

Phylogenetic and Morphological Characterization of *Cladosporium perangustum* Associated with Flyspeck on Shine Muscat Grapes in South Korea

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ABSTRACT

The Shine Muscat is a table grape, popular in South Korea for its unique mango-flavor taste. Flyspeck is a disease that is characterized by small, black, and circular specks on the grape cuticle was first observed in several commercial orchards in Sangju, South Korea, in August 2019. Here we identified the causal agent of flyspeck based on an advanced diagnosis approach, comprised of both morphological and molecular analyses. Morphological characteristics of the cultures isolated from grape flyspeck were identical to the fungus *Cladosporium perangustum*. The concatenated sequences of ITS, ACT, and *EF1-α* were used for molecular phylogenetic analysis, BLAST searches along with Bayesian inference-based phylogeny, confirmed that the causal agent of grape flyspeck is *C. perangustum*. The cultured fungal isolates also produced flyspeck symptoms on healthy fruits in pathogenicity tests. To the best of my knowledge, this is the first documented evidence of any *Cladosporium* sp. producing flyspeck symptoms on any plant.

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1. Introduction

Shine Muscat is a popular grape variety in South Korea, known for its large, seedless berry, mango flavor, and sweet taste. Because of increasing demand, the market price of Shine Muscat is higher than that of other table grapes. Consequently, more farmers are cultivating Shine Muscat grapes, and production has been increasing since 2016 [1].

However, fungal diseases, such as anthracnose and bunch rot, are a destructive challenge for Shine Muscat cultivation [2,3]. Recently, flyspeck disease was observed at several commercial orchards in Sangju, South Korea. Flyspeck is characterized by small black spots on the grape that resembles fly excreta, which easily rubs off, and is thought to be caused by fungal species that superficially colonize the fruit surface (Figure 1). Though flyspeck does not cause physical damage to grapes, it greatly reduces marketability, so disease management is critical in orchards. To date, several species of fungi including *Zygothia jamaicensis* and *Schizothyrium pomi* (more than 10 genera) have been associated with flyspeck on different hosts, including apples, pears, carnations, and bananas [4–6]. The identification of the causal agent(s) of flyspeck on grapes is a prerequisite to developing sustainable disease

management strategies in fruit orchards. Here we identify the causal agent of this newly emerging disease by employing both morphological and molecular approaches.

Shine Muscat fruits showing typical flyspeck symptoms were brought to the laboratory, rinsed with sterile distilled water for one minute, and dried in the hood. Segments of grape peels containing black spots were transferred to water agar (WA) and incubated at 25°C in the dark. Mycelium growth was visible after one week of incubation. WA plugs containing actively growing mycelia (5 mm in diameter) were then transferred to potato dextrose agar (Difco, Becton Dickinson, Sparks, MD). A total of four isolates were obtained, examined for morphological characteristics, and stored in 10% glycerol at –70°C.

Fourteen-day-old colonies were examined by microscopy and found to be gray-olivaceous and olivaceous-black when viewed from the reverse side (Figure 2(A,B)). Conidiophores were macronematous or semi-macronematous, erect, filiform to cylindrical, 0–5 septate, and 28.4–107.7 μm × 3.0–6.5 μm in size (av. ± SD: 63.1 μm ± 22.5 μm × 4.7 μm ± 0.9 μm). Ramoconidia were cylindrical-oblong, mostly aseptate, with truncate bases and 16.1–33.8 μm × 4.8–5.3 μm (av. ± SD: 22.8 μm ± 5.2 μm × 4.2 μm ± 0.6 μm). The base was

