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A New Record of *Elaphomyces guangdongensis* (Elaphomycetaceae, Eurotiales, Fungi) from Taiwan

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Abstract During the mycological survey in Central Taiwan in 2009, a few specimens of the genus *Elaphomyces* were collected in two localities. Their unusually black peridium and habitat in *Pasania* stands are characteristics not known from any other species of *Elaphomyces*. More detailed morphological observations, however, revealed that they are *E. guangdongensis*, a species known only from southeastern China and associated with *Castanopsis.* This is the first report of *E. guangdongensis* outside the type locality, and also the first record of the species from *Pasania* stands. Macroscopic and microscopic (both LM and SEM) illustrations as well as detailed descriptions of the specimens from Taiwan are provided.

Key words : Ascomycota, biogeography, *Castanopsis*, ectomycorrhizae, fungi, *Pasania*, taxonomy, truffle.

Introduction

The genus *Elaphomyces* (Elaphomycetaceae, Eurotiales, Ascomycota) is an ectomycorrhizal fungus (Theodorou and Reddell, 1991) known from both Northern and Southern Hemispheres (Castellano *et al.,* 1989). It is known as a "truffle-like" fungus because fruit bodies are usually produced below-ground (hypogeous). The higher-level phylogeny of the kingdom Fungi revealed that truffle-like forms have evolved multiple times in distantly related lineages (Hibbett and Thorn, 2001; Hosaka *et al.,* 2006; Landvik *et al.,* 1996). Most truffle-like fungi, however, belong to one of two major phyla, i.e., Basidiomycota and Ascomycota.

Within the Basidiomycota, it is strongly suggested that hypogeous, truffle-like forms have evolved multiple times from above-ground (epigeous) ancestors (Hibbett and Thorn, 2001; Peintner *et al.,* 2001). It is hypothesized that the trend of evolution from epigeous to hypogeous forms is an adaptation for spore dispersal by small animals (Thiers, 1984). Indeed, some truffle-like fungi produce chemical odors to attract small animals and insects (Pacioni *et al.,* 1991). Furthermore, many animals both from Northern and Southern Hemispheres are highly dependent on fruit bodies of such truffle-like fungi for their diet (Claridge, 2002; Fogel and Trappe, 1978; Johnson, 1996; Malajczuk *et al.,* 1987).

Within Ascomycota, to which *Elaphomyces* belongs, a vast majority of truffle-like taxa belong to the family Tuberaceae and related taxa in the Pezizales, including the well-known edible truffles in the genus *Tuber* (Hansen and Pfister, 2006). The phylogenetic position and taxonomic treatment of *Elaphomyces*, however, have been controversial until relatively recently (Trappe, 1979). One of the first phylogenetic studies with

Elaphomyces was done by Landvik *et al.* (1996). Their study clearly demonstrated that *Elaphomyces* is another independent lineage of the truffle-like form, and the genus is closely related to ubiquitous molds such as *Penicillium* and *Aspergillus*. More recently, an additional genus with epigeous fruit bodies and an ectomycorrhizal habitat, i.e., *Pseudotulostoma*, has been described from Guyana (Miller *et al.,* 2001; Henkel *et al.,* 2006) and Japan (Asai *et al.,* 2004) and demonstrated to be closely related to *Elaphomyces* (Masuya and Asai, 2004; Geiser *et al.,* 2006). Currently, *Elaphomyces* and *Pseudotulostoma* are placed in the family Elaphomycetaceae in the order Eurotiales (Kirk *et al.,* 2008).

