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Identification of *Ciboria carunculoides* RS103V, a Fungus Causing Popcorn Disease on Mulberry Fruits in Korea

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The popcorn disease caused by sclerotia forming fungi reduces the productivity of mulberry fruits in world wide. In Korea, only two species (*Ciboria shiraiana* and *Scleromitrula shiraiana*) have been reported as the major causal organisms and their morphological features are also largely unknown. Hereby, we report the first identification of another species (i.e. *Ciboria carunculoides*) in Korea and detailed features of their anamorphic stage. Fungi dominantly associated with sclerotia were purely isolated from infected mulberry fruits under the microscope. PCR-amplified DNA encoding 5.8S rRNA displayed 100% similarity to *Ciboria carunculoides*. The anamorphic features exhibited the absence of true mycelia. Instead, very short, aseptated, branched conidiophores were directly emerged from sclerotia. Phialides were usually three in number from each conidiophore, ampuliform to navicular in shape, slightly curved and tapering towards the apex. Conidia were produced from phialides and mostly found as one celled, pear shaped, not hyaline with smooth to uneven surface walled. Diversely modified features in phialides formed pseudo-mycelial structures around the host tissue. Combined all, current study is the first report of *C. carunculoides* isolated in Korea and the foremost detailed description of its anamorph stage.

Keywords **: Anamorph, *Ciboria carunculoides*, Mulberry, Popcorn disease, South Korea**

Mulberry fruits are produced from morus tree, genus of flowing plants which are cultivated in many temperate regions in East Asia. In 2011, 6,752 tons of mulberries were produced from 1,750 ha of upland in Korea ('statistical report #11416' in MAFRA, Korea). Recently, mulberry productivity is potentially threatened by the 'popcorn disease' caused by sclerotia forming fungi (Hong *et al.*, 2007; Kishi, 1998; Kohn and Nagasawa, 1984; Whetzel and Wolf, 1945). In Korea, two species of fungi, *Ciboria shiraiana* and *Scleromitrula shiraiana*, have been reported to be causal pathogens for popcorn disease (Cho and Shin, 2004; Hong *et al.*, 2007). Another Sclerotiniaceae, *Ciboria carunculoides* was known to be observed in southeastern of United States. The typical symptoms of the disease are developments of swollen and mummified white fruits which in turn formation of black sclerotia (Kishi, 1998; Kohn and Nagasawa, 1984; Whetzel and Wolf, 1945). One of the main obstacles of investigating this disease is that the fungal strains are mostly 'absolute obligates' so recalcitrant for *in vitro* culturing. Nonetheless, Hong *et al.* (2007) depicted the teleomorphic features such as apothecia, asci, ascospores and paraphyses of *C. shiraiana* and *S. shiraiana* isolated from mulberry orchards located in Korea. Anamorphic characteristics like conidia of *S. shiraiana* were also disclosed, but in a limited scale (Hong *et al.*, 2007). Combined the previous studies, the information on characteristics of the causal fungal strains is largely lacking to understand the pathogenicity of Sclerotiniaceae and develop the strategy for preventing the incidence of popcorn disease in mulberry. Therefore, further isolation and precise examination of disease causing fungal strains would be of great interest.

In current study, we firstly isolated *C. carunculoides* RS103V from the morus orchards in Korea. We also described the detailed morphology and quantitative measurements on anamorphic organs of the pathogenic fungus using scanning electron microscope. The potential aspects of each anamorphic structure were briefly

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discussed. Taken together, our study provided the advanced insight into the characteristics of fungi associated with popcorn disease of mulberry.

