

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

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Cordyceps (= *Cordyceps sensu lato*) fungi are rather well known as entomopathogens and also for their medicinal importance in curing many diseases. Many cordyceps species with widespread distribution throughout China have been recorded earlier. However, their taxonomy, hosts, life cycles and the plants on which the host insects reside in 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.

XUEFENG Mountain (literal meaning ‘mountain with snowy top’, because its top is covered with snow for most

of the year) is located in west-central Hunan Province, China. It is 350 km long and 80–120 km wide with altitude varying between 240 and 1934 m amsl (Figure 1). The annual average temperature in this region is 12.7°C (varying from –10.1°C to 27.3°C), with relative humidity of more than 87% and abundant rainfall (annual average rainfall of 1810 mm). With heavy fog and frost, winter is rather dry and cold while summer is humid and cool¹.

Cordyceps (= *Cordyceps sensu lato*)² fungi are one of the most important groups of invertebrate pathogens^{2,3}. Currently, they include more than 900 species² belonging to three families (Cordycipitaceae, Ophiocordycipitaceae and partial Clavicipitaceae) in the order Hypocreales⁴. To date, more than 140 species are reported from China⁵. For a long time, Cordyceps research mainly focused on species diversity, medicinal values and biological control^{2–5}, however, biology, ecology and association with hosts are rather poorly understood.

The common name ‘Xuefeng Cordyceps’, previously known as ‘immortal herbs’, had originally been recorded in the middle or late Tang Dynasty (about 1200 years ago), and the species has been intensively used to cure various cancers and other diseases, improve the physique and extend human longevity⁶. Although considered as a precious traditional Chinese medicine (TCM) by local Yao people, this common name was not formally reported until *Ophiocordyceps xuefengensis* T. C. Wen *et al.*, the first species was introduced^{7,8}. Molecular phylogenetic analysis indicated that *O. xuefengensis* is closely related with the important TCM *Ophiocordyceps sinensis* (Berk.) G. H. Sung *et al.* (Dong Chong Xia Cao; winter worm, summer herb)⁷, also their hosts are all Hepialidae larvae⁹. From then on, the common name Xuefeng Cordyceps came into use, in spite of the second species *O. ramosissimum* T. C. Wen *et al.* being reported, which shares the same host insect that resides on the same plant as the former¹⁰.

Rather than a precious TCM, to a greater extent Xuefeng Cordyceps represent a kind of Chinese traditional culture. In this study, we analyse the species diversity of Xuefeng Cordyceps, and identify their host insects and plants on which the insects reside. We also study the biology and ecology of Xuefeng Cordyceps and their host insects. To recognize the regional cordyceps correctly, and inherit the Chinese traditional culture properly, this study provides important taxonomic, biological and ecological information.

Explorations were made in the Xuefeng Mountain region. Specimens, including the cordyceps, host insects (larvae and pupae), and plants on which the insects resided were observed in the wild and brought to the laboratory for identification. A camera (Nikon Coolpix P520) and a compound microscope (Nikon Eclipse 80i) connected with a camera (Cannon EOS 600D) were used to take photographs. Measurements were made using Tarosoft (R) Image Frame Work software.