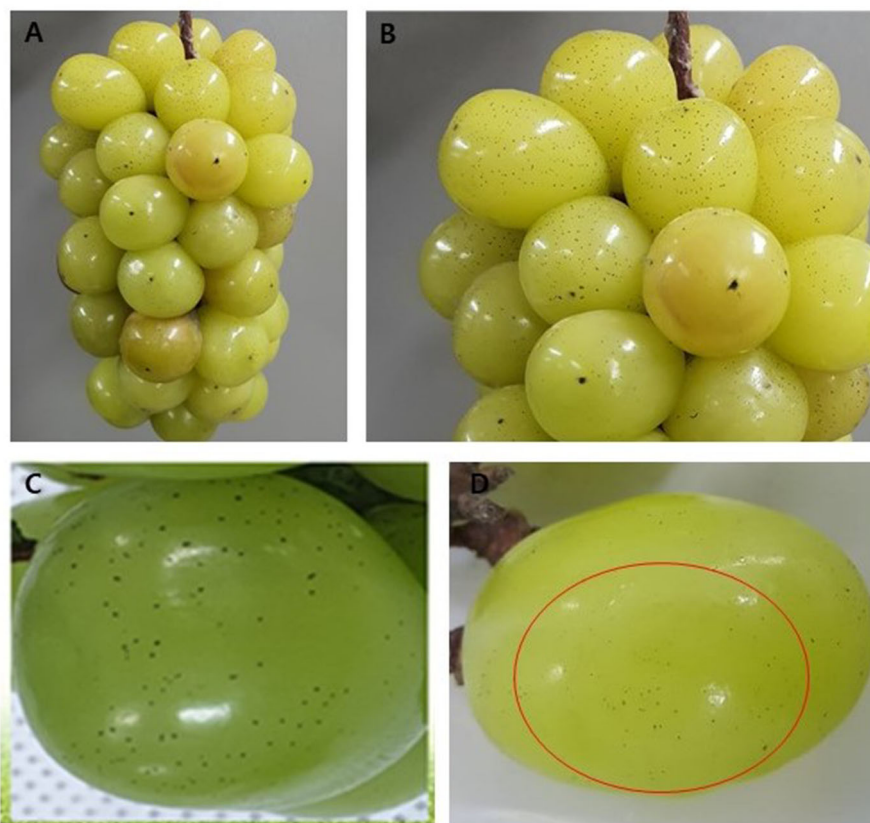


Figure 1. Symptoms of flyspeck on Shine Muscat fruits. (A, B) Symptoms on naturally infected grapes; (C, D) Flyspeck symptoms on artificially inoculated fruit.

1.6–3.7 μm (av. \pm SD: 3.1 μm \pm 0.8 μm) in diameter. Secondary ramoconidia were mostly cylindrical, few were oblong and 8.4–20.3 μm \times 3.3–4.4 μm (av. \pm SD: 13.82 μm \pm 2.9 \times 3.1 μm \pm 0.9 μm). Intercalary conidia were varied in shape, including ellipsoid, ovoid, subcylindrical, and 6.2–15.1 μm \times 2.1–4.1 μm (av. \pm SD: 9.8 μm \pm 1.8 μm \times 3.3 μm \pm 0.4 μm). Terminal conidia were globose and 2.7–4.4 μm \times 1.7–3.8 μm (av. \pm SD: 3.7 μm \pm 0.5 μm \times 2.7 μm \pm 0.6 μm) in size (Figure 2(C–F)). The morphological characteristics of the flyspeck isolates were almost identical to those of *Cladosporium* spp., described by Bensch et al. [7] (Table 1).

For molecular identification, genomic DNA was extracted from seven-day-old colonies of isolates SM1 and SM4 using a HiGene™ Genomic DNA Prep Kit (BIOFACT, Yuseong-Gu, and Daejeon, Korea). The internal transcribed spacer (ITS), actin (*ACT*), and translation elongation factor 1- α (*EF1- α*) genes were targeted for amplification, sequencing, and species identification. Primer pairs ITS1/ITS4, EF1-728F/EF1-986R and ACT-512F/ACT-783R were used to amplify and sequence fragments of ITS, *EF1- α* , and *ACT*, respectively [8,9]. The PCR conditions were as follows: an initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, primer annealing at 53 °C for ITS, 58 °C for *ACT* and 56 °C for *EF1- α* for 30 s, primer extension

72 °C for 1 min, and a final extension at 72 °C for 5 min. The success of PCR reactions was confirmed by visualizing in 1% agarose gels (w/v) stained with loading star dye (6 \times) (Dyebio, Gyeonggi, Korea) in and viewed under a LED illuminator. The confirmed PCR products were purified using the HiGene™ PCR Purification Kit (Yuseong-Gu, Daejeon, Korea) prior to sequencing. Purified PCR products were sent to Macrogen, Inc. (Seoul, Korea) for sequencing. The generated DNA sequences were analyzed in SeqMan v. 7.1 from the Lasergene package (DNASTAR, Inc. Madison, WI, USA) to obtain consensus sequences and deposited them in GenBank. The GenBank accession number for each gene isolated from the Shine Muscat flyspeck cultures is shown in Table 2. The sequences from these isolates were compared to those in the National Center for Biotechnology Information (NCBI) database using BLAST search tools to find the closest fungal species. BLASTn analysis showed that the sequence fragments obtained from the fungal isolates were 99.9, 99.5, and 99.6% homologous to the ITS, *EF1- α* , and *ACT* genes from *Cladosporium per-angustum* (strain CBS 125996), respectively. Next, the combined sequences of ITS, *EF1- α* , and *ACT* from the fungal isolates, and the reference *Cladosporium* spp. described by Bensch et al. [7], were used for phylogenetic analysis. Single gene