It is noteworthy that species of the genus *Elaphocordyceps* G.H. Sung & Spatafora are known to be specific parasites of *Elaphomyces* (Sung *et al.,* 2007; Nikoh and Fukatsu, 2000). More than 18 species of *Elaphocordyceps* are known to be parasites of *Elaphomyces* (Sung *et al*., 2007), and the monophyly of *Elaphocordyceps* indicates some level of co-evolutionary history between *Elaphomyces* and *Elaphocordyceps*. Because the fruit bodies of *Elaphomyces* are produced below-ground and not easily obtained during the fieldwork, finding the fruit bodies of *Elaphocordyceps* is possibly a good indicator of the presence of *Elaphomyces.* Indeed, some species of *Elaphomyces*, such as *E. nopporensis* S. Imai and *E. japonica* Lloyd, have been reported exclusively as a host of *Elaphocordyceps* (Imai, 1929).

During the fieldwork in Central Taiwan in 2009, a few specimens of *Elaphomyces* with peculiar morphology were collected from *Pasania* stands and mixed stands of *Pasania*, *Castanopsis*, and *Quercus* spp. Morphological examinations strongly indicated that the specimens belong to *E. guangdongensis* B.C. Zhang (Zhang, 1991), a species hitherto known only from the type locality, southeastern China. To our knowledge, this is the first report of *E. guangdongensis* in Taiwan, and also from *Pasania* stands. The collections from Taiwan are described and illustrated with a discussion on the significance of this finding in the biogeography of *Elaphomyces*.

Materials and Methods

Collecting sites, collecting scheme, and curation of specimens

Fieldwork was conducted by KH in the year of 2009 (November 7–14). The main collecting sites were located in central Taiwan, including Nantou County and Taichung County. A general collection scheme (Castellano *et al.*, 1989) for hypogeous, truffle-like fungi was followed. At each collecting site, fruit bodies were collected just under the leaf litter by using a garden rake. At the same time, potential ectomycorrhizal host trees near fruit bodies, mostly Pinaceae and Fagaceae, were identified to species. If alternative ectomycorrhizal hosts were present near fruit bodies, those were also recorded.

Each specimen was photographed and macroscopic observation was conducted. All specimens were cut into half and dried with low heat and good air circulation. In addition to dried materials, small fragments of glebal tissue from freshly collected samples were soaked in DMSO buffer (Seutin *et al.*, 1991) with an addition of 100 mM Tris-HCl (pH 8.0) and 0.1 M sodium sulfite (Na_2SO_3) under 4°C, following the procedures of Hosaka (2009) and Hosaka and Castellano (2008).

More detailed identification and description were conducted after returning to the mycology lab at the National Museum of Nature and Science, Japan. Specimens collected during the fieldwork were deposited at the fungal herbarium of the National Museum of Nature and Science, Tsukuba, Japan (TNS).

Light and stereo microscopy

For light microscopic observations, a small portion from the gleba was mounted in water, 3% or 5% (w/v) KOH and 30% ethanol solution on glass slides. Those samples were examined with a Leica DM LB microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany) under Nomars-

ki interference contrast. More than forty randomly selected ascospores were measured under a light microscope at 1000× magnification. Surface features of the peridium were also observed by a stereo microscope at 6× magnification.

Scanning electron microscopy

The surface features of the ascospores were observed by scanning electron microscopy (SEM). For SEM, a small portion from the gleba was dusted onto double-sided adhesive tape on a specimen holder and coated with platinum-palladium using an E-1030 Ion Sputter Coater (Hitachi, Tokyo, Japan). They were examined with a S-4200 SEM (Hitachi, Tokyo, Japan) operating at 20 kV.

DNA Preparation, PCR and sequencing

DNA from the specimens collected by KH was extracted from the tissue fragments stored in DMSO buffer. Tissues were ground under liquid nitrogen using a mortar and pestle. DNA extractions used either the Qiagen Plant kit (Qiagen, Mississauga, ON, Canada) following the manufacture's protocols or modified CTAB extraction followed by glass milk purification methods as summarized by Hosaka (2009) and Hosaka and Castellano (2008). DNA from the holotype specimen was extracted differently because no immature glebal tissues were available and only the powdery spore mass was present. The spore mass was subjected to bead-beating following the protocol of Hosaka and Castellano (2008), followed by modified CTAB extraction and glass milk purification as above.