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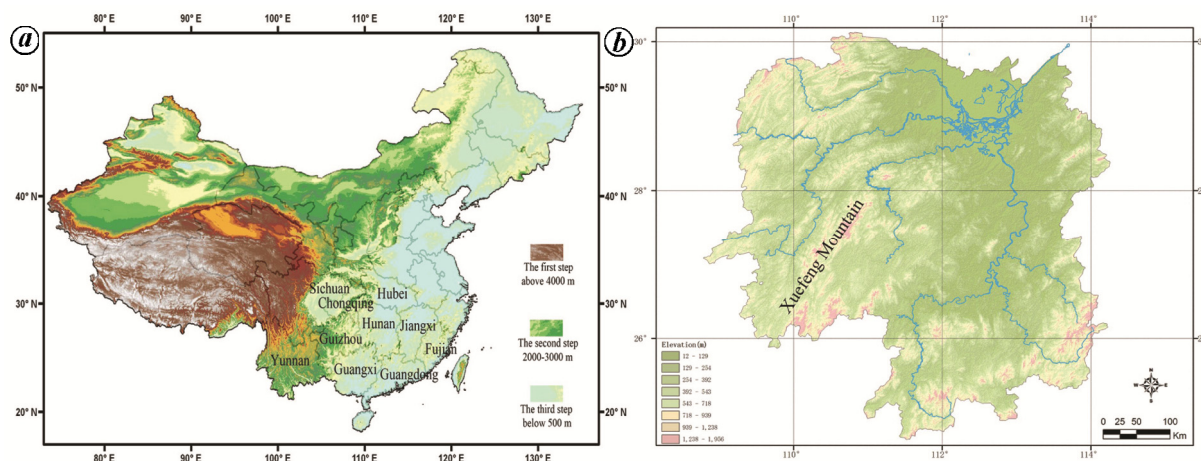


Figure 1. Topographic maps of China (a) and Hunan Province, China (b).

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.TM, Omega Biotech, CA, USA). The primer pairs ITS4/ITS5 were used for PCR amplification¹¹, 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^{12,13}. 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^{15,16}.

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. cyrtophyllum* Turcz. (Lamiales: Lamiaceae), and their hosts are all larvae of *Endoclita davidi* Poujade (Lepidoptera: Hepialidae). The five species are recorded as follows:

(1) *Beauveria bassiana* (Bals.-Criv.) Vuill.¹⁷. *Botrytis bassiana* Bals.-Criv., *Spicaria bassiana* (Bals.-Criv.) Vuill., *Penicillium bassianum* (Bals.-Criv.) Biourge. 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^{17,18}, 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*¹⁹. 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*.

(2) *Metarhizium guizhouense* Q.T. Chen & H.L. Guo^{20,21}. *Metarhizium taii* Z.Q. Liang & A. Y. Liu, *Metarhizium taii* var. *Chongqingensis* Y.H. Yang *et al.*, *Metarhizium taii* var. *taii* Z.Q. Liang & A.Y. Liu.

Sexual morph, *Cordyceps taii* Z. Q. Liang & A. Y. Liu, *Metacordyceps taii* (Z.Q. Liang & A.Y. Liu) G.H. Sung *et al.* Material examined: GACP 1229 (Figure 2d) and GACP 1230.

Notes: *M. guizhouense* was originally isolated from a dead Hepialidae larva in Guizhou, China²⁰. Liang *et al.*²² introduced *C. taii* and its asexual morph *Metarhizium taii* also on Lepidoptera larvae from Guizhou. Sung *et al.*⁴ introduced the genus *Metacordyceps* and revised *C. taii* to *Metacordyceps taii*. Kepler *et al.*²³ revised *Metacordyceps* as a synonym of *Metarhizium*. Huang *et al.*²⁴ treated *Metarhizium taii*/*C. taii* as synonyms of *Metarhizium anisopliae* (Metschn.) Sorokĭn based on ITS sequence data, but these placements were rejected by Bischoff *et al.*²¹,

