Xuefeng Cordyceps: insights into species diversity, life cycle and host association


1School of Life Sciences, Huaibei Normal University, HB 235000, AH, China
2Engineering Research Center of Southwest Bio-Pharmaceutical Resources, Ministry of Education, Guizhou University, GY 550025, GZ, China
3Centre of Excellence in Fungal Research, Mae Fah Luang University, CR 57100, Thailand
4Department of Health Sciences, Faculty of Science, University of Mauritius, Reduit 80837, Mauritius
5Institute of Chinese Materia Medica, Hunan Academy of Chinese Medicine, CS 410013, China

Cordyceps (= Cordyceps sensu lato) fungi are rather well known as entomopathogens and widespread distribution throughout China have been recorded earlier. However, their taxonomy, hosts, life cycles and the plants on which the host insects reside are poorly understood, and have sometimes led to confusion in the proper identification of the species. In this study, Xuefeng Cordyceps collected from Xuefeng Mountain, Hunan, China are identified and an assessment of their life cycles, proper hosts and plants they are associated with is made through morphological and DNA sequence-based analyses. Morphological characterization reveals that Ophiocordyceps xuefengensis from this region as commonly accepted by mycologists and local people. Species collected also include Beauveria bassiana (sexual and asexual), Metacordyceps taii (syn. of Metarhizium guizhouense), Ophiocordyceps macroacicularis and Ophiocordyceps ramosissimum. Our result challenges previous taxonomic arrangement with regard to host identity. The hosts, previously mistaken for larvae of Endoclita nodus, are corrected herein as larvae of Endoclita davidi (Lepidoptera: Hepialidae). Ribosomal DNA sequence analyses based on the ITS regions also confirm that Clerodendrum cyrtophyllum (Lamiaceae) is the plant on which the host insects reside. Morphological illustrations, where appropriate, are given for the cordyceps species, hosts and plants. The infection mechanism and life cycle of the cordyceps are also outlined.

Keywords: Clerodendrum cyrtophyllum, cordyceps, Endoclita davidi, traditional Chinese medicine.

*For correspondence. (e-mail: tingchiwen@yahoo.com)
The voucher specimens are deposited in the following institutions: (i) GACP, the Herbarium of Guizhou Agricultural College, Guiyang, Guizhou, China. (ii) GZUH, the Herbarium of Guizhou University (the original Herbarium of Guizhou Agricultural College, GACP). (iii) MFLU, Centre of Excellence in Fungal Research, Mae Fah Luang University, Chiang Rai, Thailand.

Total genomic DNAs were extracted from sclerotia of the cordyceps and leaves of the plants to confirm fungus and plant identification respectively, using Fungal DNA MiniKit (E.Z.N.A.™, Omega Biotech, CA, USA). The primer pairs ITS4/ITS5 were used for PCR amplification and sequencing of ITS gene of the Cordyceps and plants. The PCR products were sequenced (GenScript Biotechnology Co., Nanjing, China). Alignments were checked visually and improved manually with care according to sequence diagrams. ClustalX1.81, ContigExpress (Invitrogen, Carlsbad, CA, USA), Chromas230 (http://www.technelysium.com.au/chromas.html) and Mega6.06 biology software were used for sequence assembly and alignment.

Blast searches were performed to reveal the closest matches of the host plants in the GenBank database that would allow the selection of appropriate taxa for phylogenetic analyses. Maximum likelihood (ML) tree was generated using RAxML ver. 8.2.8, employing a GTRGAMMA model of nucleotide substitution; other details are provided in the literature. Maximum parsimony (MP) tree was constructed with PAUP*4.0b10 (ref. 14) and using the heuristic search option with TBR branch swapping and a bootstrap test of 1000 replicates; further details are outlined in the literature.