DNA sequence data were obtained from the internal transcribed spacer regions (ITS) and large subunit (LSU) of the nuclear ribosomal DNA. For amplifying the ITS region, the primer combination of ITS5 and ITS4 (White *et al.,* 1990) was used. For amplifying the LSU, the combination of LR0R and LR5 (Vilgalys and Hester, 1990) was used. PCR reactions were carried out using 20 μl reaction volumes each containing: 1 μl Genomic DNA, 1 μl dNTP, (4 mM), 1 μl of each primer (8 μM), 0.5 units of Taq polymerase (TaKaRa, Tokyo, Japan), 2 μl $MgCl_2$ (25 mM), 2 μl Bovine Serum Albumin (BSA). Cycling parameters were 1 cycle of 94°C for 3 min, 30 cycles of 94°C for 1 min, 51°C for 30 s and 72°C for 1 min, with a final extension at 72°C for 15 min. PCR products were electrophoresed in 1% agarose gels stained with ethidium bromide and visualized under UV light. When amplification bands were confirmed, PCR products were then purified using the ExoSap-IT (Millipore, Molsheim, France) and directly sequenced using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems Inc., Norwalk, CT, USA), following the manufacturer's instructions.

Results and Discussion

DNA Preparation, PCR and sequencing

The ITS sequences were successfully obtained from two specimens (KH-TW09-030 & 031, with GenBank accession numbers HM357249 and HM357250, respectively). In addition, LSU sequence was successfully obtained from one specimen (KH-TW09-031 with GenBank accession number HM357248). No amplifications, however, were successful from the holotype specimen of *E. guangdongensis* (HKAS 60250) despite several attempts of DNA extraction and PCR (e.g., using different extraction methods, concentrating extracted DNA using SpeedVac, and amplifying shorter fragments). Although the holotype specimen is less than 30 years old, heavy application of fumigation chemicals in many herbaria in China (Fuqiang Yu, personal communication) might have influenced the quality of DNA. This needs to be solved by using internal primers to amplify even shorter fragments, and/or by designing taxon specific primers for *Elaphomyces*.

Two ITS sequences were identical. A comparison to an unpublished dataset of 207 Elaphomycetaceae ITS sequences, including 43 black *Elaphomyces* species (Hannah Reynolds, Duke University) showed no match for the Taiwanese specimens. No sequences from *E. guangdongensis*, *E. carbonaceus,* or similar taxa are currently

available from the public database. Although sequence data are lacking, almost identical morphological characters, geographical proximity (southeastern China and Taiwan), and similarity in possible mycorrhizal hosts (Fagaceae) indicate that the holotype specimen from southeastern China and three specimens from Taiwan are conspecific. Future study is necessary to collect fresh materials of *E. guangdongensis* from the type locality to confirm their taxonomic identity.

Taxonomy

Elaphomyces guangdongensis B.C. Zhang, Mycol. Res. 95: 975. 1991. [Fig. 1–4]

Ascoma globose, subglobose, often irregularly flattened, dark brown to black, 0.8–2.2 cm diam., surface with small black conical warts (Fig. 1A, C, D, Fig. 3A, B) having pale yellowish to orangish mycelial strands (Fig. 3A, B) and often covered by thick soil crusts (Fig. 1B, C, Fig. 2), which are not easily separable from ascoma. Hyphae in the mycelial strands brownish under the compound microscope, 2–4 μm diam., with walls thin and smooth, septate (Fig. 3D). **Peridium** inner layer 0.8–2 mm thick, fleshy and pliable, white when young, brown to dark brown when mature, eventually disintegrating and leaving only outer layer (Fig. 2); outer layer 350–600 μm thick including warts, carbonaceous, fragile, composed of hyphae about 4.5–5 μm diam., blackish brown to dark brown, with walls 1 μm thick; warts about 350 μm high, 150–500 μm broad at the base, covered with parallel hyphae, surface often covered by exposed spores and appearing grainy under stereo microscope (Fig. 3A, B). **Gleba** brown with cottony texture, somewhat radially arranged when young, but soon becoming purplish black, a powdery spore mass (Fig.