Identification of *C. carunculoides*. About 500 popcorn infected mulberry fruits were collected during May–July 2013 in Iksan, South Korea. To obtain and propagate pure fungal isolate, twelve different media (e.g. RBA, PDA, MYA, NA, WA, LBA, R2A, TSA, CzDA, CMA, OMA and young mulberry fruit extract) were used by tissue planting method. However, no *Ciboria* genus was successfully propagated in any of our selected artificial media, probably due to lacking conditions yet to be reported. Alternatively, a small amount of fungal strains associated with sclerotia were directly isolated under the microscope. First, popcorn diseased druplets were harvested (Fig. 1A and 1B). Then, 25 slides were prepared with inner blackish tissue of infected druplets (Fig. 1C) or sclerotia (Fig. 1D). In addition, surgically detached samples from infected druplets and sclerotia were stained with lactophenol blue solution (Fig. 1E and 1F) and subjected to microscopic observation. We found that most of samples displayed the dominant distribution of asexual structures of one fungal strain tightly associated with sclerotia (Fig. 1E). Noticeably, phialide-like structures and conidia were observed (Fig. 1F). Some phialides clearly displayed septated and hyline pattern whereas conidia were unicellular and non-hyaline (Fig. 1F). DNA was also extracted from the swollen tissue of the infected mulberry fruits (Fig. 1A). Only the infected blackish tissues (25 mg) inside the white druplets were subjected to DNA extraction (Exgene™ Plant SV miniprep kit, GeneAll, Korea). To amplify fragment of the nuclear ribosomal DNA encompassing internal transcribed spacers (ITS1 and ITS4) in 5.8S rRNA region, universal primers ITS1 (598 bp, 5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (607 bp, 5'-CCTCCGCTTATTGATATGC-3') (White *et al.,* 1990) were used. PCR reactions were performed using 5X Green GoTaq® master mix (Takara, Japan). The thermocyclic protocol was followed as previously described (Martin and Winka, 2000). PCR products were visualized by agarose electrophoresis and purified using the Dokdo-Prep™ Gel Extraction Kit (ELPIS Biotech, Korea), followed by nucleotide sequencing (Cosmogentech, Korea). To find the mostly similar taxa available in GenBank, the retrieved nucleotide sequences were subjected to BLASTN (http://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis. The combined sequences of ITS exhibited 100% similarity to *C. carunculoides* strain ms89 (genebank accession; HQ833454.1) and *C. carunculoides* strain ms92 (HQ833457.1) which were isolated in China. To differentiate previous isolates, we designated query strain as *C. carunculoides* RS103V.

Sample preparation for SEM (Scanning Electron Microscope). For electron-microscopic observation of *C. carunculoides* RS103V, fifteen whitish swollen druplets were surface sterilized (75% ethanol for 5 min) and then pilled for the specific collection of the blackish

