IDENTIFICATION OF *TOLYPOCLADIUM PARADOXUM* AND *OPHIOCORDYCEPS YAKUSIMENSIS* FROM CHINA

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ABSTRACT

Two *Cordyceps* species pathogenic to cicada were collected from Zhejiang province, eastern China and identified using morphological and molecular approaches. Morphological characters showed that they were similar to *Tolypocladium paradoxum* and *Ophiocordyceps yakusimensis* respectively. Phylogenetic analyses of *18S-28S rDNA* internal transcribed spacer ITS4/ITS5 sequence or nuclear ribosomal large subunit (26S rDNA) D1/D2 domain sequences analyses further confirmed the morphological identification results respectively

Keywords: *Tolypocladium paradoxum*, *Ophiocordyceps yakusimensis*, *Cordyceps*, phylogeny

INTRODUCTION

The genus *Cordyceps* was earlier classified in the Clavicipitaceae, Clavicipitales, and then reclassified into three families, viz., *Clavicipitaceae*, *Cordycipitaceae* and *Ophiocordycipitaceae* of Hypocreales [1]. Its members are mostly invertebrate pathogens, some of them infect other fungi, mainly *Elaphomyces*, and a few species infect plants [2]. Since its discovery as vegetable fly (well explained by W. Watson in 1763) which was later on classified by Fries as *Cordyceps sobolifera* (the most popular cicada fungus), many interesting specimens have been found [3] and some species are also recently found in China [4-7].

Isaria cicadae (Vegetable cicada), mainly produced in southern forest areas of China, is considered as a famous Chinese herbal medicine. A survey was conducted on the distribution of *Isaria cicadae* resources in Zhejiang bamboo groves and *Isaria cicadae* was found growing on *Platylomia pieli*. Two other types of *Cordyceps* sp. were also found.

To clarify the taxonomy of these two species, detailed studies on the morphology of their teleomorphs and anamorphs were carried out, and comparison was made on their phylogenetic relationships using sequences of two regions of rDNA [internal transcribed spacer ITS4–5.8S-ITS5 or large subunit (LSU) D1/D2].

MATERIALS AND METHODS

Materials

The specimens13AJ6 and 13AJ28 were collected in Anji County, Zhejiang Province, China in 2013, and the isolates obtained were named as BA0332 and BA0351 respectively. The specimens and isolates are deposited in the Mycological Collection of BioAsia Institute of Life Sciences.

Morphological examinations

Morphological observations were conducted by light microscopy. To measure teleomorphic structures such as perithecia, asci, and part spores, fresh materials were mounted in one drop of water on glass slides and observed with an Olympus BX51 microscope using CellSens Dimension software. Microscopic examinations of anamorphic characters were made from cultures maintained on Czapek agar and potato sucrose agar (PSA) at 25 ^{o}C in the dark for more than 14 d.

DNA Extraction

For DNA isolation, the stromata were washed with distilled water and cut to collect contamination-free tissue. Cultured isolates were incubated in potato sucrose broth (PSB) in shake flask at 25 °C for 1 week to collect mycelium. Total DNA was extracted, using N96 Plant Genomic DNA Kit (Tiangen, Beijing, China), according to the manufacturers' instructions.

PCR

The internal transcribed spacer (ITS) of rDNA was amplified by polymerase chain reaction (PCR) using Taq DNA Polymerase (Tiangen, Beijing, China) as a single fragment with the standard primer pairs ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [8] for specimen 13AJ6 and isolate BA0332. Amplification was performed as follows: pre-denaturation at 95°C for 5 min, denaturation 94 °C for 30s, annealing 56 °C for 30s, extension at 72 °C for 50s, 30 PCR cycles, and then extension at 72 °C for 7 min. The divergent D1/D2 domain of the LSU rDNA gene was amplified with primers NL1 (52 -GCATATCAATA AGCGGAGGAAAAG-32) and NL4 (52 -GGTCCGTG TTTCAAGACGG-32) (Cletus and Christie, 1998)[9] for specimen 13AJ28 and isolate BA0351. Amplification was performed as follows: 36 PCR cycles with annealing at 52 °C, extension at 72 °C for 2 min, and denaturation at 94 °C for 1 min.

The PCR products obtained were purified with a Gel/PCR DNA fragments extraction kit (Tiangen, Beijing, China).