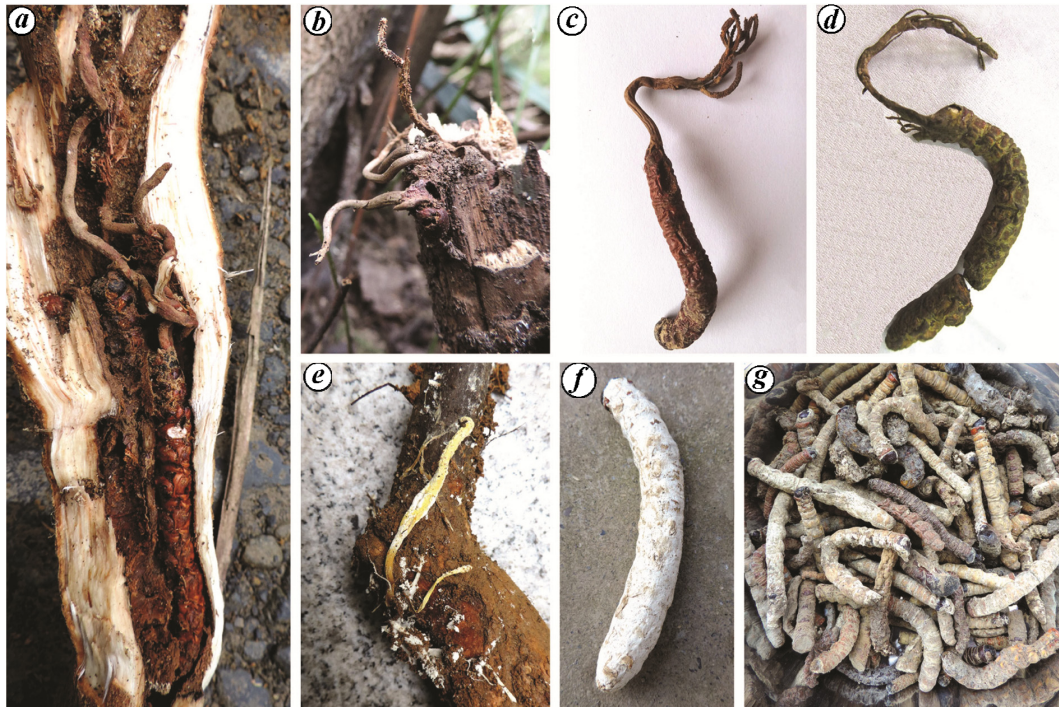


Figure 2. Species of Xuefeng Cordyceps. **a, b**, *Ophiocordyceps xuefengensis*. **c**, *Ophiocordyceps ramosissimum*. **d**, *Metacordyceps taii* (syn. of *Metarhizium guizhouense*). **e, f**, *Beauveria bassiana*, sexual and asexual morphs. **g**, Ossified insects.

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 Guizhou². This species can be collected all year around in Guizhou, but its fruiting body stage only occurs between May and August². 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 and 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 partspore observed. Asexual morph: Hirsutella-like^{25,26}.