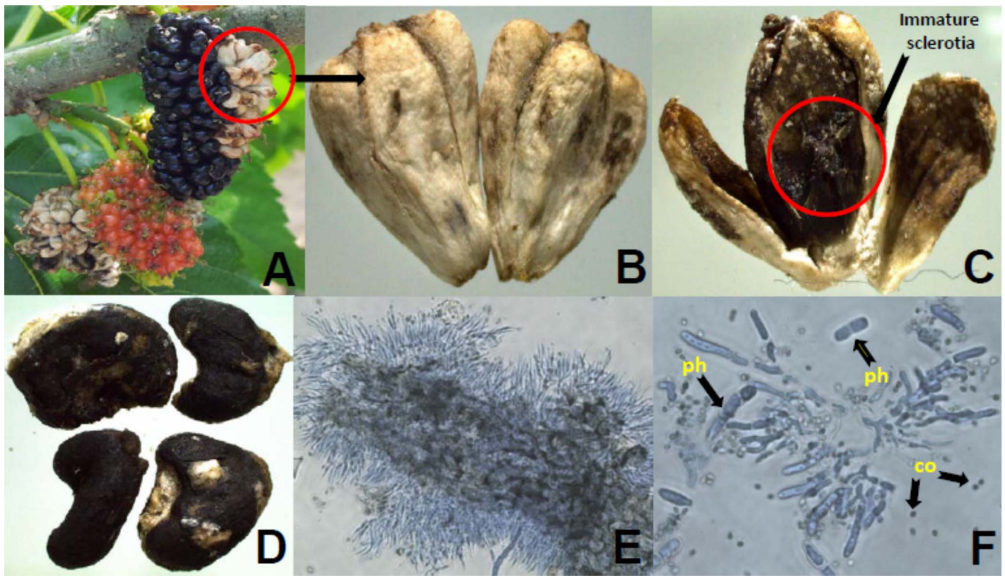

Fig. 1. Isolation of *Ciboria carunculoides* RS103V from popcorn diseased mulberry fruits. **A**: Infected fruits formed white mummified druplets, the typical symptoms of popcorn disease, **B**: Isolated druplets of infected fruits, **C**: Inner tissues of infected fruits, Immature sclerotia were circled, **D**: Mature sclerotia produced from the infected druplets, **E**: Fungal structures on the surface of sclerotia, **F**: Microscopic observation of the fungi structure present in the immature sclerotia. ph, phialides; co, conidia.

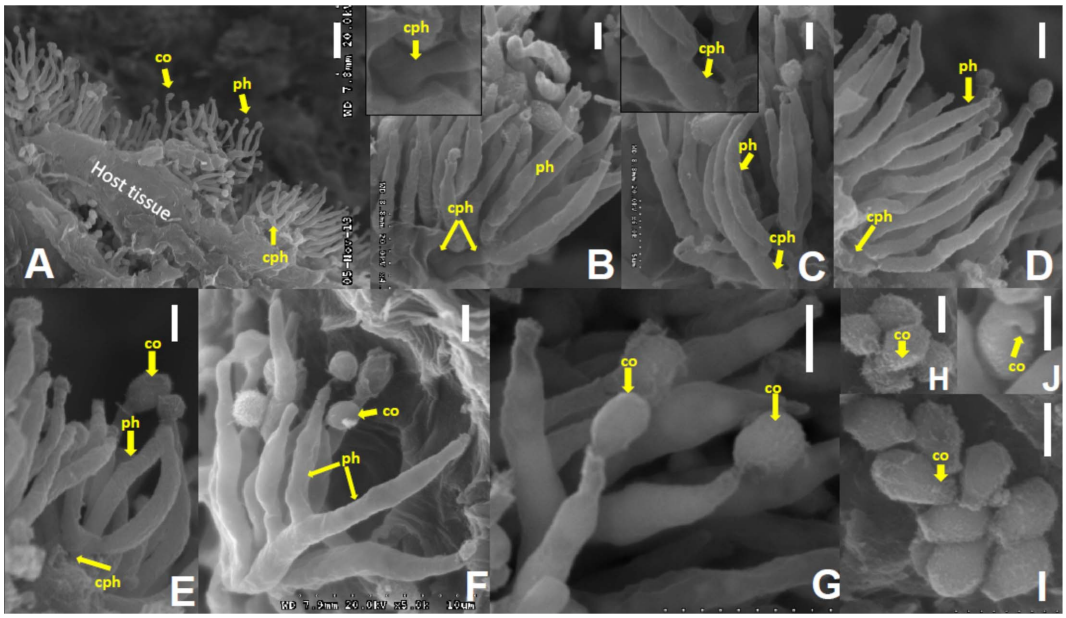

Fig. 2. Morphological features of anamorphic stages of *Ciboria carunculoides* RS103V. **A**: Fungal colonies emerged from the host tissue, **B**: Branched conidiophore, **C**: Upright conidiophore, **D**: Short conidiophore and typical shape of phialides, **E**: Exceptionally short conidiophore, **F**: Smooth walled conidia formed on phialides, **G**: Dispersing mature conidia with rough walls, **H**, **I**: Dispersed conidia on the host tissue, **J**: Germinating conidia with germ tube, Bars: 2 μm. **B**, **C**, **D**, and **E**: Insets were enlarged images of yellow boxes. ph, phialides; co, conidia; cph, conidiophore.