Molecular Phylogenetic Analyses

The sequences were obtained from both the type specimen and strains, and submitted to GenBank blasting. Sequences were edited in ClustalX ver. 1.83 software [10]. ClustalX was used to generate the evolutionary distances and the similarity values and to perform the neighbor-joining (NJ) analysis from *K*nuc values. A Neighbor Joining tree (NJ tree) was constructed using Mega 4. The percentages expressed above the branches were frequencies with which a given branch appeared in 1000 bootstrap replications [11]. Species included in this study and other GenBank accession numbers of internal transcribed spacer ITS4–5.8S-ITS5 or large subunit (LSU) D1/D2 were shown in Table 1.

RESULTS AND DISCUSSION

Morphological characters of *Tolypocladium paradoxum* (Kobayasi) Quandt,Kepler & Spatafora

Stroma raising from host of head, fleshy, dark brown or purple brown, erect, solitary, 1-2 root, mostly 1 root, unbranched, clavate or oblate, surface war, fertile portion clavate, long 26-48 mm, thick 4-11 mm; stem cylindrical shape, light brown, long 9-45 mm, thick 5-7 mm, without infertility top (Fig. 1 A).

Perithecia ovate to elliptic, brown, 520-690 × 250-360 μm, vertical deep buried. The wall thickness 23.0-33.5 μm, orifice nearly circular, diameter 34-80 μm (Fig. 1 B). Ascus serpentine, a pointed end, (200) 300-340 × 6-11 μm; ascus cap hat shaped, deeply lobed, orange, 4.2-8 μm high, 5.5-9 μm thick (Fig. 1 C). Ascospore ribbon shaped, numbering 8, which fractured after maturing. Secondary ascospores short rectangular, colorless, 4-7 × 2-3 μm.

In the medium of PSA, colonies are round velvet, pale yellow or abaxially white, the diameter reached 29.1mm after 14 d at 25□ (Fig. 2 A). On Czapek medium white, it reached 19.4mm after 14 d at 25□ (Fig. 2 B). Conidiophores erect, separated 1-2 branches, solitary or wheel was born in the fertile hypha, 13.2-52.5×1.2-2.6 μm, sporogenous cells cone, located in the top coremium, 5.0-10.8 ×1.2-2.3 μm. Conidia polyhedron like, 2.5-4.7 × 2.4-3.7 μm (Fig. 2 C).

There are a few *Elaphocordyceps* species known to be pathogenic on cicada, namely *E. inegoensis*, *E. paradoxa*, and *E. toriharamontana*. However, *E. inegoensis* has perithecia superficial or apparently half-immersed, stalks cylindrical, almost with same diameter with fertile part. *E. toriharamontana* has perithecia rectangularly immersed, oblong, with somewhat long neck (Kobayasi and Shimizu, 1963)[12]. The genus *Elaphocordyceps* and *Chaunopycnis* are emended

