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Original Article

Developing characteristics and relationships of *Shiraia bambusicola* with Bamboo

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Abstract

Growth of *Shiraia bambusicola* was observed in the wild during its developing process, and its morphological characteristics and relationships with bamboo tissue were observed by light microscopy and SEM. The fungal stromata were found only on top stalks of the previous year branches. Parasitic relationship of the fungus with the bamboo could not be indicated from the morphological evidence. The fungus grew around the interspace of bamboo leaf sheaths, showed the tissue specificity on leaf sheath of several bamboo genera. A possible life cycle of *S. bambusicola* was proposed and discussed. The fungus was confirmed to have bitunicate asci with pseudoparaphyses thus should be placed in the Pleoporales order and not in the Dothidiales. Asexual and sexual developments of *S. bambusicola* were observed and for the first time four stages of ascus and ascospore development were described. The four stages consisted of ascus primordium formation, ascus elongation, young ascospore formation, and ascospore maturation.

Keywords: life cycle, morphological relationship, tissue specificity, development stage, *Shiraia bambusicola*

1. Introduction

Shiraia bambusicola P. Hennings has been reported as a parasitic fungus on bamboo. The fungus is of medical importance because of its metabolite, hypocrellin, which has promising application in the photodynamic therapy (PDT) for anticancer treatment (Zhang *et al.*, 1998; Yang *et al.*, 2001; Deininger *et al.*, 2002). However, the fungus and its biological characteristics are not sufficiently known making its applications rather limited.

The genus *Shiraia* was first established by Hennings (1900) and placed in family Nectriaceae of Ascomycetes, but Saccardo (1902) categorized it into the family Hypocreace based on its large fleshy ascocarp. Description of earlier mycologists (Hennings 1900; Teng, 1934; Yamamoto and Okada, 1966) on asci of the fungus was unitunicate, but Amano (1980) found that the genus having bitunicate asci rather than unitunicate and suggested placement of the genus in the family Pleosporaceae (Pleosporales). Gu *et al.* (1991) observed some microstructure characteristics of the fungus by using light and scanning electron microscope (SEM) and also suggested the asci as unitunicate. Kirk *et al.* (2001) placed the fungus as genus *Shiraia* with family *incertae sedis* in Dothideales (Dothideomycetes) in *the Dictionary of the Fungi*, 9th Edition. Based on 18S rDNA and ITS-5.8S rDNA sequences analysis, Cheng *et al.* (2004) classified the genus into Pleosporales as Amano (1980) proposed but suggested it in the family Phaeosphaeriaceae. So far, *S. bambusicola* is the only species in the genus *Shiraia*.

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S. bambusicola is found distributing in some regions of southern China and Japan (Kishi *et al.*, 1991). It grows on branches of bamboo and forms warty-like or tuber-like stromata, which emerges on the bamboo at the end of April to beginning of May. The suitable time for the stromal growth and development is at the end of May to beginning of June when a lot of spores are produced during this period. Some factors, such as temperature, rainfall and relative humidity, influence emergence and transmission of the fungus. At present, reported host plants of *S. bambusicola* are some species in five genera of bamboo in China. They are *Bambusa, Fargesia, Phyllostachys, Brachystachyum* and *Pleioblastus* (Xue, 1995; Hui *et al.*, 1996; Lai and Fu, 2000; Cheng *et al.*, 2004). Lai and Fu (2000) suggested that *Brachystachyum densiflorum* and *B. densiflorum* var.*villosum* were the main hosts of the fungus through many years observation in the wild.

The stroma of *S. bambusicola* is the direct resource for extracting of hypocrellin. However, natural distribution of the fungus is very limited and without successfully artificial cultivation so far. The fungus still emerges and perishes itself in the wild. With human demand for space continuously expanding, the wild resource of *S. bambusicola* is facing inevitable reduction. The previous research has not provided enough information to protect and properly utilize of the fungal resources. In fact, there are still many questions waiting to be answered for the fungus: Does it need a specific host? What is its life cycle? What relationship is between the fungus and its host? Where does it obtain the nutrition from?

