# *Stephensia*（Pyronemataceae，Pezizales），a New Record of Chinese Hypogeous Ascomyctete*

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Abstract：*Stephensia bombycina*，collected from Chenggong County，central Yunnan，China，is fruited by a plant of *Gastrodia elata*，with *Pinus yunnanensis* and *Corylus* sp. nearby. This is the first report of the genus *Stephensia* in China. The Chinese sample has the following key characters of *S. bombycina*：brownish tomentose surface of the ascomata，interior with meandering canals converging or communicating with a well-defined central cavity，globose，hyaline，eggutulate spores 18 – 25 μm in diam.，and peridium with a pseudoparenchymatous cortex. ITS sequence of the Chinese sample is 99% similar to those of North American and European samples and LSU sequence of this sample has only three base pairs different from that of the North American sample. Phylogenetically，this species is closely related to the hypogeous genus *Hydnocystis* and epigeous genus *Geopxyis*.

Key words：hypogeous acscomycete；*Stephensia bombycina*；taxonomy
CLC number：Q949.325 Document code：A Article ID：1672-3538（2016）02-0069-07
DOI：10.13341/j.jfr.2014.1111

# 中国地下子囊菌新记录属——史蒂芬块菌属*

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摘 要：文中首次报道了史蒂芬块菌属 *Stephensia* 在中国的分布。史蒂芬块菌发现于滇中地区呈贡县，生于 1 株栽培的天麻旁边，附近长有云南松和榛属植物。标本的特征与文献中对于该种的描述一致，即子囊果表面具褐色绒毛，产孢组织有曲折的脉沟，脉沟向中心辐聚，中心有时形成空腔，孢子球形，直径 18 ~ 25 μm，无油滴，包被（外囊盘被）角胞组织。中国标本的 ITS 序列与北美和欧洲样品有 99% 的相似性，其 LSU 序列仅有 3 个碱基与北美样品不同。史蒂芬块菌在分子系统上与腔囊块菌属 *Hydnocystis* 和地杯菌属 *Geopxyis* 近缘。

关键词：地下子囊菌；史蒂芬块菌；分类学

* **Funding**：The Joint Funds of the National Science Foundation of China and Yunnan Province Government（No. U1202262）；the National Natural Science Foundation of China（No. 30470011，31270075）；International Cooperation Yunnan Program of Innovation to Strengthen Provinces by Science & Technology（No. 2009AC013）；Key Laboratory for Plant Diversity and Biogeography of Eastern Asia，Kunming Institute of Botany，Chinese Academy of Sciences（No. 0806361121）

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**Date received**：2016-01-21

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**中图分类号**: Q949.325 **文献标识码**: A **文章编号**: 1672-3538(2016)02-0069-07
DOI: 10.13341/j.jfr.2014.1111
**引文格式**: 王晓进,刘培贵,王立松,等. 中国地下子囊菌新记录属——史蒂芬块菌属[J]. 菌物研究,2016,14(2):69-75.

The generic name *Stephensia* Tul. & C. Tul. has been applied to nine truffle-like taxa (Index Fungorum, http://www.indexfungorum.org/Names/Names.asp)[1-2]. Among them, *S. arenivaga* Cooke & Massee was excluded from the genus and used to erect *Elderia* McLennan by McLennan[3]. Another species, *S. varia* Rodway, was moved to *Labyrinthomyces* Boedijn (Tuberaceae) by Trappe[2]. *Stephensia crocea* Quél. was reduced by Fisher[4] to a variety of *S. bombycina* (Vittad.) Tul. & C. Tul. (type species of the genus), but this treatment is debatable due to the different colors and spore sizes between these two species[5]. De Varies[5] supposed *S. shanorii* (Gilkey) Gilkey to be conspecific with *S. crocea*, but molecular data[6] clearly showed that they are different. Consequently, now seven species should be considered in *Stephensia*: *S. bombycina*, *S. bynumii* Trappe et al., *S. colomboi* De Vito, *S. crocea*, *S. shanorii*, *S. peyronelii* Mattirolo and *S. sumatrana* Boedijn. The genus has a distribution in Europe, North America and Asia[2]. The phylogenetic positions of *S. bombycina* and *S. shanorii* have been tested by molecular data, and it showed that they are closely related with epigeous discomycetes *Geopyxis* (Pers.) Sacc. and *Tarzetta* (Cooke) Lambotte and hypogeous *Hydnocystis* Tul. & C. Tul. and *Paurocotylis* Berk. in Pyronemataceae[6-8]. *Geopyxis*, *Tarzetta* and *Hydnocystis* have been included in the Pyronemataceae mycota of China[9].

