation between its copy num-

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ber and host cellular count, i.e. biomass (Chaturvedi *et al.*, 2011). Therefore, *in situ* detections become the promising approaches, and copy number of ITS nrDNA rather than the fungal weight is regarded as more reasonable index to quantify *O. sinensis* biomass, similar to other culture-independent fungi (Krak *et al.*, 2012). Taken together, copy number of ITS segment was used for qPCR assay of *O. sinensis* in the current study. Here, we develop a qPCR method to quantify the amount of *O. sinensis* in the host. After validating the sensitivity and specificity of this assay, colonization level of this fungus was explored in four tissues of the host caterpillars.

Materials and Methods

Study sites and sampling

The Segyi La Mountain lies in the southeast of Tibetan Plateau. One of the most excellent habitats of *O. sinensis* distributes on this mountain, where "Characteristic Resources Scientific Workstation of Sun Yat-sen University" (Alt. 4156 m, 29°36′ N, 94°35′ E) was founded for the biological studies in natural ecological environment. Some familiar fungi isolated from this habitat and then cultured at 18°C, such as *O. sinensis*, *Beauveria* sp., *Paecilomyces hepiali*, *Paecilomyces* sp., and *Lecanicillium pui*. Fifteen larvae of *Thitarodes pui* were collected in the same sites.

DNA preparation

Genomic DNA from the pure culture of *O. sinensis*, *Beauveria* sp., *P. hepiali*, *Paecilomyces* sp., and *L. pui* was respectively extracted using the Multisource Genomic DNA Miniprep kit (Axygen, USA). The caterpillar was dissected into four parts, including body-wall, fat-body, haemolymph and intestinal-wall, from which genomic DNAs were obtained with the above method. After the agarose gel electrophoresis, the quality and concentration of all DNAs were measured by a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). DNA solution was quantified by the following formula: DNA concentration = OD_{260} × extinction coefficient (50 μg/ml) × dilution factor. The copy number of the desired gene was calculated according to another formula: copy number = (DNA amount × 6.022 × 10^{23}) / (length × 1×10^9 × 660).

qPCR primer design and validation

ITS sequences from *O. sinensis* (GenBank accession no. HM135167) and its concomitant species, including *Beauveria* sp. (HM135176), *P. hepiali* (HM135170), *Paecilomyces* sp. (HM135164), *L. pui* (JF794472), *O. ryogamiensis* (JN943345), *O. cuboidea* (JN943333), *O. appendiculata* (JN943325), and *O. prolifica* (JN943339), were aligned to select the species-specific ITS amplicon with the computer tool Vector NTI Suit 8. Meanwhile, the complete ITS region was imported into the Primer Premier 5.0 tool, and search criteria were set as following: PCR product size was 80 bp to 120 bp, Primer length 19 bp to 22 bp, and all the rest parameters were default values. Thus, five candidate primer pairs were designed based on the above results, and subsequently they were evaluated the possibility of dimer polymers and hairpin structures by DNAStar software package. Each primer pair and various primer combinations were tested by using DNA templates of *O. sinensis* and other concomitant fungi for species specificity and expression level, for amplification efficiency of ITS region and for supreme avoidance of primer dimmers. At last, qPCR primers were designed successfully and then synthesized by Life Technologies Corporation.

qPCR amplification

20 μl of qPCR mixture was composed of 5 μl of genomic DNA, 10 μl of SYBR Green PCR Master Mix (2×) (Toyobo, Japan), and 0.5 μl (100 μmol/L) of each forward and reverse primer. The qPCR ran according to the temperature-time profile as following: denaturation of 95°C for 5 min, 40 cycles of 95°C for 30 sec, 60°C for 15 sec and 72°C for 32 sec, and dissociation curves analysis at 60°C to 95°C. The reactions were performed in an Applied Biosystems 7500 Real-Time PCR System (Life Technologies Corporation, USA), and data were collected and analyzed using SDS Software v1.3 for 7500 (Life Technologies Corporation).

Specificity test of qPCR condition

The specificity of the proposed qPCR condition, especially the designed primer pairs, was evaluated for *O. sinensis*-specific assay using end-point PCR amplification analysis. Conventional concentration of genomic DNA from *O. sinensis* (positive control) and double distilled water (negative control), together with other preponderant fungi *B. bassiana*, *P. hepiali*, *Paecilomyces* sp., *L. pui*, served for templates, respectively. After each qPCR operation, the primer pair and related program were estimated for specificity and sensitivity by analyzing the melting curves.

Calibration curve construction

High-quality DNA of *O. sinensis* was used as the standard sample to construct the calibration curve, and copy number of ITS gene was designated to measure the fungal biomass. The acquired DNA stock solution reached 390.5 ng/μl, whose copy number was determined 3.73×10^{12} and subsequently serially diluted by 10-fold increment (yielding solutions of 3.73×10^{11}, 3.73×10^{10}, 3.73×10^{9}, 3.73×10^{8}, and 3.73×10^{7}). On this basis, the regression equation and its curve were obtained by plotting C_T value vs. the nominal copy numbers of *O. sinensis* standard samples. The linearity relationship was demonstrated by a correlation coefficient (R^2) higher than 0.999.