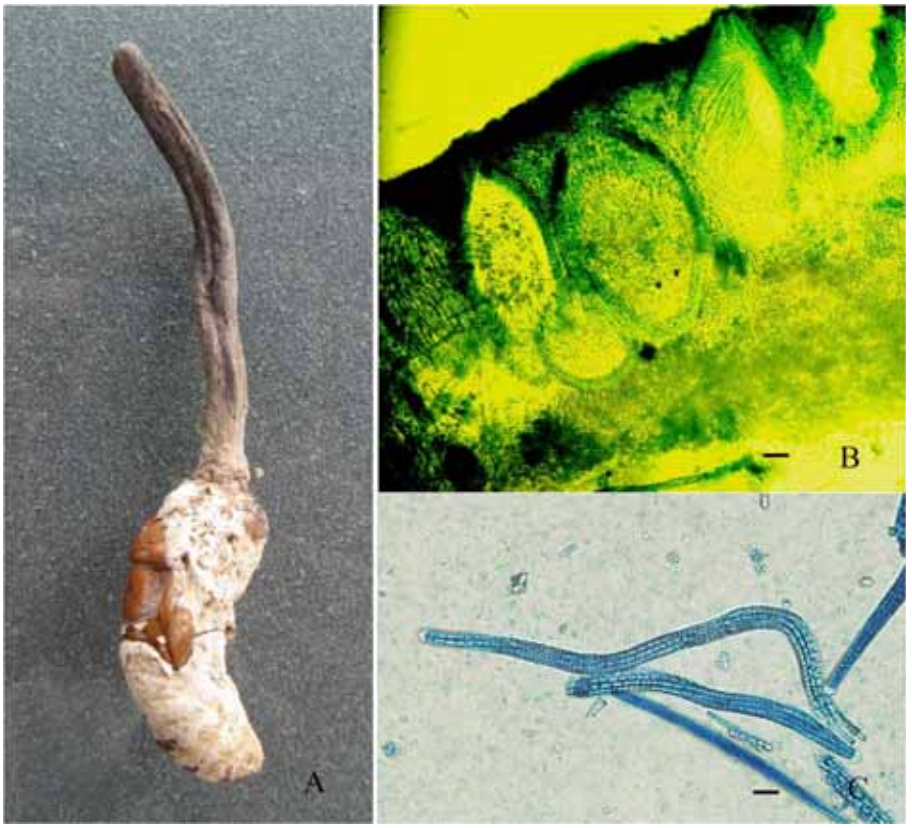

Figure 1. A: Specimen of AJ6; B: Perithecia Bar=100 µm; C: Ascus Bars=10 µm

Figure 2. Colonies of the isolate BA0351 on PSA and Czapek media. A: Colony on PSA medium; B: Colony on Czapek medium; C:Conidiogenous structure of BA0351, Bar=10 µm

as new combinations within the genus *Tolypocladium* [13] recently, here we named the specimens 13AJ6 and isolate BA0332 as *Tolypocladium paradoxum.*

Morphological characters of *Ophiocordyceps yakusimensis* (Kobayasi) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora

Stroma 8-11 cm long, 1.5-2 mm thick, stalk simple or dichotomous with short branch on the upper part, slender cylindric, irregularly contorted (Fig. 3 A). Perithecia vertical buried, spindle shaped or long elliptic, long 620-790 µm, wide 120-200 µm, wall thickness of 22-40 µm; orifice circular, diameter 48-97 µm, center slightly convex (Fig. 3 B). Ascus cylindrical, the lower end acuminate, 180-300 × 3-6.5 µm; ascus cap is flat globose, 3-5 ×5-7 µm (Fig. 3 C). Secondary ascospores short column, 2-3.5× 1-1.8 µm.

Colonies grow slowly on PSA, smooth, gray white, the old culture produced a large number of conidia, appearance silt like yellow, diameter 9.7 mm after 14 days at 25 °C (Fig. 4 A). On Czapek medium, colonies smooth, gray white, 4.9 mm after 14 days at 25 °C (Fig. 4 B).