Key characters of *Stephensia* include brownish tomentose ascomata surface, truffle interior with meandering canals, globose smooth spores (except for *S. peyronelii* with ellipsoid spores), and non-amyloid asci[10] (http://www.natruffling.org/ascokey.htm). Outermost layer of peridium (ectal excipulum) can be of *textura intricata* (e.g. in *S. bynumii*[6]) or *textura angularis* (pseudoparenchymatous) (as in *S. bombycina* and *S. crocea*)[5,11-12]. Members of the genus might be confused with those of *Elderia* (Pezizaceae) or young ascomata of *Labycinthomyces* (Tuberaceae). *Elderia* was supposed to be congeneric with *Stephensia*[2], but molecular data showed that these two species were phylogenetically distinct[13].

In the early summer of 2015 several truffleascomata were accidentally harvested in a private garden near Kunming, Yunnan, when the owner was digging the rhizome of *Gastrodia elata* (Chinese famous herb medicine "Tianma"). After sequencing the sample and studying its morphology, we confirmed that it represents *S. bombycina*, a species originally described from Europe. Comparing ITS and 28S sequences obtained from the Chinese sample and retrieved from GenBank, we demonstrated the generic variation within *S. bombycina*. The morphological description and result on phylogenetic analyses are provided herein.

## 1 Materials and methods

### 1.1 Site description

The study site is located at Chenggong County, Yunnan, China (N24° 58′ 12.85″, E102° 51′ 57.99″, elevation 1 972 m). Six ascomata were found at 10 – 15 cm depth below the ground, around 30 cm away from the stem of a plant of Tianma (inoculated with mycelium of *Armillariella* sp.), which was planted one year ago. The six ascomata grew in a small group by a trunk of *Cupressus torulosa* that was cut off two years ago (Fig. 2-A). The site was on a shady slope, with a Yunnan pine (*Pinus yunnanensis*) five meters away and two hazels (*Corylus* sp.) 7 m away (in different directions). The substrate is red soil without fertilizing.

### 1.2 Morphological observation

Macroscopic observations are based on fresh and dried material. Driedascoma was sectioned with a stainless razor blade. Slides were prepared by mounting the tissue in 5% or 10% KOH. Flame-heating was used to remove bubbles in the tissue when necessary. Reactions were tested using Melzer's reagent and Cotton Blue. Slides were made by hand under a Leica L2 stereoscope and observed and photographed under a Leica DM2500 microscope

with a DFC450C camera installed in it. Thirty spores that come from different asci or dissociate outside the asci were measured from the mature ascoma (as judged by the presence of well-developed thick-walled spores).

### 1.3 DNA extraction, PCR and Phylogenetic analyses

Total DNA was extracted from driedgleba with a modified CTAB protocol [14]. The primer pairs ITS1F + ITS4 and LR0R + LR5 were used to amply the ITS region and part of the 28S respectively [15] (R. Vilgalys lab, http://www.biology.duke.edu/fungi/mycolab/primers.htm). PCR amplification was performed with Takara © DNA polymerase (Dalian, China) using the following protocol (25 μL reaction mixture): 2.5 μL buffer, 2.5 μL 0.1% BSA, 2 μL 2.5 mmol/L dNTPs, 0.5 μL 10 μmol/L of forward and reverse primers, 0.2 μL 5U μL Taq polymerase, 5 μL total DNA solution (419.4 ng/μL), and 12 μL $ddH_2O$. The following PCR programs were used: 5 min at 94.0℃, 38 cycles of 1 min at 94.0℃, 1 min 30 s at 53.0℃, and 2 min at 72.0℃, and a final extension of 72.0℃ for 10 min. The PCR products, collected by gel electrophoresis, were purified and sequenced at Sangon Biotech Corporation, Shanghai, China. Sequences were deposited in GenBank with accession numbers: KU556814 for ITS and KU556815 for 28S.