In the Xuefeng Mountain region, we have so far found five Xuefeng Cordyceps species. These were dug out from basal trunks or roots of the plant Cl. cyrrophyllum Turcz. (Lamiales: Lamiaceae), and their hosts are all larvae of Endocliita davidi Poujade (Lepidoptera: Hepialidae). The five species are recorded as follows:

1. **Beauveria bassiana** (Bals.-Criv.) Vuill. Botrytis bassiana Bals.-Criv., Spicaria bassiana (Bals.-Criv.) Vuill., Penicillium bassianum (Bals.-Criv.) Bourge. Sexual morph, Cordyceps bassiana Z.Z. Li et al. Material examined: Asexual morph – GACP 15101201, GACP 15101206 (Figure 2f); sexual morph – GACP 14071004, GACP 16080601 (Figure 2e).

   Notes: Though the asexual *B. bassiana* and the sexual *C. bassiana* are conspecific, herein we habitually separate them for discussions. *B. bassiana* is common in the wild, but it has seldom been found in trunks or roots of plants. Notably, in all entomopathogenic fungi, only *B. bassiana* and *O. sinensis* are included in the Chinese Pharmacopoeia. Unlike the common *B. bassiana*, *C. bassiana* is rarely collected in the wild. A third *C. bassiana* specimen (GACP 16041002) also grew on a Hepialidae larva, but it was dug out from soil (Guizhou, China) instead of from a tree trunk or root. The sequencing results further substantiate that *C. bassiana* is the sexual morph of *B. bassiana*.


   Notes: *M. guizhouense* was originally isolated from a dead Hepialidae larva in Guizhou, China. *Metarhizium taii* and its asexual morph *Metarhizium taii* also on Lepidoptera larvae from Guizhou. Sung et al. introduced the genus *Metacordyceps* and revised *C. taii* to *Metacordyceps taii*. Kepler et al. revised *Metacordyceps* as a synonym of *Metarhizium*. Huang et al. treated *Metarhizium taii/C. taii* as synonyms of *Metarhizium anisopliae* (Metschn.) Sorokin based on ITS sequence data, but these placements were rejected by Bischoff et al.
who treated Metacordyceps taii/Metarhizium taii as synonyms of M. guizhouense based on a multilocus phylogeny. Evidences from hosts, geographical distributions, morphologies and molecular phylogeny are all supportive of M. guizhouense, Metacordyceps taii (C. taii) and Metarhizium taii to be conspecific.

Metacordyceps taii is widely distributed in the southwestern provinces of China and has a high yield, especially in Guizhou. This species can be collected all year round in Guizhou, but its fruiting body stage only occurs between May and August. Generally it grows from heads of larvae or pupae of Hepialidae in the soil. This study shows that Metacordyceps taii can also grow in tree trunks or roots.

(3) Ophiocordyceps macroacicularis S. Ban, T. Sakane & Nakagiri, 2015 (ref. 25). Material examined: GACP 15092001, GACP 15092002, GACP 15092003, GACP 15092004. These four specimens were dug out within the same plant. Specimens from other regions: GACP SG0301–GACP SG0312 (12 specimens), March to May 2016, Guizhou, China, growing from heads of larvae of Endoclita sp. living in basal tree trunks or roots, collected by local people.

Redescription – Sexual morph (Figure 3): Stromata – 1–2, seldom fasciculate, slender and long, cylindrical at base, then gradually thinning upwards, unbranched, relatively smooth, brown, 80–220 × 0.1–2.5 mm, growing from heads of lepidopteran larvae living in basal tree trunks or roots. Stipe – lower part (below middle) sterile, sometimes distal part also sterile due to being too slender. Perithecia – superficial, vertically placed to the stipe, ovoid or compressed, apices a little coned, yellowish-brown to brown, covering middle to sub-distal part of the stipe, but sparse or absent on distal part, 390–820 × 250–440 μm (537.4 × 324.7 μm on average). Asci – hyaline, cylindrical, eight-spored, 240–310 μm long (267.6 μm on average), apical cap conspicuous and thick, 5.0–7.2 (5.6 μm on average) in diameter. Ascospores – hyaline, needle-shaped, septate indistinctly, but 10–20 septa barely visible in old–mature specimens, 200–300 × 2.0–3.0 μm (253.8 × 2.5 μm on average); no parts of spore observed. Asexual morph: Hirsutella-like25,26.