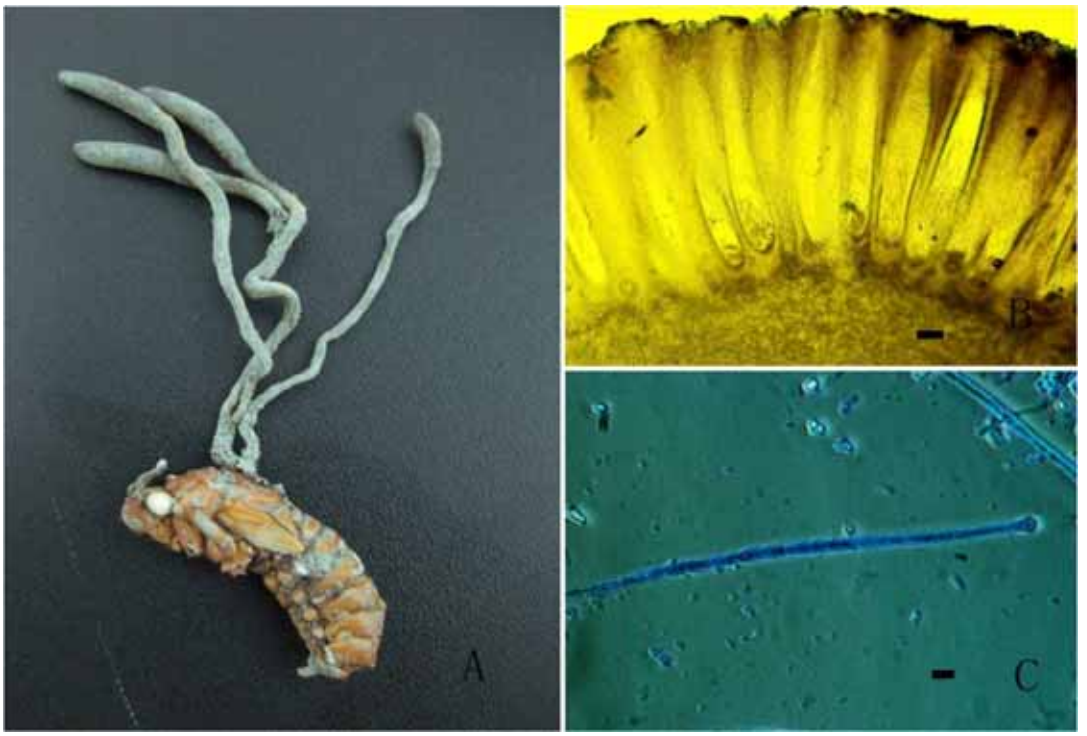

Figure 3. A: specimen of AJ28; B: perithecia Bar=100 µm; C: ascusBar=10 µm

Conidiophores sympodial branched, surface rough, 75-261 × 3.8-6.8 µm. Sporogenous cells ellipsoid, with dilated base, 5.0-10.9 × 2.5-5.3 µm, and a slender neck, 1.50-2.50 µm (Fig. 4C). Conidia are solitary, long shuttle shape, both ends slightly, long oval, colorless, 5.4-10.0×2.5-4 µm (Fig. 4 D).

There are a few *Ophiocordyceps* species known to be cicada pathogens, namely *O. sobolifera*, *O. longissima*, *O. heteropoda*, and *O. yakusimensis.* However, *O. sobolifera* stroma is generally single on head of host, fertile part not distinct from stalk, stromata clavate, usual with conidial form as side branches. *O. longissima* fertile part is distinct from stalk, perithecia ovoid. *O. heteropoda* stroma is thick cylindrical, fertile part ovoid, ascospore 6-7.7 × 0.9-1 µm.

Phylogenetic relationships of specimen/ isolateBA0332 *Tolypocladium paradoxum*

DNA fragments of approximately 600 to 700 bp were amplified by PCR using primers ITS4 and ITS5. The fragment contained ITS4-5.8S-ITS5 regions of rDNA. A fragment of ITS4-5.8S-ITS5 rDNA nucleotides was submitted to GenBank. A BLAST search in NCBI (www.ncbi.nih.gov/blast) showed these sequences to be most similar to *Tolypocladium* sp. KF696558.1 (99%) and *Elaphocordyceps paradoxa* KF356192.1 (99%). A phylogenetic tree was generated from 7 aligned sequences with an equal character. This tree showed that both the specimen and the fungal isolate locate in the same clade with other reference fungal sequences from *Tolypocladium* sp. KF696558.1 and *Elaphocordyceps paradoxa* KF356192.1 (Fig. 5).

Phylogenetic relationships of isolate BA0351*Ophiocordyceps yakusimensis*

DNA fragments of approximately 600 to 700 bp were amplified by PCR using primers NL1and NL4. The fragment contained the LSU D1/D2 regions of rDNA. A fragment of LSU rDNA nucleotides was submitted to GenBank. A BLAST search in NCBI showed this sequence to be most similar to *Ophiocordyceps yakusimensis* KF836760.1 (99%). A phylogenetic tree was generated from 8 aligned sequences with an equal character. This tree showed that both the specimens and the fungal isolate locate in the same clade with other reference fungal sequences *Ophiocordyceps yakusimensis* KF836760.1 (Fig.6). The tree shows *Ophiocordyceps yakusimensis, Metacordyceps yakusimensis, Ophiocordyceps longissima* and *Ophiocordyceps sobolifera* have very closely relatedness.

Figure 4. Colonies of the isolate BA0351 on PSA and Czapek media. A: Colony on PSA medium; B: Colony on Czapek medium; C: Conidiogenous structure of BA0351,D: Conidia of BA0351; Bar=10 µm

CONCLUSION

Morphological characters and phylogenetic analyses confirmed that the two species of *Cordyceps* were *Tolypocladium paradoxum* and *Ophiocordyceps yakusimensis*.

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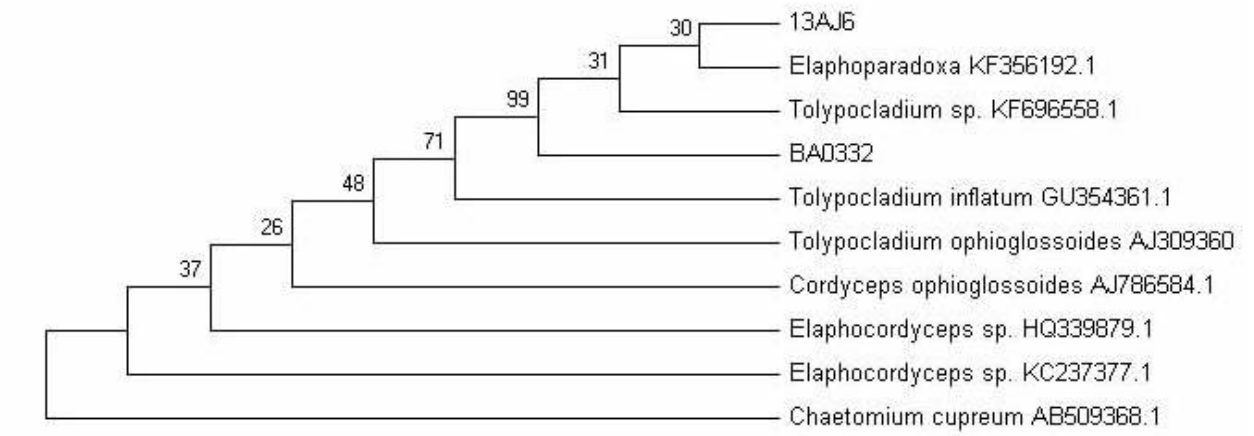