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

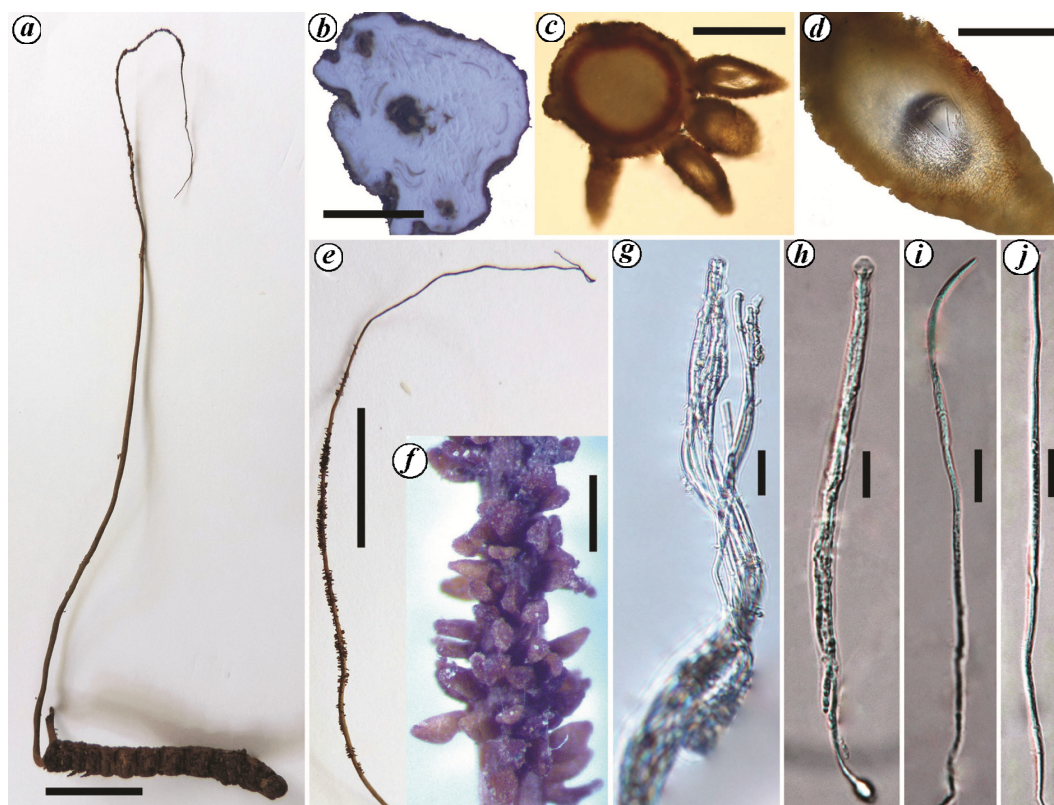


Figure 3. *Ophiocordyceps macroacicularis*. **a**, Overview of stroma and the host. **b**, Section of ossified insect. **c**, Section of stroma. **d**, Section of ascocoma. **e**, Distal part of stroma. **f**, Brown, superficial ascocoma on stroma. **g**, A bunch of asci twisted together. **h**, Mature ascus with ascospores. **i, j**, Hyaline and filiform ascospores with indistinct septa. Scale bar: **a, e** = 20 mm, **b** = 2 mm, **c** = 500 μ m, **d** = 200 μ m, **f** = 100 μ m, **g** = 40 μ m, **h-j** = 20 μ m.

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 2c), 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 2g) 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 2b), GACP 16092301 and GACP 16092302 (Figure 2a).

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 Mountain 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 4i), are typical characteristics which confirm that the species is *E. davidi* (= *E. gigantodius* (Chu & Wang), = *P. gigantodius* Chu & Wang, = *E. nankingi* (Daniel), = *P. nankingi* Daniel; Figure 4)^{29–31}, instead of *E. nodus* that had formerly been identified and reported.