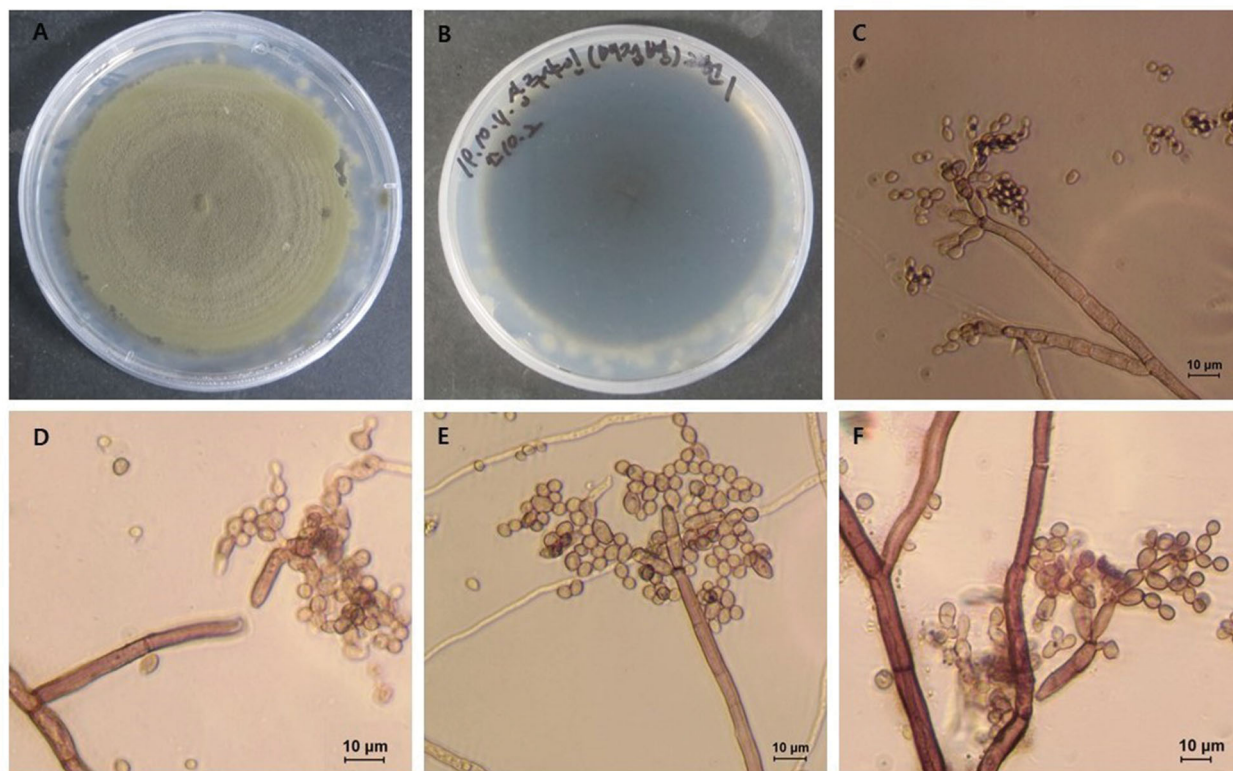


Figure 2. Morphological characteristics of *Cladosporium perangustum* (SM1). (A, B) Fourteen-day old colony; (C–F) Macronematous conidiophores and conidial chains. Scale bar = 10 µm.

Table 1. Comparative study of morphological characteristics of present and previously identified isolates of *Cladosporium perangustum*.

