

# Genetic Evidence Disapproves Coevolution between *O.sinensis* and Host Insects from Qing-Tibetan Plateau

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## Original article

**Keywords:** co-evolution, *Ophiocordyceps sinensis*, ITS, COI

**Posted Date:** June 23rd, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-593174/v1>

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# Abstract

Host-parasite coevolution is driven by the selective pressures each partner conferring on the other to merely maintain parity with other evolving species. *Ophiocordyceps sinensis* represents one of excellent model host-parasite complex, allowing us to explore evolutionary relationship between host and parasite. Although previous studies have demonstrated coevolution between *O. sinensis* and host insects, the incongruence in phylogenetic relationship of both is incompatible. In this study, we used ITS gene of *O. sinensis* and COI gene of host insects to evaluate the phylogenetic relationship, estimate the divergence time, infer demographic history, analyze identity by state distance matrix, and simultaneously explore evolutionary relationship or address the evolutionary pattern. We found distinct phylogenetic topology between *O. sinensis* and host insects from the main distribution of China. Additionally, the branch length ratio, demographic inference, and IBS analysis consistently suggested independent evolution between *O. sinensis* and host insects rather than arms race coevolution. However, populations of both *O. sinensis* and host insects from Qinghai lake showed similar evolution rate and phylogenetic topology, which might be associated with the formation of Qinghai Lake. Together, the data presented here provided new insights into host-parasite evolutionary relationship.

## Key Points

*O. sinensis* and host insects evolve across the main land of Tibetan Plateau with only exception found in Qinghai Lake.

The differentiation of *O. sinensis* is at least partially associated with uplift of Tibetan Plateau.

## Introduction

Host-parasite coevolution is a special form of interactions including reciprocal adaptive genetic changes in the particular host and parasite species. It is driven by the selective pressures each partner conferring on the other, in the form of evolutionary arms race (Milutinovic et al. 2016; Neiman and Fields 2016).

Parasitic fungi and their particular host insects are the most pervasive coevolution pattern detected in natural world (Joop and Vilcinskis 2016). For example, *Tribolium castaneum* is resistant to *Beauveria bassiana* infection by secreting microbicides (Pedrini et al. 2015); *Drosophila melanogaster* exits substantial genetic variation of in resistance to infection of *Entomophthora muscae* (Wang et al. 2020).

One of the most prominent parasitic fungi is *Ophiocordyceps sinensis* (syn. *Cordyceps sinensis*), a genus of ascomycete (Sung et al. 2007). It parasitizes mainly on the larvae of *Thitarodes* and converts it into sclerotium, from which fungus fruiting body grows (Wang and Yao 2011; Zhang et al. 2012). Arms race is believed to occur between *O. sinensis* and its host, due to the comparable correlations between genetic distance and latitude-dependent distribution of both fungi and host, using *ITS* sequences of *O. sinensis* and *COI* sequences of the host insects from the same complex of host cadaver and stroma (Quan et al. 2014b; Zhang et al. 2015). Alternatively, neutral evolution could drive similar

correlation between genetic distance and geographic distance in both host-parasite species. Moreover, this fungal invasion occurs rarely in nature, which succeeds only moderately in experimental conditions via multiple assisted methods (Li et al. 2019; Peng et al. 2013). Reciprocal selective forces should hardly influence the whole populations, let alone drive arms race. Therefore, it was reasonable to interrogate the driving force of interactions during the evolution of *O.sinensis* and *Thitarodes* each.

## Materials And Methods

### Samples

Here, we collected 29 *Ophiocordyceps sinensis* samples from Qinghai, and Yunnan. A 504 bp length polymerase chain reaction product within ITS region was amplified for *O.sinensis* using primers designed in previous research (White et al. 1990) (5'-F: TCCTCCGCTTATTGATATGC; 3'-R: GGAACAATGCTGAAAATGAAGG). We also obtained 617 bp length *COI* sequences from corresponding host insects with our designed primers (5'-F: AATTGGTTAAATTCTACTAATG; 3'-R: CAATAGGAGGAAATCGACCTT). For specified topology and wider coverage in geographic analysis, we combined an additional 33 *ITS* and 33 *COI* sequences downloaded from NCBI website, with accession Nos. listed Table S1. The integrated samples covered the main habitats in China (**Fig. 1a**).

### Phylogenetic analysis

These sequences were assembled and checked with SeqMan7.1 (Burland 2000). All of them were analyzed in MEGAX including alignment, computing pairwise distances and constructing the phylogenetic tree using maximum-likelihood method based on Kimura 2-parameter model (bootstrap 1000) (Edgar 2004; Kimura 1980; Kumar et al. 2018).