The quantification assay of *O. sinensis* in caterpillars

Genomic DNA from different tissues of caterpillar was respectively manipulated in terms of the above qPCR-based primers and condition, and parasitical amounts of *O. sinensis* were calculated by conversation relation in the calibration curve. Three samples were collected in the habitat of *O. sinensis* in December, and each sample was analyzed in triplicate through the described qPCR method.

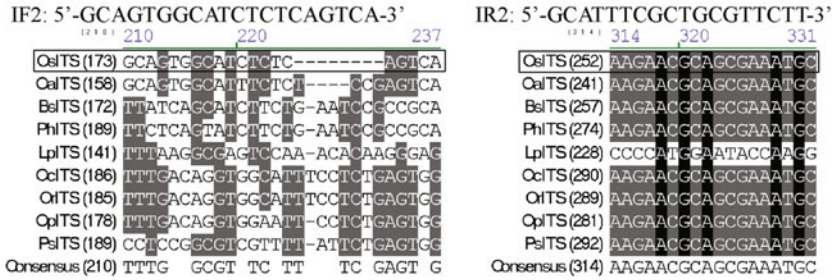

Fig. 1. Alignment of ITS sequences from *O. sinensis* and its related speices. The primers employed in this qPCR system were indicated in the panes. The GenBank accession nos. were listed as following: *O. sinensis* (HM135167), *Beauveria* sp. (HM135176), *P. hepiali* (HM135170), *Paecilomyces* sp. (HM135164), *L. pui* (JF794472), *O. ryogamiensis* (JN943345), *O. cuboidea* (JN943333), *O. appendiculata* (JN943325), and *O. prolifica* (JN943339).

Data analyses

SPSS software ver. 18.0 was used for statistical analyses with significance as $P<0.05$. Both Microsoft Excel 2003 and Microcal Origin 6.0 were operated in graphic drawing. In order to determine the relationship of C_T values and fungal biomass with DNA copy numbers, liner regression and determination coefficient were calculated using the Proc Reg method.

Results and Discussion

Development of designed qPCR primers

Both reliable primers and suitable amplification are of principle importance for any PCR-based assay. The ITS gene was chosen in virtue of its high conservation on the level of species, and used often as the genetic marker to identify the different fungal species (Wei *et al.*, 2006). Among the fungi in the current study, *Beauveria* sp., *P. hepiali*, *Paecilomyces* sp., and *L. pui* were the known dominant species in the habitat of *O. sinensis*, coupled with *O. ryogamiensis*, *O. cuboidea*, *O. appendiculata*, and *O. prolifica* as the relative species of *O. sinensis*.

Based on multiple alignment analysis of nucleotide sequences of OsITS and ITSs from other related species (Fig. 1), several variable regions were selected for the primer candidates. After *in silico* and experimental tests, a pair of specific primer was designed for real-time qPCR system, consisting of a forward (IF2: 5′-GCAGTGGCATCTCTCAGTCA-3′) and a reverse (IR2: 5′-GCATTTCGCTGCGTTCTT-3′) with 97 bp amplified product of *O. sinensis* species (Fig. 1). The crucial factor for the species specificity was the forward primer (IF2), which possessed of the unique oligonucleotide sequence to *O. sinensis* among nine testing species. Although a certain similarity was found between *O. sinensis* and *O. appendiculata*, the latter has not been found on the Tibetan Plateau.

Specificity of the qPCR assay

The theoretical specificity of the qPCR-based system was determined by comparing the desired sequence of ITS region with that available in the NCBI/GenBank Database. The computer tool BLASTn was used to search and align

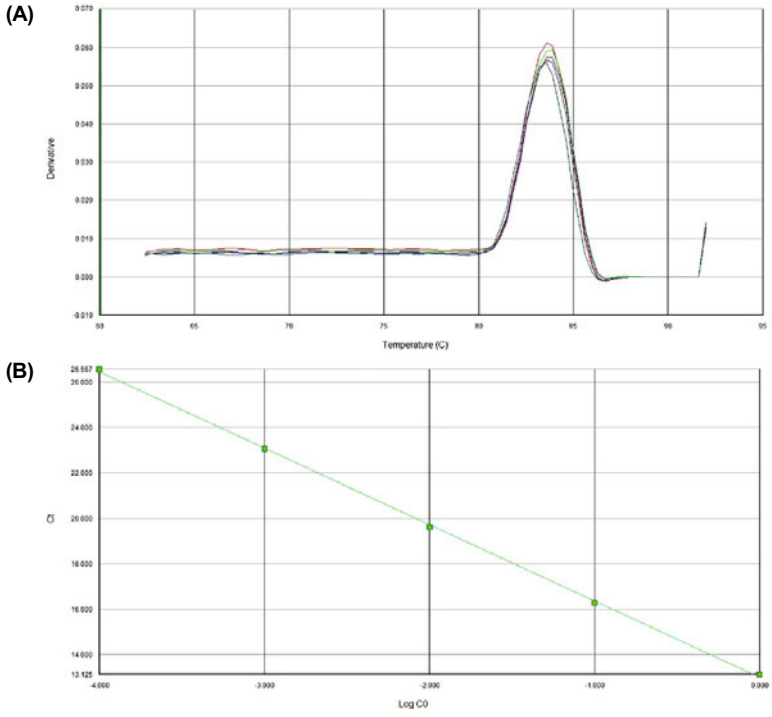

Fig. 2. Calibration curve construction. (A) Melting curves of the standard samples; (B) Calibration curve in the developed qPCR system.