Characteristics	Present isolate	<i>Cladosporium perangustum</i> [7]
Colony	Gray-olivaceous to olivaceous-black	Gray-olivaceous to olivaceous, olivaceous-gray or iron-gray.
Conidiophores		
Shape	Macronematous or semimacronematous, erect, filiform to cylindrical, and 0-5 septate.	Macronematous, semimacronematous or micronematous, erect, straight or slightly flexuous, filiform to narrowly cylindrical-oblong, and 0-6 septate.
Size	28.4–107.7 × 3.0–6.5 µm (av. ± SD: 63.1 ± 22.5 × 4.7 ± 0.9)	12–130 × 2–3.5 µm
Ramoconidia		
Shape	Cylindrical-oblong, mostly aseptate, and truncate bases.	Cylindrical-oblong, rarely septate, and truncate bases
Size	16.1–33.8 × 4.8–5.3 µm (av. ± SD: 22.8 ± 5.2 × 4.2 ± 0.6). Base: 1.6–3.7 (av. ± SD: 3.1 ± 0.8)	25–45 × 2.5–3 (–4.5) µm Base: 2–2.5 (–4) µm wide
Secondary ramoconidia		
Shape	Mostly cylindrical, few are oblong	Narrowly ellipsoid to cylindrical-oblong and septate
Size	8.4–20.3 × 3.3–4.4 µm (av. ± SD: 13.82 ± 2.9 × 3.1 ± 0.9)	6–30 × 2–3 µm (av. ± SD: 17.8 ± 7.4 × 2.5 ± 0.4)
Intercalary conidia		
Shape	Ellipsoid, ovoid, and subcylindrical	Ovoid, limoniform to ellipsoid, somewhat fusiform or subcylindrical
Size	6.2–15.1 × 2.1–4.1 µm (av. ± SD: 9.8 ± 1.8 × 3.3 ± 0.4)	4–16 × 2–3 µm (av. ± SD: 8.7 ± 3.8 × 2.5 ± 0.4)
Terminal conidia		
Shape	Globose	Globose, subglobose or ovoid to obovoid
Size	2.7–4.4 × 1.7–3.8 µm (av. ± SD: 3.7 ± 0.5 × 2.7 ± 0.6)	2–4 × 2–2.5 µm (av. ± SD: 3.1 ± 0.6 × 2.1 ± 0.2)

sequences from 33 taxa (*Cladosporium* spp. reference isolates from Bensch et al. [7]) were aligned using MEGA v. 6.0 [10] and then concatenated with Mesquite v. 2.75 [11]. A 50% majority-rule consensus tree was constructed using MrBayes 3.2.10 [12] and viewed in FigTree v1. 3.1 [13]. The phylogenetic

Table 2. List of fungal isolates present and their Genbank accession numbers.

Isolates	Host	Accession number		
		ITS	EF-1α	ACT
SM1	Shine Muscat	LC573980	LC573984	LC573982
SM4	Shine Muscat	LC573981	LC573985	LC573983