### IBS analysis between fungal and host insects

We used the pheatmap package in R to draw the heatmaps of ITS and COI genes based on the IBS distance matrix to show the IBS correlation within each fungal sample and corresponding infected insect and observe the differentiation within and among different groups of *O.sinensis* and host insects (Tibetan Plateau, Qinghai Lake, Nyingchi, Yunnan). Then we conducted correlation analysis *using Spearman method in R* to observe the correlation of IBS distance between *O.sinensis* and corresponding host insect.

### Analysis of the genetic structure

After alignment, we used the software DNAsp v6 to calculate the DNA polymorphism and visualize the single nucleotide polymorphism (SNP) sites (Rozas et al. 2017). Principle component analyses were performed using GenAIEx v6.502 through the SNP of ITS and COI sequences (Peakall and Smouse 2012).

### Divergence time estimates

To estimate the divergence time of either *O. sinensis* and host insects, we used the Bayesian method implemented in the program BEAST2 v2.6.2 (Barido-Sottani et al. 2017; Bouckaert et al. 2019).

For analysis of *O. sinensis*, the fossil evidence of *Paleoophiocordyceps coccophagus* (99-105million years ago, Mya) was used as the crown calibration point (Kumar et al. 2017; Sung et al. 2008; Zhang et al. 2015). For host insects, we used a standard insect mtDNA clock of 2.3% pairwise sequence divergence per million years because there are no fossil records or unambiguous biogeographic events that could be used to calibrate the host insect tree (Brower 1994; Zhang et al. 2015).

The relaxed uncorrelated lognormal clock model and the strict clock model was used for the fungus data set and the host insect data set, respectively (Zhang et al. 2015). We set the Calibrated Yule model for fungus data set (Heled and Drummond 2012) and Coalescent Constant Population for the host insect data set as the tree priors (Heled 2012). Markov Chain Monte Carlo (MCMC) analyses were run for 10 million generations with parameters sampled every 2000 generations. Convergence of the runs was assessed using Tracer v1.7.1 to analyze the output of BEAST, which indicated most parameter valued had effective sample size well above 200 (Rambaut et al. 2018). Then TreeAnnotator v2.6.2 was used to calculate the mean ages of all the nodes, the corresponding 95% highest posterior density interval (HPD) ranges and the posterior clade probability for each node with the target tree type of Maximum clade credibility tree (Bouckaert et al. 2014). Finally, the chronogram was visualized using FigTree v1.4.4 (<http://tree.bio.ed.ac.uk/software/figtree/>).

## Demographic inference

The historical population dynamics of *O. sinensis* and host insects were inferred by coalescent-based Bayesian skyline plots (BSP) implemented in BEAST2 v2.6.2 (Bouckaert et al. 2019; Drummond et al. 2005). To plot the population history, load the log file in Tracer v1.7 (Rambaut et al. 2018) and select the menu Analysis/Bayesian Skyline Reconstruction.

# Results

## Genetic structure and phylogenetic relationship

Non-parameter principal component analysis (PCA) manifested four genetic clusters for stroma samples (*O. sinensis*) corresponding to their geographic distribution: Tibetan Plateau group except Nyingchi (TPo), Qinghai Lake group (QLo), Yunnan group (YNo), and Nyingchi group (NCo) (**Fig. 1b**). This geographic-dependent separation remained partially in host samples (*Thitarodes*), such as Qinghai Lake group (QLt) and Nyingchi group (Nct). Exceptions were Yunnan group (Ynt) and Tibetan Plateau group except Nyingchi (TPt), which clustered with each other thoroughly (**Fig. 1c**). A parallel affinities were recovered in phylogenetic trees constructed via maximum-likelihood (ML) methods, using *Ophiocordyceps irangiensis* and *Ogygioses* as outgroup for stroma and host, respectively (**Fig. 1d-e**).

Coevolution between parasite and host is defined as the extent to which their phylogenetic trees are congruent (Legendre et al. 2002). In spite of several researches that demonstrated coevolution between *O. sinensis* and host insects (Zhang et al. 2015), we observed incongruous phylogenetic relationship within the complex of host cadaver and stroma. Specifically, stroma phylogeny inferred a close relationship between YNo and NCo, which formed a sister branch with TPo and QLo (**Fig. 1d**). Meanwhile, YNt clustered with TPt, which formed a sister branch with QLt. NYt was basal to all of them (**Fig. 1e**). Considering the geographic distance and the mobility of insects, this incongruence may infer a mixture event between YNt and TPt. Parasite and corresponding host driven by arms race should experience similar divergence time. However, the branch length ratio of TPo/NCo (1.04) was nearly 2 times to that of TPt/NCo (0.53), indicating different evolutionary trajectories with different time-scale in each *O. sinensis* and *Thitarodes*.