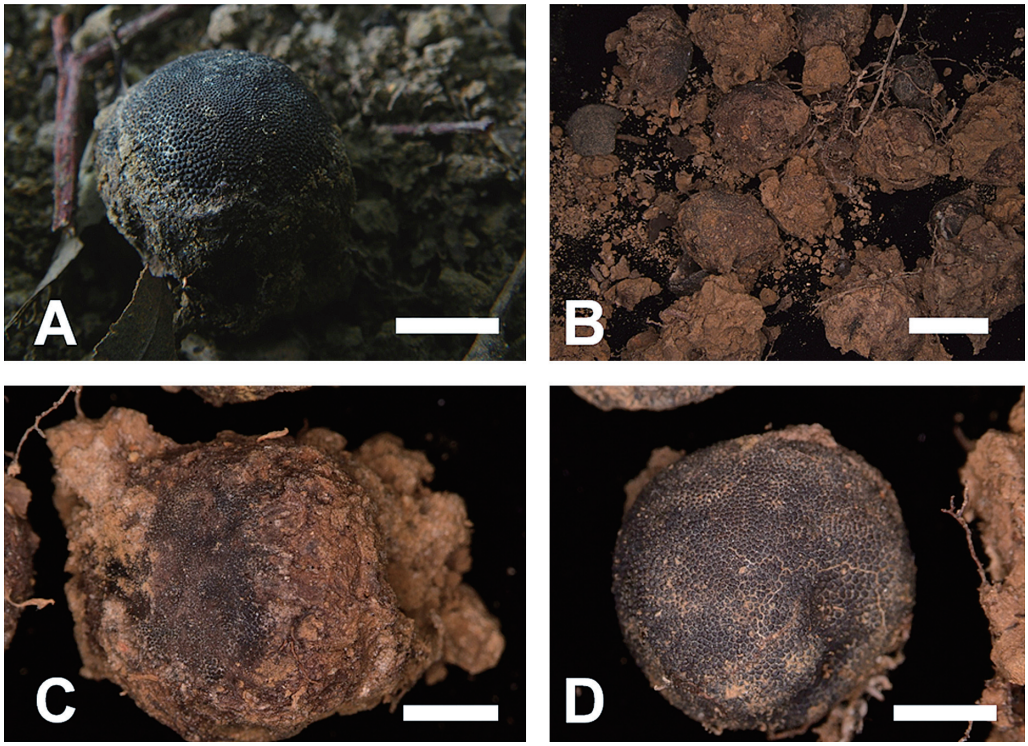

Fig. 1. Ascomata of *Elaphomyces guangdongensis*. A. Subepigeous habit in Lianhuachih Research Center, Taiwan (KH-TW09-030). B. Fresh ascomata just excavated from the ground (KH-TW09-031). Note thick soil crusts enclosing each fruit body. C. Close-up of a fresh fruit body, with partially exposed peridium (KH-TW09-031). D. Close-up of a fresh fruit body, with almost completely exposed peridium. Bars＝1 cm.

2). **Asci** could not be observed. **Ascospores** globose, dark brown to blackish brown, 16.5–19 (–22.5) μm diam. including ornamentation; ornamentation composed of parallel to spiral ridges when viewed under light microscope (Fig. 3C), under SEM parallel deep ridges formed by rows of joined spines buried in gelatinous material (Fig. 4A–D).

Habitat: Hypogeous to subepigeous under leaf litter but with little understory, in pure stands of *Pasania cornea* and in mixed woods of *Pasania cornea, Castanopsis uraiana, Quercus* spp., *Calocedrus* sp. and *Cryptomeria japonica*.

Presumable ectomycorrhizal hosts: *Pasania cornea, Castanopsis uraiana,* and *Quercus* spp.

Fruiting season: November.