In this report we intend to use light microscopy and SEM as tools to observe relationships between *S. bambusicola* with its hosts and its morphological characteristics during its life process. While we did not carry out discussion on placement of the fungus, the morphological characteristics are important references of the taxonomy. According to observation results, we summarize life cycle process of *S. bambusicola*.

2. Materials and Methods

2.1 Observation in the wild

Growth of *S. bambusicola* was observed in Zhejiang in PR China during May to June in 2008 and 2009, which was the period when the fungus started to form stroma in the wild. Stroma forming time, growing site, host species, habitat and temperature were recorded. Stromata in different growing stages were collected and stored in formalin/acetic acid/alcohol (FAA) fixative (containing 50% alcohol 90 ml, glacial acetic acid 5 ml and formalin 5 ml) for further microscopic observation.

2.2 Observation with light microscope

The stromata collected from *Pleioblastus amarus* bamboo were cut into thin slices by hand then put into a drop of water or, dyed with lactophenol cotton blue solution or Hoechst 33258 for observation.

2.3 Observation with scanning electron microscope

The SEM samples were prepared by using improved tertiary butyl alcohol freeze-drying method of Inoué and Osatake (1988) and Gao *et al.* (1989). The stromata of *S. bambusicola* collected from *P. amarus* were cut into small pieces and fixed with 0.5% glutaraldehyde at 4°C for 24 hrs. Fixed specimens were rinsed with 0.1 M phosphate buffer pH 7.2 for 15 min three times. Subsequently, they were further fixed with 1% osmium tetroxide in 0.1 M phosphate buffer pH 7.2 for 1 hr at room temperature. After fixation, the specimens were dehydrated through a graded series of frozen tertiary butyl alcohol for 15 min each in 50%, 70%, 80%, 90%, and 95% of the tertiary butyl alcohol, respectively, and for 20 min in that of 100%, and then further vacuum freeze dehydrated with a Hitachi ES-2030 freeze dryer. The specimens were masked by ion sputtering (Hitachi E-1010) and observed with a Hitachi S-3400N SEM.

3. Results and Discussion

3.1 Observation in the wild

Four species of bamboo belonging to three different genera were found being hosts of *S. bambusicola*. They were *Pleioblastus amarus*, *Phyllostachys praecox*, *Brachystachyum* sp., and *Phyllostachys* sp. The fungal stromata were found only on top stalks of the previous year branches. From our observation the fungus has never been found on new shoot branches. During the fungal growing season, from May to June, the temperature were about 22 to 26°C with plenty rainfall and high relative humidity.

3.2 Microscopic observation

As shown in Figure 1 the stromata of *S. bambusicola* were pinkish irregular tuberculate surrounding the host bamboo branches (Figure 1a). Ascocarps and conidia were formed in a stroma (Figure 1b). Pseudothecia with opening ostioles after maturation were immersed in the stroma tissue near its surface. Within the pseudothecia, asci were found parallel arranged around the pseudoparaphyses (Figure 1c). Most bitunicate asci contained six ascospores or rarely four ascospores (Figure 1d, e). A significant number of conidia were produced near the host stalk during the enlargement of the stroma and secreted on to the stromal surface. Conidiophore was short and difficult to see (Figure 1f). Spore mass was yellow. Conidia and ascospores were dictyospores with transverse and longitudinal septa. The conidia were 53~79 μm × 26~31 μm in size growing on a very short conidiophore, while the ascospores were 55~80 μm × 14~25 μm (Figure 1g, 1h).