## Divergence time

To infer the causative factors that promoted divergence in *O. sinensis* based on ITS sequence and *Thitarodes* based on COI sequences, respectively, we estimated the time of the most recent common ancestor (TMRCA) for each divergence node, using the commonly used fossil calibration point and nucleotide substitution rate for fungal and insects, respectively. Specifically, for *O. sinensis*, the TMRCA of QLo and TPo was 1.15 Mya (**Fig. 1f**), coincide with Kunlun-Huanghe movements (1.2-0.6 Mya) (Li and Fang 1998). The TMRCA of all *O. sinensis* clades was 1.71 Mya (**Fig. 1f**), parallel to the period of Scene C of Tibetan movements (1.7 Mya) (Li and Fang 1998). Previous study also revealed that population divergence of *O. sinensis* was associated with latitude-based geographical variation (Quan et al. 2014a). These results indicated that the lifestyle of *O. sinensis* might be sensitive to latitude variance and their differentiation was at least partially influenced by Tibetan Plateau uplift exhumation events. The TMRCA estimates for host insects ranged from 0.303 to 0.0007 Mya (**Fig. 1g**), which were much younger than that of *O. sinensis*. This could be due to the absence of appropriate fossil calibration for insects, hereafter we did not consider the TMRCA of insects.

## IBS correlation between fungal and host insect

Another evidence of coevolution was previously illustrated as a positive correlation between genetic distance and geographic distance within *O. sinensis* and *Thitarodes*, respectively (Quan et al. 2014b). However, this could be a consequence of either neutral evolution or other adaptive evolution within each species. A direct evidence for coevolution could be a positive correlation between *O. sinensis* and *Thitarodes* from different geographic distributions. Here we assessed the identity-by-similarity (IBS) matrix within *O. sinensis* based on ITS sequence in Table S2 and *Thitarodes* based on COI sequence in Table S3, respectively. Ranked from lowest to highest genetic differentiation between *O. sinensis* within groups are Nyingchi, Yunnan, Qinghai Lake and Tibetan Plateau, while that between host insects within groups are Qinghai Lake, Yunnan, Tibetan Plateau and Nyingchi (**Fig. 1h-i**), showing that the fungal may not co-evolve with the host insects.

## Demographic inference

Parasite and corresponding host driven by arms race should experience similar divergence time and demographic histories. However, the branch length ratio of TPt/Nct (1.04) was nearly 2 times to that of TPo/NCo (0.53). Moreover, BSP inference (Drummond et al. 2005) inferred a conspicuous increase of effective population size in *O.sinensis* and a moderate reduction in *Thitarodes* (Fig. 1j-k). This discrepancy of dynamic demography further indicated rare influence of arms race on the evolution of interactions between *O.sinensis* and *Thitarodes*.

## Discussion

Although evidences mentioned above disapproved coevolution between *O.sinensis* based on ITS gene and its host *Thitarodes* based on COI gene for the major part of their habitats, Qinghai Lake stood out as an exception. QLo formed a unique clade that diverged from the lineage to TPo, after the separation of NCo lineage (Fig. 1d). The same phylogenetic topology occurred in Qt (Fig. 1e). Moreover, the branch length ratio of QLo/NCo was 0.67 in *O.sinensis*, which was comparable to Qt/Nct in host insects (0.71). The IBS of QHo and QHt are higher correlated (Fig. 1l). Additionally, both QLo and Qt showed similarly constant population size since 0.0006 Mya (Fig. 1k). Collectively, these evidences suggested a possibility that there could be some factors affecting both *O.sinensis* and *Thitarodes* simultaneously and therefore driving similar evolutionary pattern.

As mentioned above, the divergence of *O.sinensis* was sensitive to latitude. Considering that the Qinghai Lake basin was fractured from the northwest to the southeast during the QTP uplift in the late Tertiary (Madsen et al. 2008), it was reasonable to assume that the divergence of QLo was associated with the occurrence of Qinghai Lake. Indeed, we estimated the divergence time of Qinghai Lake group to be 1.15 Mya, which was consistent with the formation of Qinghai Lake (2-0.2 Mya) (Madsen et al. 2008). During the middle Pleistocene, vast crustal movements caused the area to sink substantially, forming the lake (Madsen et al. 2008). The regional climate, rainfall and plant distributions in this area may be shaped by the lake and the topography of the basin. Therefore, both *O.sinensis* and its host insects in this region may evolve parallelly due to the long-term geographical isolation under neutral evolution, or even the adaptation to the distinct ecological environment.