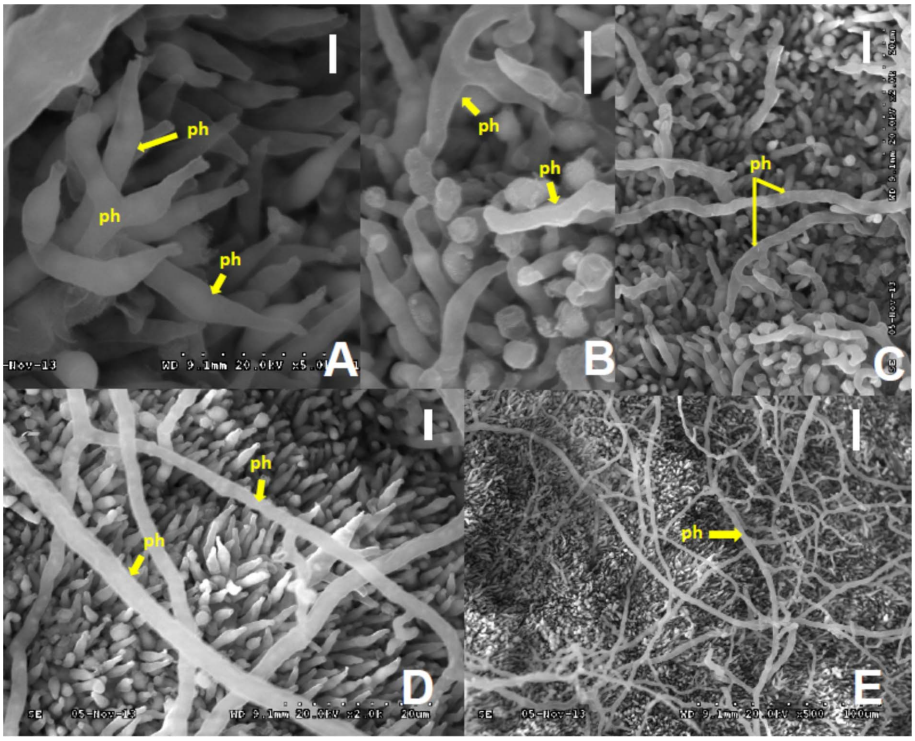

Fig. 3. Morphological features of modified phialides of *Ciboria carunculoides* RS103V. **A**: Typical ampuliform or navicular shape of phialides with some variations in branching and thickness, **B**: Atypical phialides elongated in horizon, **C**: Phialides excessively outgrown, **D**: Trailing phialides elongated over the lawn of typical phialides, **E**: Mycelia-like formation of atypical phialides. ph, phialides; co, conidia; cph, conidiophore.

inner tissue. Specimens for SEM were prepared as follows. For primary fixation, samples were placed at 4ºC for 4 hrs in 2% paraformaldehyde, 2% glutaraldehyde, 0.05 M sodium cacodylate buffer (pH 7.2). Then, they were washed at 4ºC for 10 mins three times with 0.05 M sodium cacodylate buffer (pH 7.2). Post-fixation was performed at 4ºC for 2 hrs with 1% osmium tetroxide in 0.05 M sodium cacodylate buffer (pH 7.2), followed by washing at room temperature two times briefly with distilled water (10 mins each). Subsequently, samples were stained with 0.05% uranyl acetate and dehydrated by gradually increasing the concentration of ethanol. After further dehydrated with HMDS (hexamethyldisilazane) or tetramethylsilane and subsequently in critical point dryer equilibrated with isoamyl acetate 100%, samples were mounted for the observation.

Morphological features of anamorphic stage of *C. carunculoides* RS103V. To further understand features of the anamorphic stages of C. *carunculoides* RS103V, the fungal samples attached to sclerotia were precisely examined using SEM (Fig. 2). Conidia, phialides, conidiophore-like structures were developed from the host tissue and formed the colonies (Fig. 2A). Conidiophores displayed no definite shape. Instead, they were developed to have branched (Fig. 2B), unbranched (Fig. 2C), or be very short in length (Fig. 2D). In addition, conidiophore also exhibited sub-erect, hyaline and aseptated pattern (data not shown). In some cases, the length of conidiophore was too short to delineate (Fig. 2E). Nonetheless, coniodiophore were measured as (1.4) 4.6–5.61 × 1.4–1.47 μm (Fig. 2B to 2D). Bunch of phialides (usually three or more) were developed from conidiophores terminally but not in a whorl shape (Fig. 2D), non verticillate, usually three in number on each conidiophore which was smooth-walled without clamps. Phialides were ampuliform to navicular in shape and measured as (5.1) 9.57–13.34 × 1.2–1.58 μm. Mostly, they enlarged terminally with some exceptionally modified branches (Fig. 2F). However, tip of the phialide always remain tapered (Fig. 2F). Mature phialides developed pear shaped conidia at the open apex (Fig. 2F). The conidia were also non-catenulate and tuberculate (data not shown). The sizes of conidia were measured as 1.69–3.44 × (1.54) 1.7–2.68 μm. At the early stage, all conidia surface was smooth (Fig. 2F) but gradually became uneven to rough when detached from phialide apex (Fig. 2G to 2I). Inside the host tissue, the dispersed conidia were germinated through developing germ tube (Fig. 2J).