Distribution: Known only from southeastern China (Guangdong) and central Taiwan (Nantou County).

Materials examined: Holotype: **China**, Guangdong, Boluo, Mt. Luofoushan Natural Reserve, 22 November 1988, B.-C. Zhang 568 (HMAS 60250). Other collections: **Taiwan**, Nantou Co., Yuchi Township, Lianhuachih Research Center [23°55′18.7″N, 120°53′14.1″E], alt. 678 m, 9 November 2009, K. Hosaka (KH-TW09-030, KH-TW09-031; TNS); Nantou Co., Sun Moon Lake, Hanbi trail [23°51′46.3″N, 120°54′35.8″E], alt. 775 m, 10 November 2009, K. Hosaka (KH-TW09-038; TNS).

Remarks: This is the first record of *E. guangdongensis* from Taiwan. As far as we know, this is the second locality officially reported, besides the type locality in Guangdong Province, China, for this species. This is a significant finding because two specimens (KH-TW09-030 & 031) were collected from pure stands of *Pasania cornea*, which is most likely to be an ectomycorrhizal host of this species. The type specimen was recorded from *Castanopsis* stands, and it indicates that the species can form ectomycorrhizae with at least two distinct tree genera belonging to Fagaceae. Mycorrhizal hosts of KH-TW09-038 could not be identified because

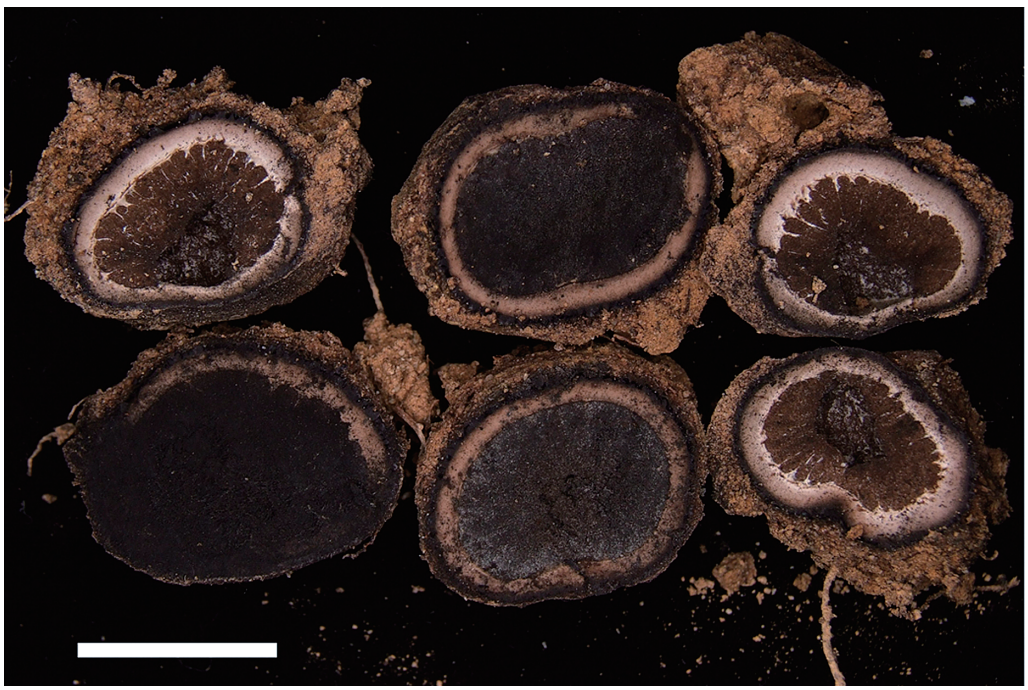

Fig. 2. Cross sections of fresh fruit bodies of *Elaphomyces guangdongensis* (KH-TW09-030). Glebal color changes from brown when immature (right and top left) to a purplish black, powdery mass when matured. Note thick soil crusts covering each fruit body. Bar = 1 cm.

not only *Pasania*, but also *Castanopsis uraiana* and *Quercus* spp. were present at the site. We cannot eliminate the possibility that the species can also form ectomycorrhizae with *Quercus* spp. It is possible that *E. guangdongensis* can associate with a broad range of host trees in Fagaceae.