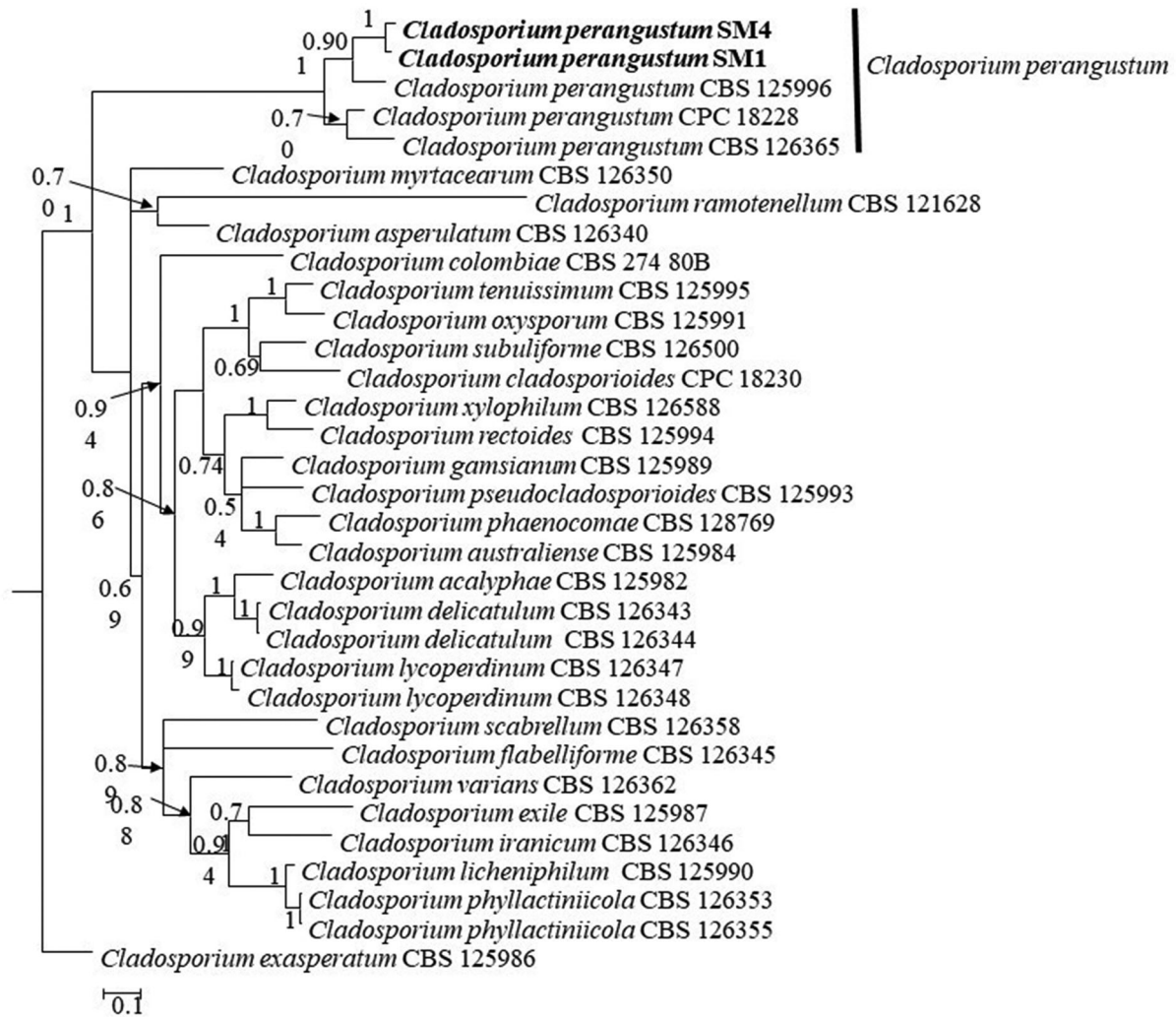


Figure 3. A 50% majority-rule consensus tree (Bayesian inference) using a combined dataset of ITS, *EF1-a* and *ACT* sequences. The posterior probability (>0.5) are given at the nodes. The scale bar shows the number of substitutions per site.

tree shows that genes found in the Shine Muscat flyspeck isolates form a distinct clade with *C. perangustum*, with high posterior probability (Figure 3).

To test *C. perangustum* pathogenicity on grapes, mature asymptomatic Shine Muscat berries were surface sterilized with 70% ethanol and dried. Spore suspensions were prepared from 25-day old flyspeck cultures. Approximately 15 mL of sterilized distilled water was added to each plate and spores were loosened by scraping the agar surface with a sterile wire loop. The suspension was filtered through Kimtech tissue paper (Kimberly–Clark Professional), and the spore concentration was estimated using a hemocytometer. Healthy fruits were inoculated by swabbing with an inoculum suspension containing 1×10^6 spores mL^{-1} using a sterilized brush. This process was repeated for each isolate and control berries were inoculated with sterile water alone. Samples were kept in a plastic box and incubated at 25 °C in the dark. This experiment was replicated 12 times, with one berry considered as one replication. At 20 days post-incubation, flyspeck symptoms (Figure 1(C,D)) were observed on all inoculated

berries, identical to the symptoms seen in the orchard, while the control berries remained healthy. Furthermore, flyspeck symptoms were found on 100% of the inoculated berries. The causal agent was re-isolated from the flyspeck and identified as *C. perangustum* based on morphological characters.

Cladosporium is a common genus of fungi, containing more than 722 named species [14,15]. Bensch et al. [16] revised the taxonomy of *Cladosporium* and recognized 169 species. Due to the saprophytic and pathogenic nature of fungal species within *Cladosporium*, this genus has a wide host range [14–16]. *Cladosporium sphaerospermum* has previously been reported as the causal agent of sooty mold in blueberries, while *Cladosporium cladosporioides* is the causal agent of the sooty spot on *Citrus unshiu* (mandarins) [17,18]. This study identified *C. perangustum* as the causal agent of flyspeck on Shine Muscat grapes. Previously, *C. perangustum* was implicated in the wood discoloration of pine and larch in South Korea, and leaf spot of *Myrica rubra* in China [14,19]. The symptoms produced by *C. perangustum* on grapes are identical to flyspeck.

This is the first report of flyspeck being caused by any *Cladosporium* species anywhere. No *Cladosporium* species had previously been documented as producing flyspeck symptoms on any plant. *Cladosporium* spp. are common surface inhabitants on fruit, but have not been associated previously with flyspeck symptoms. Therefore, this report is important not only to alert Korean farmers, crop managers, and plant pathologists about an emerging disease and pathogen but also as an expansion of scientific knowledge of diversity in the SBFS complex, which includes >100 species in many genera.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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