In conclusion, we found incongruent phylogenetic topology between *O.sinensis* and host insects *Thitarodes* from the main distribution of China. This together with varied branch length ratio, different demographic dynamics, and uncorrelated IBS matrix consistently suggested independent evolution between *O.sinensis* and *Thitarodes* rather than coevolved arms race. The appearance of Qinghai Lake might promoted island isolation and thus drove the divergence of both *O.sinensis* and *Thitarodes* around Qinghai Lake from other lineages.

In our study, we used the ITS sequence of *O.sinensis* with 504bp length and COI sequence of *Thitarodes* with 617bp length to analyze, leading to the results being considered tentative. However, previous study has revealed that although the sequence is short, the results are relatively reliable if the sample size is

large enough (Ma et al. 2020). Consequently, our study was limited, but it also provided new insights into host-parasite coevolution.

## **Declarations**

### **Funding**

This work was supported by the Strategic Priority Research Program of the Chinese Academy of Sciences (XDA2004010302), Second Tibetan Plateau Scientific Expedition and Research (STEP) Program (2019QZKK0501), National Natural Science Foundation of China (31860305), Sino-Africa Joint Research Center, Chinese Academy of Sciences (SAJC201611), and Research Center for Ecology and Environment of Central Asia, Chinese Academy of Sciences. Y.L. was supported by the Young Academic and Technical Leader Raising Foundation of Yunnan Province.

### **Conflicts of interest**

The authors declare no competing interest.

### **Availability of data and material**

All data generated or analyzed during this study are included in this published article and its supplementary information files.

### **Ethics approval and consent to participate**

Not applicable

### **Consent for publication**

Not applicable

### **Contributions**

ST provided the samples used in study; XL conducted the experiments of DNA extraction, amplification and sequencing; ZG analyzed data and wrote the manuscript; YL provided funds, conceived, and revised the manuscript. All authors read and approved the final manuscript.

### **Acknowledgements**

This work was supported by the Strategic Priority Research Program of the Chinese Academy of Sciences (XDA2004010302), Second Tibetan Plateau Scientific Expedition and Research (STEP) Program (2019QZKK0501), National Natural Science Foundation of China (31860305), Sino-Africa Joint Research Center, Chinese Academy of Sciences (SAJC201611), and Research Center for Ecology and Environment of Central Asia, Chinese Academy of Sciences. Y.L. was supported by the Young Academic and Technical Leader Raising Foundation of Yunnan Province. Samples used in this study were provided by Prof. T.Sha,

State Key Laboratory for Conservation and Utilization of Bio-resources in Yunnan, School of Life Sciences, Yunnan University, Kunming, China. We thanked X.X Guo (Henan University, China) for drawing the samples distribution map.