Hosts: Growing from heads of larvae of Endoclita (Hepialidae) or Cossidae living in basal tree trunks or roots, such as E. davidi living in Cl. cyrtophyllum, or Cossida sp. living in Reynoutria japonica Houtt. (Polygonaceae)25. Distribution: China (Hunan (new record), Guizhou); Japan (Kyoto)25.

Notes: Our collections were identified as O. macroacicularis based on two reasons: (i) morphologically, apart from only 1–2 stromata, our collections are closely similar to O. macroacicularis from Japan25; (ii) ITS sequences of our collections (GACP 15092001, GACP 15092002 and GACP SG0301) and O. macroacicularis from Japan are highly similar (differences only one or two DNA bases).

Ban et al.25 named O. macroacicularis to distinguish it from O. emeiensis (A.Y. Liu & Z.Q. Liang) G. H. Sung et al. (ref. 27) because the latter has only 1–2 stromata and
one distinctly different ITS sequence (AJ309347, uploaded in 2002). Notably, also having 1–2 stromata, our collections are more similar to _O. emeiensis_ 27. So we consider the unique ITS sequence of _O. emeiensis_ (AJ309347) may be problematic, and _O. macroacicularis_ may be a synonym of _O. emeiensis_.

(4) _Ophiocordyceps ramosissimum_ T.C. Wen, J.C. Kang & K.D. Hyde, 2014 (ref. 10). Material examined: MFLU 12-2165 (holotype), GZUH HN8 (Figure 2 c), GZUH 2012HN2, GZUH 2012HN10 and GZUH 2012HN12 (paratypes).

Notes: Morphologically, _O. ramosissimum_ can be easily differentiated from _O. xuefengensis_ and _O. macroacicularis_ by its multi-branch stromata, although the ossified insects (Figure 2 g) or immature fruiting bodies are similar and not easy to distinguish.

(5) _Ophiocordyceps xuefengensis_ T.C. Wen, R.C. Zhu, J.C. Kang & K.D. Hyde, 2013 (ref. 7). Material examined: GZUH 2012HN14 (holotype), GZUH HN13, GZUH 2012HN11, GZUH 2012HN13, and GZUH 2012HN19 (paratypes); GACP 16090901 (Figure 2 b), GACP 16092301 and GACP 16092302 (Figure 2 a).

Notes: For their special medicinal value, ancient Yao people called these strange herbs growing on caterpillars as ‘immortal herbs’ 6,28. Since the establishment of _O. xuefengensis_, the original name ‘immortal herbs’ has automatically been replaced by the new name ‘Xuefeng Cordyceps’. In other words, Xuefeng Cordyceps represent a group of Cordyceps species, and _O. xuefengensis_ is just one of them.

Figure 4 shows host insects of Xuefeng Cordyceps. Hosts of _O. xuefengensis_ and _O. ramosissimum_ were originally identified as larvae of _E. nodus_ (Chu & Wang) (= _Phassus nodus_ Chu & Wang) 7,10, and in subsequent literature, larvae of _E. nodus_ have routinely been treated as the hosts of Xuefeng Cordyceps.