The generated sequences were firstly submitted to the Nucleotide Basic Local Alignment Search Tool (BLAST) to find sequences with high homology. To test the relationship between the Chinese sample and Trappe 3268 (whose 28S sequence in GenBank has 99% similarity with ours), we compared our ITS sequence with that of Trappe 3268 (GenBank accession: KU556813), which was generously provided by Dr. K. Hansen. Since the phylogenetic position of *S. bombycina* has been tested using 28S data [6,8], here we only made a simplified ITS dataset to show the ITS variation within *S. bombycina*. Besides those of *S. bombycina*, two samples of *H. piligera* (which was shown to be the closest relative of *S. bombycina* by Alvarado et al. [8]), two samples of *G. carbonaria*, one sample of *G. rehmii* Turnau (which was shown to the closest NCBI-hit of a *S. bombycina* sample by Stark et al. [16]), and two samples that might be conspecific with *G. rehmii* are included in the ITS dataset.

Alignments were made manually in BioEdit Version 5.0.9 [17]. Ambiguous site were totally gapped. Maximum Parsimony (MP) and Bayesian Inference (BI) analyses were performed to construct the ITS phylogeny. The dataset was not partitioned. MP analysis was conducted in PAUP* 4.0β10 [18], using a heuristic search with the following settings: starting tree(s) obtained via stepwise addition; addition sequences with random option of 1 000 replicates; tree-bisection-reconnection (TBR) branch-swapping; 1 000 replicates to calculate the bootstrap values in the consensus tree. BI analysis was conducted in MrBayes v3.2.1 [19]. GTR + I + G model was used and all parameter values, except branch lengths and tree topologies, were set unlinked. The BI analysis was conducted using four runs with four chains each for $1 \times 10^7$ generations sampling every $100^{th}$ tree. Runs were terminated when the average standard deviation of split frequencies went below 0.01. A majority rule consensus tree was built after discarding trees from a 25% burn-in. No outgroup was designated and the tree was rooted with midpoint.

## 2 Results

### 2.1 Sequences comparison and molecular phylogenetic analyses

In BLAST, there was 99% similarity between our 28S sequence DQ220435, which is from a Mexican sample Trappe 3268. There are three base pairs difference between the two sequences. There were two sequences with 99% similarity to our ITS sequence in BLAST. The two ITS sequences are from "uncultured *Geopyxis*" samples from orchid roots [16,20]: GQ223459 from root of *Gymnadenia conopsea* from Germany; the ITS sequence of HKAS 86978 has five base pairs different from this sequence; GU327418 from mycorrhiza of *Epipactis helleborine* from Czech; the ITS sequence of HKAS 86978 has four different base pairs from this sequence. Compared with the ITS sequence of Trappe 3268 (KU556813), that of HKAS 86978

(KU556814) has seven different base pairs, three of which are degenerated in KU556813.

The ITS dataset included 11 taxa and 626 characters. MP analysis generated three equally parsimonious trees (*CI* = 0.989, *RI* = 0.994, *RC* = 0.983), which only differed in the topology within *S. bombycina*. In the ITS trees generated by MP and BI analyses, the two un-identified "*Geopyxis*" samples from orchid roots, Trappe 3268 and HKAS 86978 formed a clade with MP-BP 96% and BI-PP 0.92. The two European samples from orchid roots were not grouped in a clade and instead, one of them formed a terminal clade with North American Trappe 3268 with low support in the BI analysis and one of the three equally parsimonious trees in the MP analysis (Fig. 1). The *S. bombycina* clade formed a sister of *H. piligera* clade including two Spanish samples with BI-PP 1.00 and MP-PP 100%. This relationship is consistent with that of Alvarado et al. [8].