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# Figures

Fig. 1

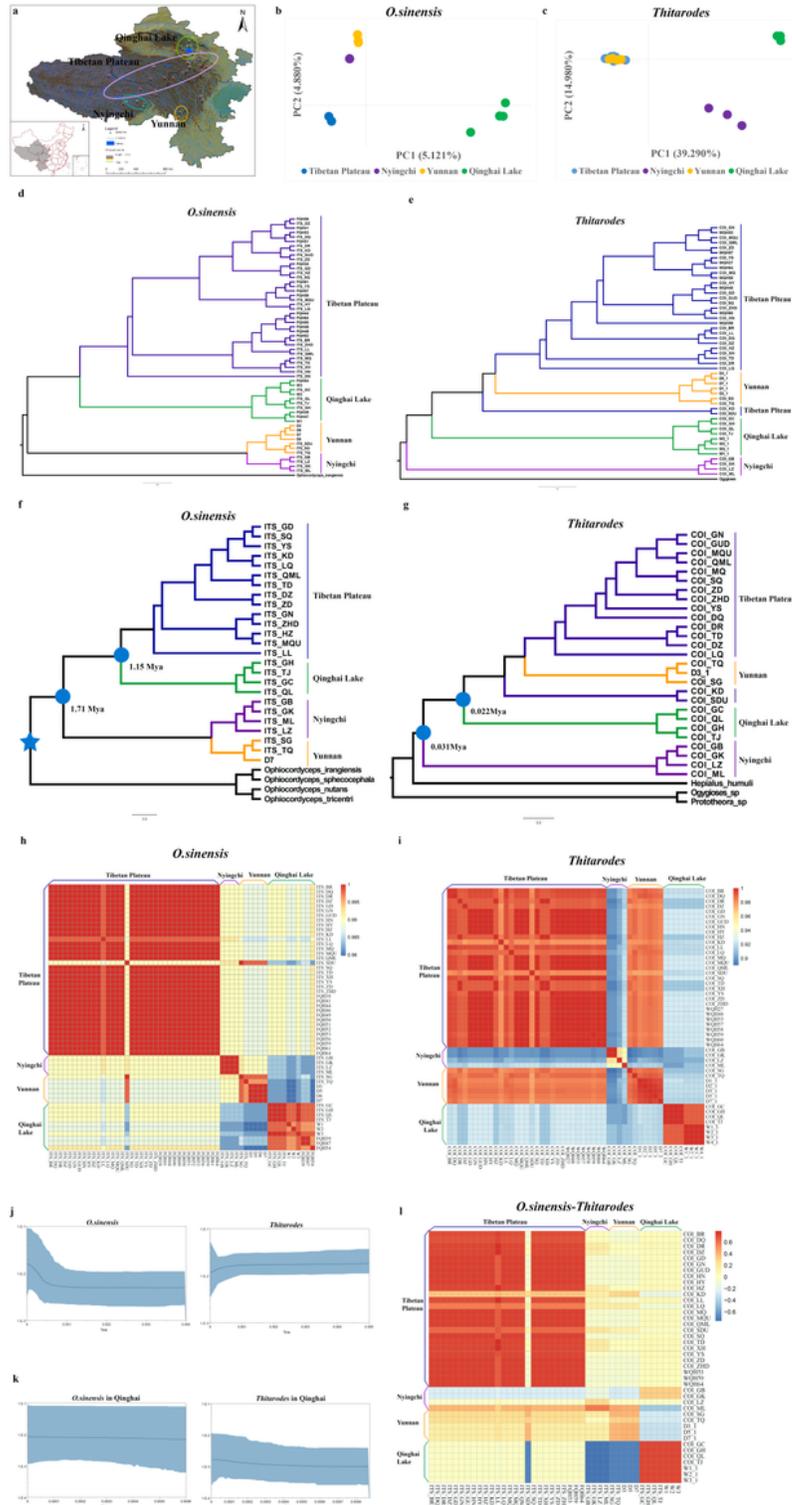


Figure 1

The phylogenetic and evolutionary relationship between *O. sinensis* and host insects. a Distribution of *Ophiocordyceps sinensis* samples used in the study. The pink ellipse represents the interior and northeast of Tibetan Plateau; the light blue ellipse represents Nyingchi; The green ellipse represents the north of

Qinghai (around the Qinghai Lake); and the orange ellipse represents Yunnan. b, c Principal components analysis (PCA) showing PC1 against PC2 using SNP of ITS and COI to show the genetic structure of *O.sinensis* (left) and host insects (right), respectively. d, e The phylogenetic tree of *O.sinensis* constructed by ITS dataset (left) and the phylogenetic tree of host insects constructed by COI dataset (right) using Maximum Likelihood method. f Estimation of the divergence time for *O.sinensis* based on the ITS data set. The fossil evidence of *Paleoophiccordyceps coccophagus* reported by Sung et al. (2008) was used as a crown calibration point for *Ophiocordyceps* (star). The nodes indicate divergence time between populations. g Estimation of divergence time for host insects based on the COI dataset. The age of host insects was calibrated by the standard insect mtDNA clock. The nodes indicate divergence time between populations. h Heatmap of the relationship within and among *O.sinensis* from different regions based on IBS distance as shown by the color scale bar on the right. i Heatmap of the relationship within and among host insects from different regions based on IBS distance as shown by the color scale bar on the right. Each cell represents an individual of the *O.sinensis* and host insects. j, k Demographic inference inferred by coalescent-based Bayesian skyline plots. j Bayesian skyline plots (BSP) of *O.sinensis* (left) and host insects (right). k Bayesian skyline plots (BSP) of *O.sinensis* (left) and host insects (right) in Qinghai Lake. The X-axis shows time in millions of years before present. The Y-axis (logarithmic scale) indicates effective population size estimates multiplied by generation time. The dashed line represents the median estimate of the effective population size, with the 95% high posterior density interval shown in blue. l IBS correlation was assessed using Spearman method between *O.sinensis* and corresponding host insects (seen in Table S4 and S5). The heatmaps of IBS correlation between each fungal sample and corresponding infected insect based on IBS distance as shown by the color scale bar on the right. Each cell represents an individual of the *O.sinensis* (x axis) and corresponding host insects (y axis). \*The abbreviation of samples was given in Table S1.

## Supplementary Files

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