Interestingly, the shapes of phialides were diversely modified (Fig. 3). Most of phialides exhibited ampuliform or navicular shape with some variations in branching and thickness (Fig. 3A). Interestingly, however, some phialides were continuously elongated horizontally (Fig. 3B) and trailed over the fungi colony (Fig. 3C and 3D). Later, these modified phialides dominated over the regular-sized phialides and formed the mycelia-like structure (Fig. 3E).

Inferred from the similar morphology, such compact mass might mislead to be regarded as true mycelia. Generally, conidia are formed from phialides or conidiophore, but never produced directly from mycelia. Therefore, observation of conidia emergence from such structure must be robust evidence to distinguish phialides from mycelia. To clarify this, we examined the surface of the newly formed sclerotia on which such net-like structure were overwhelmed (Fig. 4A). Noticeably, conidia were developed from the apex of modified and tangled net-like structures (Fig. 4B), which strongly indicate that such structure was comprised of phialides but not true mycelia. In other words, *C. carunculoides* strain RS103V developed the outgrown phialides in its anamorphic stage. Such modifications of phialides were possibly associated with sclerotia formation at the later stage.

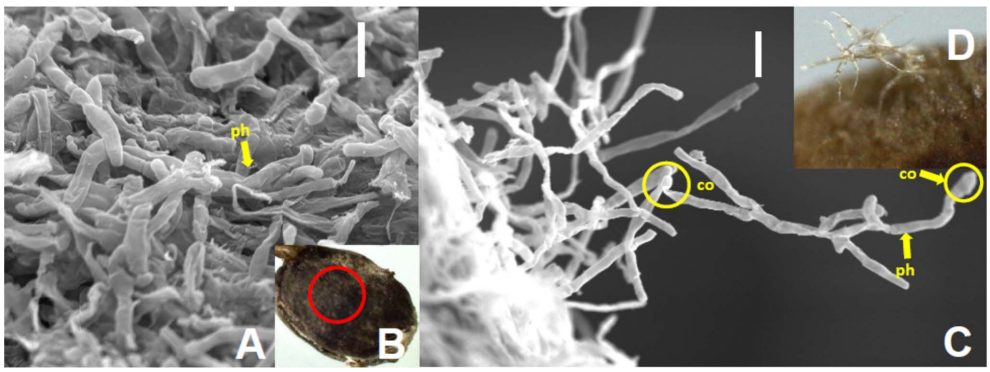

Fig. 4. Morphological features of trailing phialides *Ciboria carunculoides* RS103V. **A**: Phialides covering the surfaces of sclerotia, **B**: Another view of phialides grown over the sclerotia. ph, phialides; co, conidia; cph, conidiaphore.

Taken together, in anamorphic stages, *C. carunculoides* developed common asexual tissues like conidiophore, phialides, and conidia, but also exhibited the various morphological alterations which might be associated with the maintenance of cell viability and asexual propagations inside host tissues.

In conclusion, in this paper, we firstly reported the existence of *C. carunculoides*, a major causal fungus of popcorn disease, in Korea. Despite of technical obstacles in culturing of *Ciboria* genus in artificial media, we explicitly delineated the broad aspects of anamorphic stage of *C. carunculoides*. The current study provided the advanced insight into fungal behavior and development of effective controlling methods against mulberry popcorn disease.

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