From morphology and development of the stroma, the

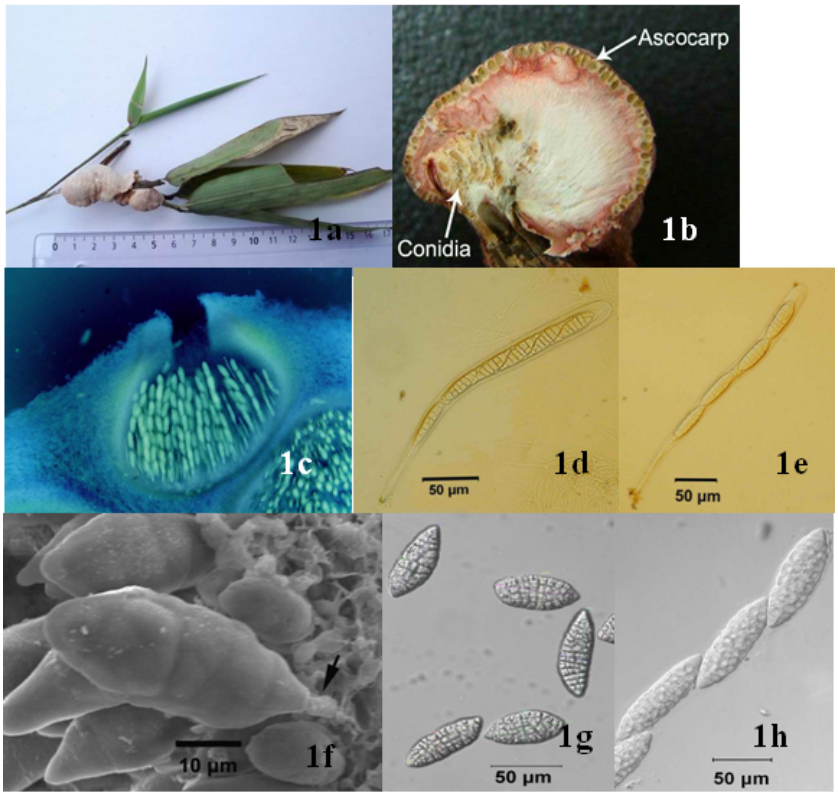

Figure 1. Morphology of *Shiraia bambusicola.*
1a. Stroma on bamboo branches.
1b. Photograph of stromal section.
1c. Photomicrograph of mature pseudothecium.
1d. Photomicrograph of ascus with 6 ascospores.
1e. Photomicrograph of ascus with 4 ascospores.
1f. SEM micrograph of conidia on a short conidiophore (arrow).
1g. Photomicrograph of conidia.
1h. Photomicrograph of ascospores.

fungus under the study was confirmed to be *S. bambusicola.* While some reported taxonomic characteristics of the fungus were inconsistent, our observation confirmed that it had bitunicate asci and produced pseudoparaphyses inside the ascocarp. These two are the distinctive characteristics of the Pleosporales (Alexopoulos, 1962). Therefore the fungus should be placed in the order Pleosporales as suggested by Amano (1980). The placement corresponds with 18S rDNA and ITS-5.8S rDNA sequences analysis on the order classification of *S. bambusicola* by Cheng *et al.* (2004).

3.3 Morphological relationship between *S. bambusicola* and bamboo tissue

From our observation, most of the mycelia of *S. bambusicola* were found to grow around the interspace of bamboo leaf sheaths, intertwined with the glandular hairs but did not penetrate the plant cuticle (Figure 2a, b). These mycelial mats transformed into stromal tissue among the leaf sheaths and covered the leaf sheath surface except the outermost layer (Figure 2c). The fungal mycelia were found just only on the present stalk containing leaf sheath but not on stalk of the next node. Moreover, the stromal tissue could be peeled off from the host leaf sheath without damaging the cuticle (Figure 2d).

The observation indicates that the fungal stromata could grow only on top branch stalks of the host bamboo. As Kuai (1996) reported the bambusicolous fungi appear to have tissue specificityFor example, *Phyllachora shiraiana* could be found on *BambusaPhyllostachys* and other several bamboo genera but was restricted to leaves. *S. bambusicola* also shows the tissue specificity on leaf sheath of several bamboo genera. From the morphological evidence observed in our study, parasitic characteristics of the fungus could not be established. The host bamboo seem to show no signs of sickness (necrotic tissue or deformation), the fungal stroma could be peeled off the bamboo stalk easily, and no success in many attempts of inoculation on bamboo. All these evidences lead us to believe that the fungus may not be parasitic to the bamboo. This however does not preclude the