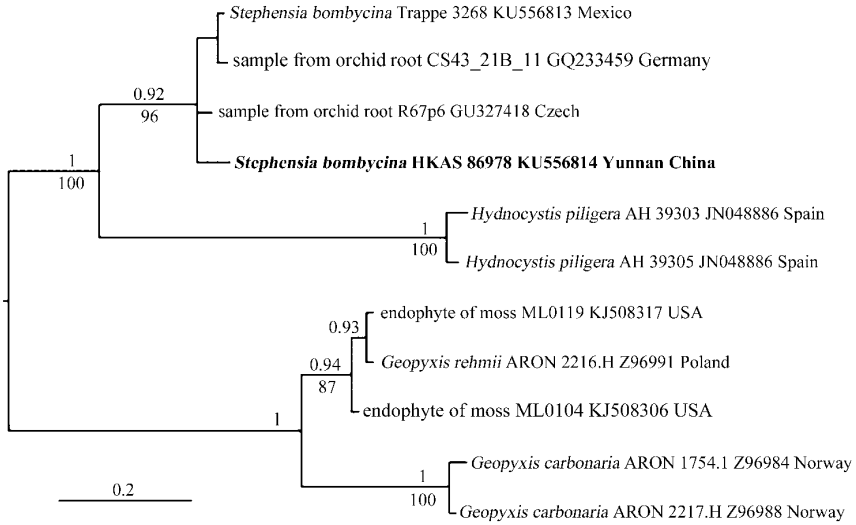



Fig. 1. Bayesian Inference (BI) phylogram of *Stephensia bombycina* and its relatives based on the ITS region, rooted with midpoint

Note: Posterior probabilities higher than 95% in the BI analysis and bootstrap proportions higher than 70% in the Maximum Parsimony (MP) analysis are indicated above and below the branches respectively. Samples are provided with sample numbers followed by GenBank accessions. Sequence generated in this study is in bold