Figure 5. Phylogenetic tree of *Tolypocladium paradoxum* speciemen 13AJ6 and isolate BA0332 and its allies using Clustal X by NJ analysis based on ITS4-5.8S-ITS5 rDNA sequences.

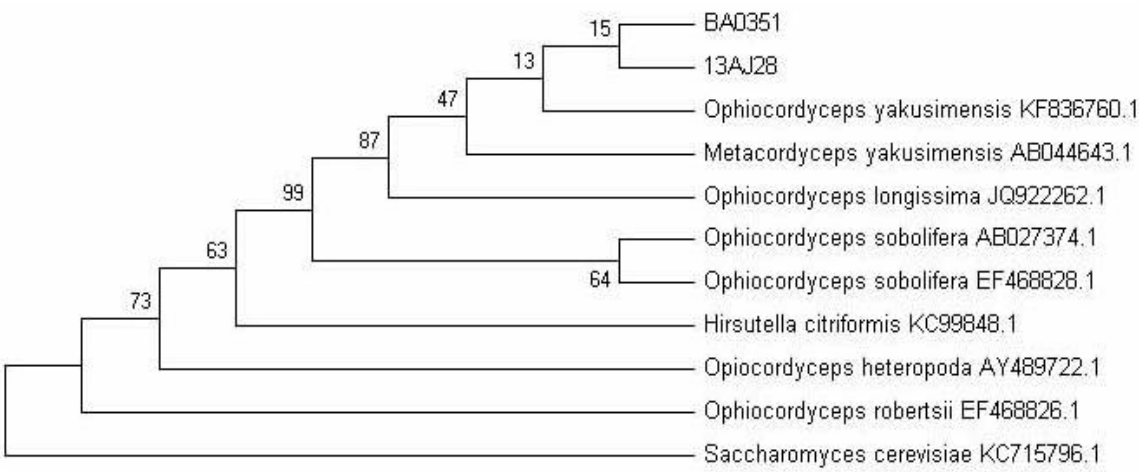

Figure 6. Phylogenetic tree of *Ophiocordyceps yakusimensis* specimen 13AJ28 and isolate BA0351 and its allies using NJ analysis based on LSU D1/D2 rDNA sequences

Table 1 *Cordyceps* and related species and their NCBI accession numbers used in this study.

Species	**GenBank accession no.**	
	Internal transcribed spacer ITS4/ITS5 sequence	**Large subunit (LSU) D1/D2 rDNA sequences**
Tolypocladium sp. YPC-2013	KF696558.1	
Cordyceps ophioglossoides	AJ309360	
Elaphocordyceps sp. 20124794d	HQ339879.1	
Elaphocordyceps paradoxa	KF356192.1	
Tolypocladium inflatum strain KVL 07-62	GU354361.1	
Cordyceps ophioglossoides	AJ786588.1	
Elaphocordyceps sp.20124521a	KC237377.1	
13AJ6	This study	
BA0332	This study	
Chaetomium cupreum	AB509368.1	
Ophiocordyceps yakusimensis strain BA0024		KF836760.1
Ophiocordyceps sobolifera strain KEW 78842		EF468828.1
Ophiocordyceps longissima strain EFCC 6814		JQ922262.1
Ophiocordyceps sobolifera		AB027374.1
Metacordyceps yakusimensis		AB044643.1
Ophiocordycepsbertsii strain KEW 27083		EF468826.1
Ophiocordyceps heteropoda		AY489722.1
13AJ28		This study
BA0351		This study
Hirsutella citriformis strain INIFAP-Hir-2		KC911848
Saccharomyces cerevisiae strain JN1N-17		KC715796.1

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