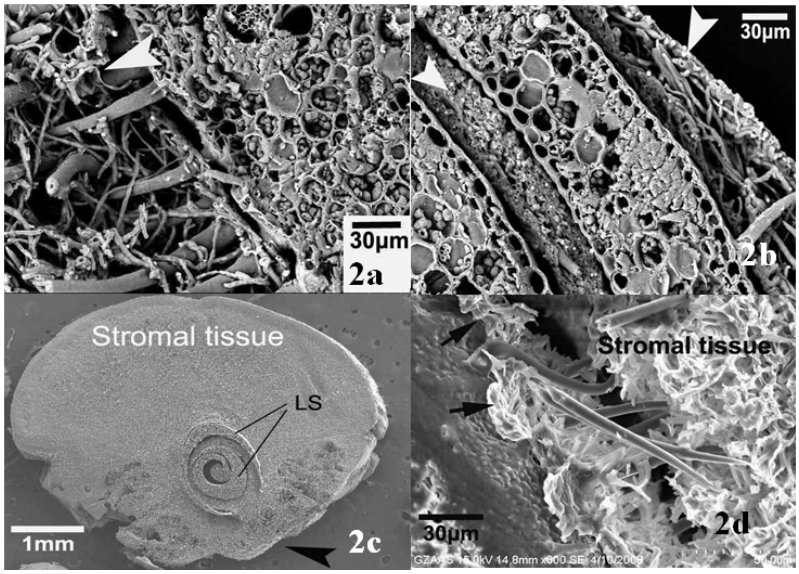

Figure 2. Scanning electron micrograph of transverse section of *Shiraia bambusicola* stroma on *Pleioblastus amarus.*
2a. Mycelia intertwines glandular hairs of the leaf sheath (arrow).
2b. Mycelium mats (arrows) packed in between 2 leaf sheaths with no sign of penetrating fungal structure.
2c. Stromal tissue surrounding the leaf sheaths (LS), the outer leaf sheath (arrow) was lost.
2d. The stromal tissue on the surface of host leaf sheath (arrows).

possibility that the fungus could infect the growing stem tip hidden inside the leaf sheath. Shoot tip necrosis or die back is a very common fungal disease of many dicotyledon species because it is easily observed. This type of symptom is hardly seen in the monocotyledon species because their growing points are obscured by the leaf sheath. Therefore to prove or disprove the parasitic relationship between *S. bambusicola* and bamboo will need more investigation.

3.4 Development of asexual and sexual stages

As described previously, the asexual and sexual spores of *S. bambusicola* could be found together in the same stroma. When the stroma was very young, some irregular cavities had started to emerge near the side of the outermost leaf sheath, in which lots of conidia were produced continuously on the cavity wall until maturation (Figure 3a). Subsequently the conidia were pushed off by the new generations of conidia and accumulated within the cavity (Figure 3b). Then the mature conidia were gradually released on to the stromal surface mostly in the side of the outermost leaf sheath or partially spilled out from the stromal crack during the developing stage (Figure 3c), finally leaving the empty cavities in the stroma (Figure 3d).

With the development of the stroma, more conidia were produced and pseudothecia began to form in the stromal tissue near surface. In our observation, there were four stages of ascus and ascospore development. First, the spherical ascus primordia emerged at the base of pseudothecium wall (Figure 3e). Then, the primordia grew gradually into cylindrical asci but no ascospores were found in the elongating ascus. During this period, protoplast covered by inner ascus wall also elongated simultaneously (Figure 3f). The third stage was the formation of the young ascospores. Transverse and longitudinal septa formed in the protoplast and a figure of six ascospores could be observed at this stage. However, the protoplast containing six ascospores was not divided and kept as a whole. A special structure of apex on inner ascus wall was globose and distinct (Figure 3g). The fourth stage was maturation of ascospores with transverse and longitudinal septa. At this stage, the ascospores grew bigger and become separated into individual six units at maturation. The special apex structure in the inner ascus wall became unclear at this period (Figure 3h). The mature ascospores finally dispersed onto stromal surface from ostiole opening of the pseudothecium (Figure 3i).