As discussed by Zhang (1991), *E. guangdongensis* is most similar to *E. carbonaceus* Corner & Hawker reported from Singapore (Corner and Hawker, 1953). However, the sizes of ascospores and peridial warts can clearly distinguish these two species. Unfortunately, no DNA sequence data are currently available from either *E. guangdongensis* or *E. carbonaceus*. Comparison of DNA data from additional materials is critical for future studies.

Biogeographical implication

Because of the geographical proximity of southeastern China and Taiwan, it is not surprising to find that they share species of ectomycorrhizal fungi. It is, however, uncertain whether *E. guangdongensis* expanded its distribution via land bridges (i.e., vicariance) or by transoceanic dispersal. Although there is only limited evidence, recent studies suggest that even belowground organisms, like truffles, can cross the oceans (Hosaka *et al.,* 2008; Wedén *et al.,* 2004) or other geographical barriers such as mountains ranges and valley systems (Murat *et al.,* 2004; Grubisha *et al.,* 2007) to expand their distributions.

Castanopsis, one of the recognized ectomycorrhizal hosts for *E. guangdongensis*, is distributed throughout the warm temperate regions of Japan,

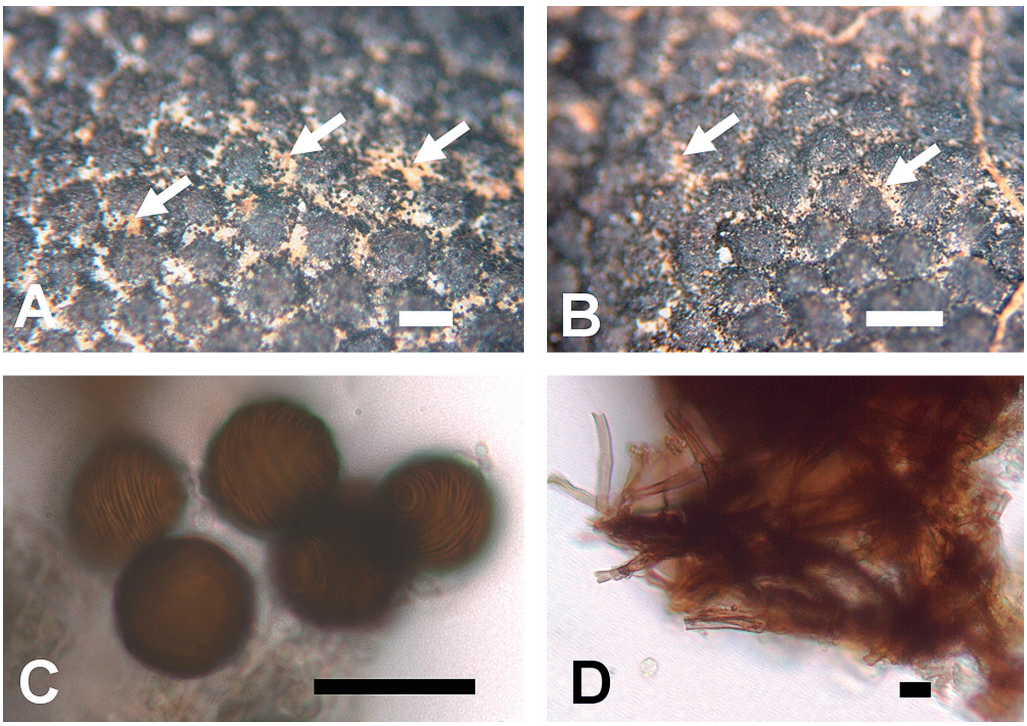

Fig. 3. Peridial surface, ascospores, and hypha of *Elaphomyces guangdongensis*. A, B. Peridial surface under stereo microscope. A. Specimen from Taiwan (KH-TW09-030). Arrows indicate pale yellowish to orangish mycelial strands. B. Holotype specimen from China (HMAS 60250). Arrows indicate yellowish to orangish mycelial strands. Bars = 500 μm. C. Ascospores under light microscope (KH-TW09-031). Note spore surface ornamented with parallel ridges. Bar = 20 μm. D. Hypha with septa in peridium under light microscope. Bar = 5 μm.