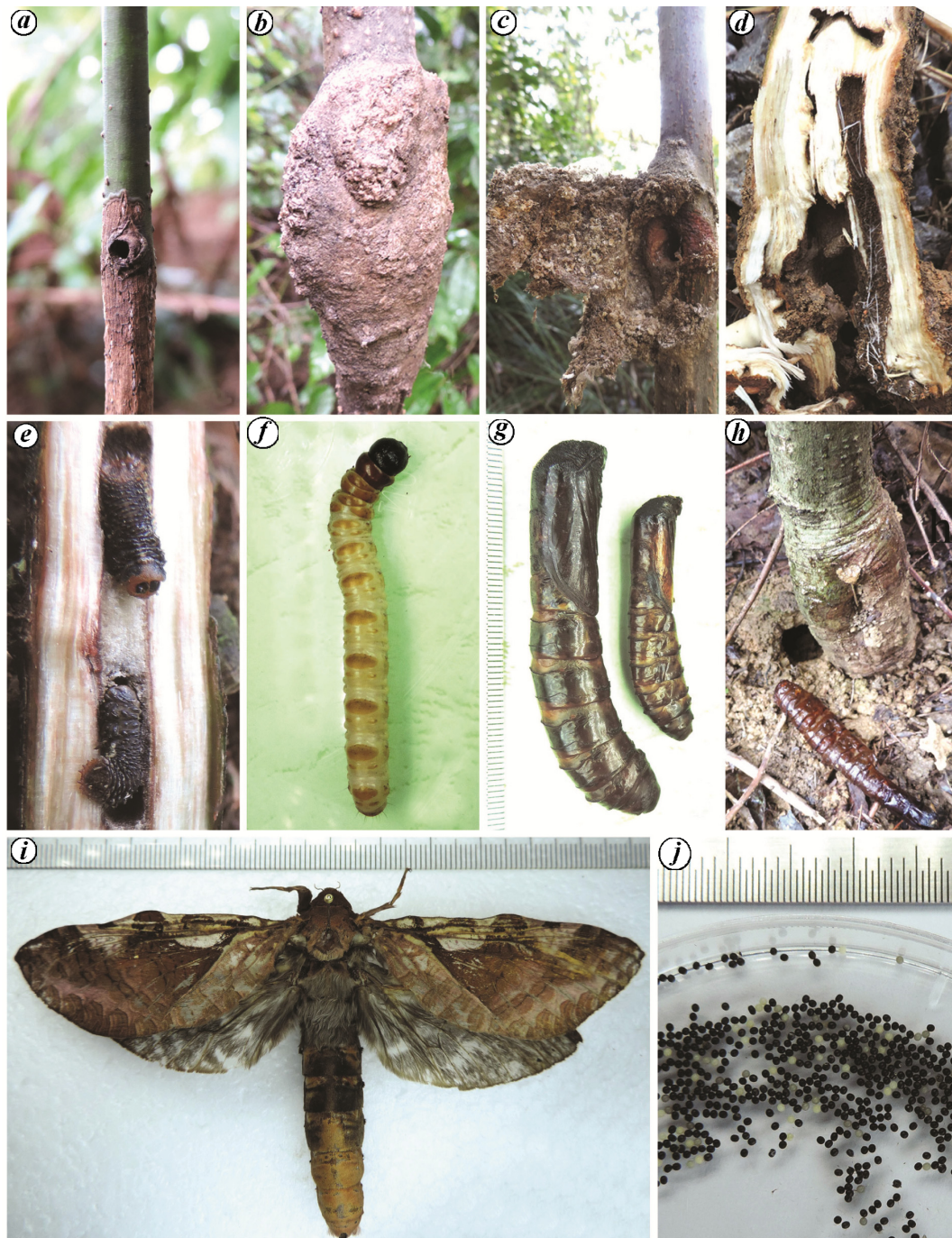


Figure 4. *Endoclita davidi* and its ecology. *a*, Upper part of a tree trunk with young larvae. *b*, *c*, Frass bags produced by middle-aged larvae. *d*, Basal trunk. *e*, Newborn larvae. *f*, Middle-aged larva. *g*, Female (left) and male (right) pupae. *h*, Pupa slough. *i*, Female adult. *j*, Eggs.

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 4 *a* 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 4 *b–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 4 *d* and *h*). Food shortage and disturbances can cause them to pupate and moult early, whereas lower temperature will delay their



Figure 5. *Clerodendrum cyrtophyllum*, the host plant of *Endoclit a davidi* in the Xuefeng Mountain region, Hunan, China. *a*, Plant. *b*, Roots of the plant with *E. davidi* larva. *c*, Fruits.