2.2 Taxonomy

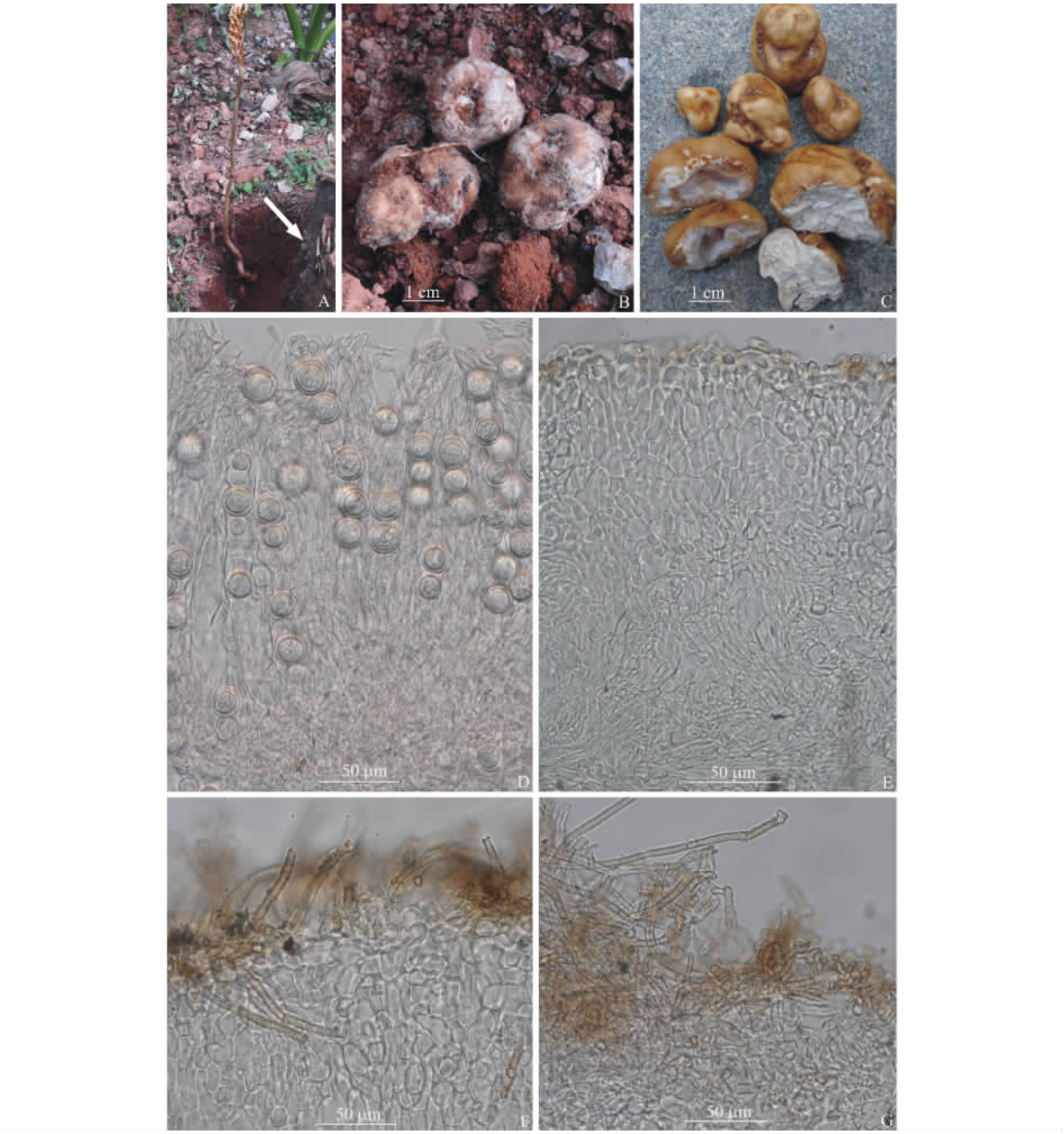
*Stephensia bombycina* (Vittad.) Tul. & C. Tul., Fungi Hyopg. : 129 (1851)

≡ *Genea bombycina* Vittad., Monogr. Tuberac. (Milano): 29 (1831)

Ascomata (Fig. 2-B, C) hypogeous, buried in soil 10 – 15 cm deep below the ground, fleshy, firm, subglobose, shallowly furrowed to partly lobed and convoluted, up to 3.0 cm in diameter, yellowish brown, in youth paler, with a scattered brown tomentum, tomentum thicker along furrows. Gleba solid, white to nearly white at maturity, with scattered dull whitish to light brown meandering canals, these canals converging into the center, sometimes forming a cavity. Odor not distinctive. Taste not recorded.

Ascospores (Fig. 2-D) globose, (17) 18 – 25 (26) μm in diam., hyaline, smooth, the wall at maturity 1.5 – 3.0 μm thick, in Melzer's reagent deep yellow to yellowish brown, not staining in Cotton Blue. Asci (Fig. 2-D) cylindrical to clavate with uniseriate spores, 160 – 230 × 20 – 26 μm including a tapering stem 40 – 80 μm long with a forked base, rounded at the apex, hyaline, initially 8-spored but often with 2 – 4 mature ones retained, non-amyloid. Paraphyses (Fig. 2-D) about as long as or slightly exceeding the asci, 4.0 – 5.0 μm in diam., the tips rounded and straight, locally slightly inflated, hyaline, septate. Subhymenium of tightly interwoven, hyaline thin-walled hyphae 2 – 3 μm in diam. Peridial tomentum (Fig. 2-F, G) of septate, brownish hyphae 4 – 6 μm in diam., occasionally with a swollen base up to 12 μm in diam., the wall 0.8