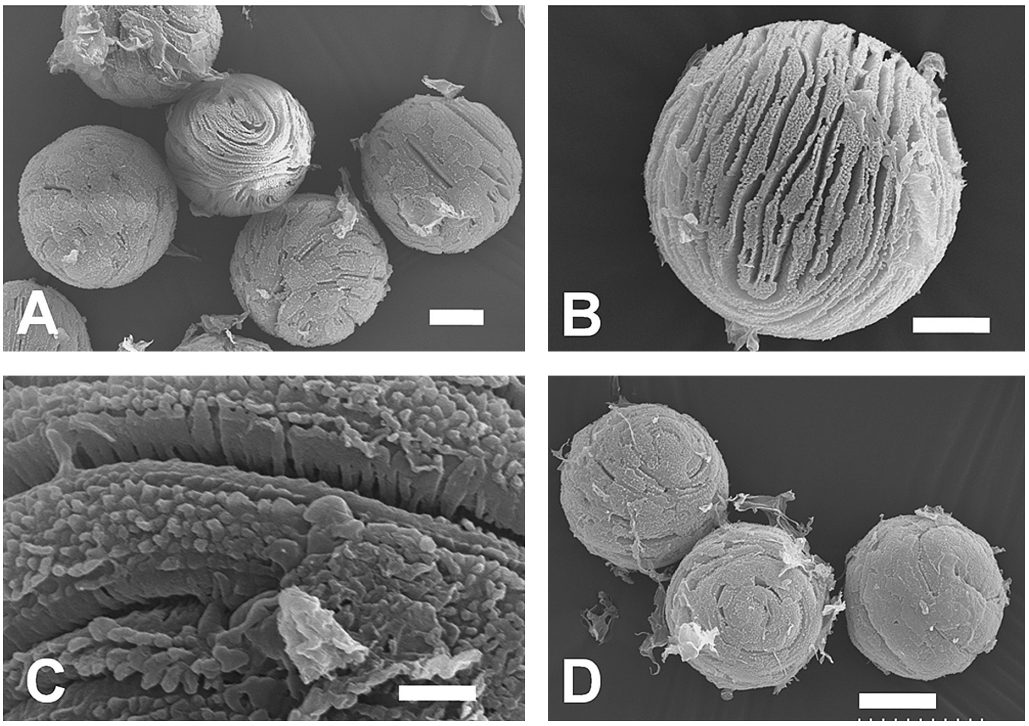

Fig. 4. Scanning electron micrographs of ascospores of *Elaphomyces guangdongensis*. A. Specimen from Taiwan (KH-TW09-030). Note spore ornamentation with parallel or spiral ridges. Bar=5 μm. B. Specimen from Taiwan (KH-TW09-031). Note distinct parallel ridges. Bar=5 μm. C. Close-up of spore ornamentation (KH-TW09-031). Note parallel deep ridges formed by rows of joined spines. Bar=1 μm. D. Holotype specimen from China (HMAS 60250). Bar=5 μm.

where 14 species of *Elaphomyces* have been reported (Kobayasi, 1960). More intensive surveys, especially from the southern parts of Japan (Ryukyu Islands, etc), may reveal a wider distribution of *E. guangdongensis*. In addition, *Castanopsis* (and other fagaceous trees) is widely distributed in tropical and subtropical areas of Southeast Asia to South Pacific (i.e., New Guinea). Surveys in these areas will be critical in clarifying the distribution of *E. guangdongensis*.

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