Normally abundant conidia of most ascomycetes are produced for reinfection and ascospores are taken as infection source for next year. However, reinfection of *S. bambusicola* on bamboo was not found or proved in our study. The conidia formed underneath the stroma followed by forming of ascospores. The release of conidia and ascospores took place when they were mature. To a certain extent this fungus shares similarity with that of *Guignardia bidwellii* (Agrios, 1997), a pathogen of black rot of grape. It is postulated that *S. bambusicola* produces conidia and ascospores in the present year, which are released and dispersed by rain water, wind or insects, then arrive and adhere to somewhere of new shooting leaf sheaths of the bamboos to overwinter. When suitable conditions arrive, the conidia, ascospores or mycelia

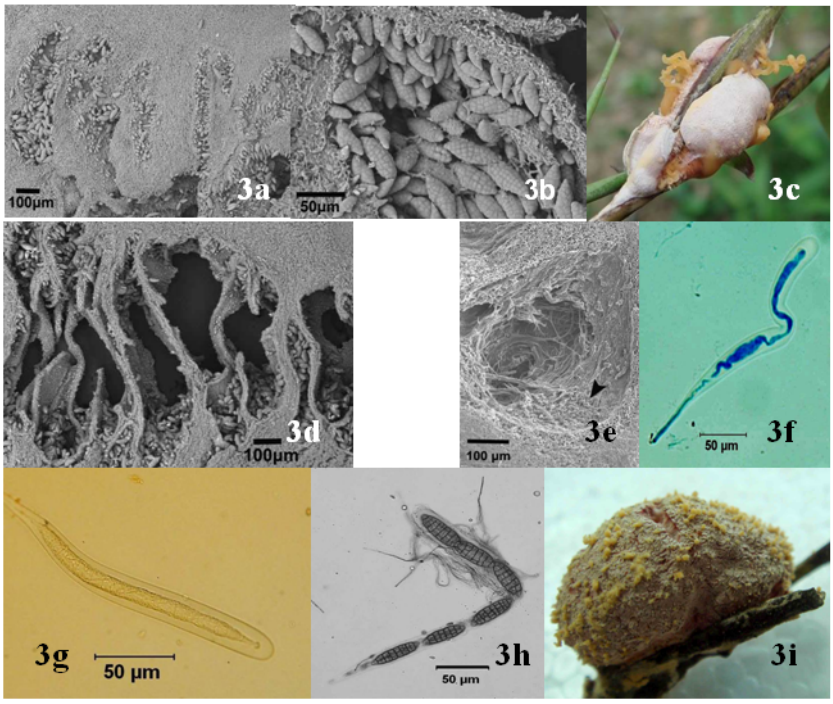

Figure 3. Developing process of asexual and sexual stages of *Shiraia bambusicola* on *Pleioblastus amarus* bamboo.
3a. Cavities containing conidia in stroma.
3b. Conidia produced on the cavity wall and mature conidia in the cavity.
3c. Conidia were released onto stroma surface as yellow conidial mass.
3d. Hollow cavities after conidia dispersed out.
3e. Section of a pseudothecium showing ascus primordia at the base of pseudothecium (arrow).
3f. Elongating ascus.
3g. Young ascospore formation.
3h. Ascus with mature ascospores.
3i. Mature ascospores released to stroma surface as yellow mass.

will grow in the leaf sheath and develop into new stromata to start another cycle of spore dissemination.

From our knowledge this is the first time that four stages of sexual development of *S. bambusicola* has been reported. Its bitunicate asci nature has also been confirmed. The unitunictate asci observed by many researchers could have resulted from the special apex structure in the inner ascus wall became unclear at ascospore maturing stage making them look unitunicate.

4. Conclusion

Stromata of *S. bambusicola* were found only on top stalks of the previous year branches without any evidence of parasitic relationship with the bamboo. The stroma grew around the interspace of bamboo leaf sheaths, showed the tissue specificity on leaf sheath of several bamboo genera. The transmission strategy and life cycle of *S. bambusicola* were proposed and described. Because the fungus has bitunicate asci and pseudoparaphyses we suggest it should be placed in the Pleosporales order.

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