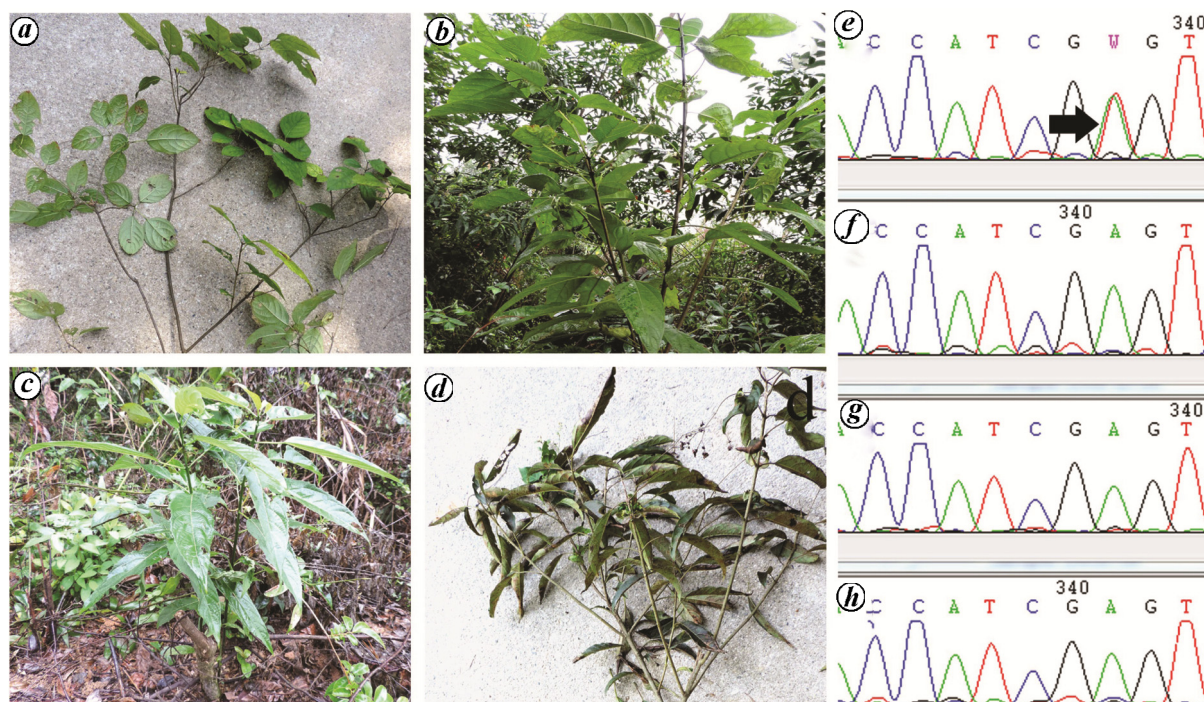


Figure 6. Morphological changes of leaves versus base variation of ITS sequence of the *Clerodendrum cyrtophyllum* plants. *a*, Oval and short leaves. *b*, *c*, Long and fat leaves. *d*, Sharp and thin leaves. *e-h*, Partial sequencing results of ITS sequences of the corresponding plants (*a-d*) with 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 4*j*). 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 in-

sects 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)³² 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 2b).

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 Hepialidae³¹, 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^{6,28} observed three plant species but did not identify them

properly. Wen *et al.*^{7,10} 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 plants³³.

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 chinense* Oliv., *Argyrea pierreana* 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. macroacicularis*, *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^{34–36} and anti-tumour effect³⁷ 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*². 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 tree⁶. 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.

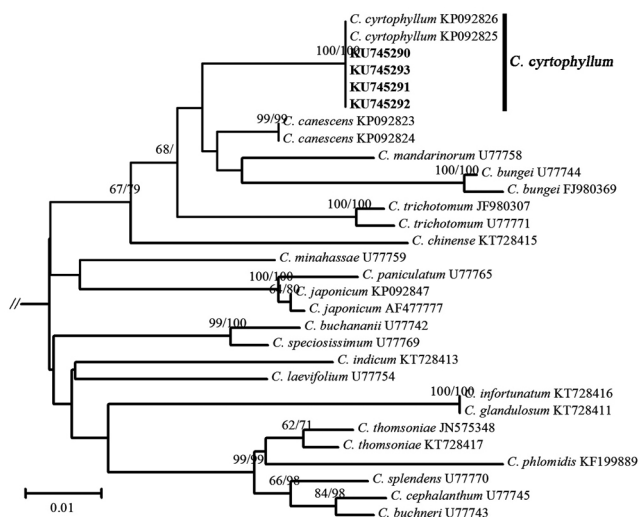


Figure 7. Maximum likelihood (ML) tree of *Clerodendrum cyrtophyllum* and its allies inferred from ITS rDNA gene sequences. The ITS gene dataset comprised 654 characters (including gaps), of which 187 were variable and 123 were parsimony-informative. Bootstrap support values greater than 60% for ML and 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.

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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).

Received 13 May 2016; revised accepted 8 July 2019

doi: 10.18520/cs/v117/i5/839-846