Recently, in the Xuefeng Mountian region, we had dug out several hundred host specimens (including larvae, pupae and ossified insects) from tree trunks/roots. After morphological identification and comparison, the insects have been confirmed as only one _Endoclita_ species. Fortunately, some pupae had moulted into adults successfully. The huge size, unique spots on the fore wing and a distinct protrusion in the middle of the anterior margin of the fore wing (Figure 4 i), are typical characteristics which confirm that the species is _E. davidi_ (= _E. giganodus_ (Chu & Wang), = _P. giganodus_ Chu & Wang, = _E. nankini_ (Daniel), = _P. nankini_ Daniel; Figure 4) 29–31, instead of _E. nodus_ that had formerly been identified and reported.
Biology and ecology: The newborn larvae eat young tree trunks or twigs and dig tunnels within them, and several larvae can live together in one tunnel (Figure 4a and e). In the following 1–2 months they continue to eat in a downward direction and become larger. The entrance of each tunnel is covered by a large frass bag (Figure 4b–d, f). These large larvae are aggressive and attack/kill one another due to fierce competition; each tunnel can be occupied by only one larva. During night with no rainfall, wind and disturbance, and under relatively high temperature, the larvae crawl out and this is what makes them more susceptible to be in contact with the ascospores/conidia of Xuefeng Cordyceps. The old–mature larvae pupate and moult in tree roots (Figure 4d and h). Food shortage and disturbances can cause them to pupate and moult early, whereas lower temperature will delay their

Figure 4. *Endoclita davidi* and its ecology. a, Upper part of a tree trunk with young larvae. b, c, Frass bags produced by middle-aged larvae. d, Basal trunk. e, Newborn larvae. f, Middle-aged larva. g, Female (left) and male (right) pupae. h, Pupa slough. i, Female adult. j, Eggs.
Figure 5. *Clerodendrum cyrtophyllum*, the host plant of *Endoclita davidi* in the Xuefeng Mountain region, Hunan, China. a, Plant. b, Roots of the plant with *E. davidi* larva. c, Fruits.

Figure 6. Morphological changes of leaves versus base variation of ITS sequence of the *Clerodendrum cyrtophyllum* plants. a, Oval and short leaves. b, c, Long and fat leaves. d, Sharp and thin leaves. e–h, Partial sequencing results of ITS sequences of the corresponding plants (a–d) with accession numbers in GenBank as KU745290, KU745291, KU745292 and KU745293 respectively.

Moulting. Shortly after moulting, the insects can fly and the females begin to lay eggs (no need to mate). They lay eggs continually, especially when vibrating their wings or on being disturbed. One female can produce several thousands of eggs; the eggs are white at first, then become milky yellow and finally black (Figure 4j). The eggs fall on grasses, fallen leaves, humus layers and soil surface, but most of them will be eaten by predatory insects, mice, birds, etc. and only a few can successfully hatch. The insects overwinter as eggs or pupae and as a result adults can be seen two times (May and September) every year. A life cycle needs one year and the adults can live for 10–15 days. We presume that the insects are capable of parthenogenesis.

Unlike hosts of most entomopathogenic fungi, larvae of *E. davidi* live in tree trunks or roots instead of the soil, that are rarely influenced by changes in the external environment. Hence environmental factors (mainly to
temperature and humidity) are not the direct cause of insects being infected by Xuefeng Cordyceps. Given that the larvae are inherently aggressive, they are easily injured or even killed during fighting. This possibly renders them more vulnerable to infections and allow Cordyceps species to become opportunistic pathogens. This also explains why the infected hosts are all larvae of irregular instars instead of pupae or adults (Figure 2g), and the reason why several Cordyceps simultaneously grew in neighbouring tunnels (Figure 2a). Apart from fighting, other injuries can also cause the larvae being infected (Figure 2h).

Xuefeng Cordyceps grow slowly due to limited humidity in the plants, but they can continuously grow because both humidity and temperature are steady. Fruiting bodies of three Ophiocordyceps species can grow for 1–3 years in their plants, which is much longer than that of B. bassiana (sexual morph) and Metacordyceps taii. The fruiting body grows along the tunnel, until it reaches the entrance of the tunnel where it will begin to sporulate. As the largest species in Heptalidae, the huge larva of E. davidi can provide sufficient nutrition for the fungus to grow, and this possibly explains why Xuefeng Cordyceps are huge. Length of the fruiting body is dependent on the length of the tunnel, and as a result some fruiting bodies can even grow up to more than 40 cm.