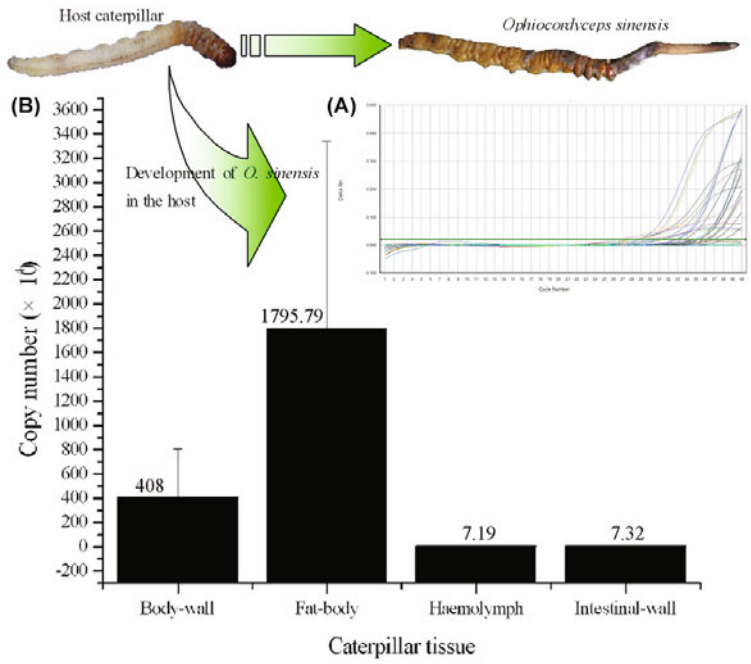

Fig. 3. Quantitative detection of *O. sinensis* in their host caterpillars. (A) Melting curves of the experimental samples; (B) The level of *O. sinensis* quantified with qPCR. C_T values were converted to copy numbers, which were shown on the column.

online, consequently showing no obvious homology with other fungi (data not shown). To increase reliability of detection and avoid false positive results, the designed primers (IF2 and IR2) were employed to amplify equivalent amount of purified DNA from *O. sinensis* and other related fungi, such as *Beauveria* sp., *P. hepiali*, *Paecilomyces* sp., and *L. pui*. After qPCR amplification reaction under the same condition, there were not any cycle threshold found in all of these concomitant species, indicating they could not disturb the *O. sinensis* assay. In contrast, as shown in the melting curve of *O. sinensis*, none of the melting peaks was observed due to unspecific products, primer dimers or hairpin. Besides, the amplification curve demonstrated the expression level and reactive condition to be suitable for *O. sinensis* assay.

System optimization and calibration curve

It is essential for the effectual qPCR system about high amplification efficiency of the target ITS segment and lack of any impure peaks in the melting curve. A series of experimental results were compared to examine the primers and initiative standard DNA. Here, 390.5 ng/μl of DNA solution was treated as the initiative sample, and the primers (IF2 and IR2) were validated for the *O. sinensis* assay. A calibration curve was established and exhibited a very good linear correlation between the fungal biomass and the C_T values (R^2 = 0.999419). Because of the possibility that just extremely low amount of *O. sinensis* colonizes in certain caterpillars, *O. sinensis* biomass may fall beyond the range of this curve, but the current R^2 coefficient was so high that the calibration curve enabled sufficient detection of *O. sinensis* biomass (Fig. 2).

Detection of *O. sinensis* in the host caterpillar

We developed an efficient procedure of DNA extraction and qPCR assay with significant specificity and sensitivity to rapidly detect the *O. sinensis*. After the quantitative assay, *O. sinensis* were detected in all caterpillar tissues (Fig. 3). The maximum content of *O. sinensis* colonized in the fat-body, and next came body-wall. Both of them were much larger than that observed in the haemolymph and intestinal-wall. Sampling and detection were carried out in December, 2010, just when caterpillars almost hibernated and thus few *O. sinensis* could enter the intestine and haemolymph as they fed. Meanwhile, the fat-body became the primary tissue of *O. sinensis* parasitization, because it was not only able to provide sufficient energy and nutrition for the development of *O. sinensis*, but also this fungus might parasitize the fat-body and then transferred to the next generation through the ovary (Suh *et al.*, 2001; Liang *et al.*, 2010).

In conclusion, a pair of species-specific ITS primers and their related qPCR program were developed with high reliability and efficiency. *O. sinensis* was detected rapidly in the host caterpillars, and this lays the methodological foundation for the revelation of occurrence and developmental mechanism of *O. sinensis*. Besides, this method targeted to the qPCR assays may be useful for further investigation of microbial ecology of this fungus.

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