–1.3 μm thick, covered with hyaline to pale brown granules. Ectal excipulum (peridial epicutis) (Fig. 2-E, F) of textura angularis, not clearly differentiated from the ental excipulum, 100 – 130 μm thick, of 6 – 8 cells thick, cells 12 – 30 × 7 – 15 μm, thick-walled with wall 0.8 – 1.2 μm thick, subglobose, ellipsoid, or irregularly inflated, more or less perpendicular to the surface, hyaline to pale yellowish brown. Ental excipulum (peridial subcutis) (Fig. 2-E) of a transitional form from textura angularis to textura prismatica, 60 – 90 μm thick, cells shortly cylindrical to sausage-shaped, rarely subglobose, 16 – 30 × 8 – 12 μm, perpendicular to the surface, hyaline, slightly thick-walled. Medullary excipulum (gleba) of textura intricata of tightly interwoven, hyaline, thin-walled hyphae, 4.0 – 6.0 μm in diam., cylindrical, septate.




A, B, C. Asocomata; D. Asci and ascospores; E. Peridium; F. Scattered brownish hypahe of peridial tomentum; G. Aggreate brownish hyphae along furrows

Fig. 2. *Stephensia bombycina*

Specimen examined: Xiya Villa, Shuihaizi village, Chenggong, Yunnan, CHINA. N24° 58′ 12. 85″, E102°51′57. 99″, elevation 1 972 m, June 3, 2015, leg. L. S. Wang, HKAS 86978 (KUN).

## 3 Discussion

Although there is minor genetic differentiation among Chinese, European (environmental) and North American samples, we identified the Chinese specimen as *S. bombycina*, based on the morphological consistency between the Chinese specimen and the documentation in literatures. Trappe et al. [10] gave a world key to the species of *Stephensia*, in which *S. bombycina* is easily distinguished from *S. bynumii*, *S. shanori* and *S. sumatrana* by the biggest spores (> 20 μm). This separation was supported by the observation on the spores of *S. bombycina*, *S. crocea* and *S. shanori* by De Vries [5]. Montecchi & Sarasini [21] gave a measurement of 21 – 28 μm and Türkoğlu & Castellano [11] gave 21 – 26 μm. Only Kers [12] gave a smaller measurement [17 – ) 18 – 20 ( – 23) μm], but this still exceeds that of *S. crocea*. We observed mature spores from the Chinese specimen, whose dimensions meet the scope given in the literatures above and that we observed from the Mexican specimen Trappe 3268 (average diameter 23. 9 μm). Gilkey [1], in the enumeration of species of *Stephensia*, keyed out *S. bombycina* as a species with "venae externae communicating with a well-defined basal (and often a central) cavity in the ascocarp", different from *S. sumatrana* with the "venae externae somewhat obscurely converging at the base of ascocarp". Kers [12] illustrated the well-developed canals converging to a basal ostiolum in *S. bombycina*. In the Chinese specimen, canals are well converged into the center of the ascomata, forming a cavity in one ascoma. The spores, peridium structure and brownish hairs all coincide with descriptions provided by Kers [12], Montecchi & Sarasini [21] and Türkoğlu & Castellano [11] on Swedish, Italian and Swedish collections respectively. Montecchi & Sarasini [21] described the odor of *S. bombycina* as "strong of cooked cauliflowers, unpleasant". Such odor is not detected on the Chinese specimen.

Although the Chinese specimen was collected by a plant of Tianma, we do not have any direct evidence proving their relationship. Nevertheless, given that the two environmental samples of *S. bombycina* are both from roots of orchids [16,20], the observations are provided. In addition, hazels, which have been recorded at least three times for the *Stephensia* species [10-12], also occur in the habitat of the Chinese specimen. More data are needed to clarify the mycorrhizal status of *S. bombycina*.

Acknowledgements: We thank Dr. K. Hansen, Swedish Museum of Natural History for allowing us to use the ITS sequence of the North American sample of S. bombycina and collecting literatures.

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