Figure 5 shows host plants of the host insects. Proper identification of the host plants of E. davidi is always controversial. In the Xuefeng Mountain region, Huang observed three plant species but did not identify them properly. Wen et al. reported Cl. cyrtophyllum as the host plant, but did not give any further taxonomic and botanical information to properly validate host identity. Given the relative importance of Cordyceps in TCM or biocontrol, it is absolutely necessary to properly name the hosts and fungi concerned. In this study, a morphological comparison supplemented by DNA sequence-based data is implemented to better support any taxonomic arrangement proposed.

Depending on tunnels and frass bags produced by larvae of E. davidi, the host plants were confirmed and collected for identification. Morphologically, the plants are similar to Cl. cyrtophyllum, but leaf shapes of the plants vary distinctly and can be classified into three types: (i) oval and short (Figure 6a); (ii) long and fat (Figure 6b and c) and (iii) sharp and thin (Figure 6d). Molecular phylogenetic analysis was used to identify these plants.

DNA sequence analyses reveal that the host plants with different leaf shapes have exactly the same ITS sequence (only one DNA base difference, see arrow in Figure 6e–h), and phylogeny generated further confirms that the host plants are all Cl. cyrtophyllum (Figure 7). It is to be noted that in the Xuefeng Mountain region, other plants such as Rhus chinensis Mill., Loropetalum chinesum Oliv., Argyreia pereirana Bois and Paulownia sp. can also harbour larvae of E. davidi which can also crawl up to eat, but a short time later they will retreat. In trunks or roots of these plants, larvae of E. davidi have been rarely, and Xuefeng Cordyceps have never been discovered.

According to the present analyses, we can summarize that Xuefeng Cordyceps refer to Cordyceps fungi that grow on larvae of E. davidi living in basal trunks or roots of Cl. cyrtophyllum in the Xuefeng Mountain region, Hunan, China. These Cordyceps fungi are associated with B. bassiana (asexual and sexual morphs), Metacordyceps taii (syn. of Metarhizium guizhouense), O. macroaculatis, O. ramosissimum, O. xuefengensis, etc. rather than only one species, viz. O. xuefengensis as commonly accepted by mycologists and local people.

With similar chemical composition and anti-tumour effect to the important TCM O. sinensis, Xuefeng Cordyceps have increasingly been accepted by researchers around the world. Their market price, even the ossified insects alone (Figure 2g), has already risen to US$ 30,000/kg (dry weight) that is exactly the price of O. sinensis. What’s more, they are huge cordyceps, generally a dry specimen has 3–5 g, while O. sinensis has only 0.2–0.6 g each. On one occasion, 120 g Xuefeng Cordyceps had at one time been dug out from a big tree. Driven by the huge economic benefits, Cl. cyrtophyllum plants are being damaged excessively and yield of Xuefeng Cordyceps was drastically decreased in recent years. However, prevailing in low altitude unlike O. sinensis, artificial culture of Xuefeng Cordyceps will probably be realized in the near future.

Figure 7. Maximum likelihood (ML) tree of Clerodendrum cyrtophyllum and its allies inferred from ITS rDNA gene sequences. The ITS gene dataset comprised 654 characters (including gaps), of which 187 were variable and 123 were parsimony-informative. Bootstrap support values greater than 60% for ML and maximum parsimony (MP) are indicated above the nodes and separated by ‘/’ (ML/MP). KU745290, KU745291, KU745292 and KU745293 refer to the host plants with different leaf shapes.
Conflict of interest: The authors declare that they have no conflict of interest.


ACKNOWLEDGEMENTS. This work was supported by the National Natural Science Foundation of China (No. 31760014), and the Science and Technology Foundations of Guizhou Province, China (No. [2019]2451-3 and No. [2016]2863).