RESEARCH ARTICLE

Ambrosiella catenulata Isolated from Ambrosia Beetles in Korean Apple Orchards

Ju-Heon Lee[1,2], Youngsoo Kim[1], Jong-Taek Park[1], Dong-Hyuk Lee[1], and Hee-Young Jung[2*]
[1]Apple Research Center, National Institute of Horticultural & Herbal Science, Gunwi 43100, Korea
[2]Department of Plant Medicine, Kyungpook National University, Daegu 41566, Korea

Corresponding author: heeyoung@knu.ac.kr

ABSTRACT

A fungus was isolated from ambrosia beetles collected using beetle traps in an apple orchard in Gunwi-gun, Daegu, South Korea. This fungal strain was termed ARI-24-A5, and was identified through morphological characterization and molecular phylogenetic analysis. After 8 d of incubation on potato dextrose agar (PDA), ARI-24-A5 exhibited gray-to-olive coloration, abundant aerial mycelia, and a colony diameter of 72.0–79.0 mm. Morphologically, the aleurioconidiophores formed monilioid chain structures, and the size of the aleurioconidia was 11.1 × 10.8 µm. For precise identification, molecular phylogenetic analysis was performed using the internal transcribed spacer (ITS) region, translation elongation factor 1-alpha (TEF1-α), small subunit of nuclear ribosomal RNA (SSU), and RNA polymerase II subunit 1 (RPB1) gene sequences. The overall analysis confirmed that ARI-24-A5 belongs to the genus *Ambrosiella*, which is known for its symbiotic relationship with ambrosia beetles. In the phylogenetic tree, ARI-24-A5 shared the same taxonomic position as *A. catenulata* and its morphological characteristics were consistent with those of this species. Therefore, ARI-24-A5 was identified as *A. catenulata*, making this the first record of this species in South Korea.

Keywords: Ambrosia beetle, *Ambrosiella catenulata*, Korean apple orchard, Symbiotic fungi

pISSN : 0253-651X
eISSN : 2383-5249

Kor. J. Mycol. 2024 December, 52(4):381-389
https://doi.org/10.4489/kjm.520416

Received: December 16, 2024
Revised: December 20, 2024
Accepted: December 20, 2024

INTRODUCTION

Ambrosia beetles (Coleoptera: Curculionidae) belong to the subfamilies Scolytinae and Platypodinae and are a highly diverse pest group, with 158 species reported in Korea [1]. These beetles cause significant damage to orchards, nurseries, and forest ecosystems, making them major pests [2]. Based on their ecological characteristics, beetles are classified into two types: bark beetles, which feed on the tree substrate itself, and ambrosia beetles, which bore tunnels into trees and cultivate ambrosia fungi within these tunnels for nutrition [3,4]. These unique ecological behaviors suggest that these pests cause long-term damage to trees through interactions with their symbiotic fungi.

Most of these beetles have evolved a wide range of symbiotic relationships with fungi. However, the degree and nature of these associations vary significantly, often exhibiting unique characteristics that are

not observed in other beetle groups [5,6]. Similarly, the relationships among bark beetles, fungi, and their tree hosts are diverse. Some bark beetles require tree mortality for reproduction [7], whereas others feed on white-rot fungi found in decaying tree trunks [8], and some live in association with fungi in dry branches [9].

In Korea, *Raffaelea quercus-mongolicae* is the causal agent of oak wilt disease and has been reported to form a symbiotic relationship with *Platypus koryoensis*, with beetle invasion triggering the disease [1]. In addition, in the United States, ambrosia beetles (*Anisandrus maiche*), which contribute to the decline of apple trees, have been collected. *Ambrosiella cleistominuta* has been isolated from the galleries and adults of this beetle and was identified as a symbiotic fungus associated with *A. maiche* [2].

As mentioned, ambrosia beetles are associated with various fungi [5,10], and Batra [11] defined 'primary ambrosia fungi' as fungi that co-evolve with the mycangia of the beetle and form dense spore layers inside the beetle tunnels, serving as a food source for the beetles. Most species of the genus *Ambrosiella*, associated with ambrosia beetles, have been reported to exhibit a strong relationship with these beetles [12]. In Korea, two species have been documented: *A. grosmanniae* KNU16-001 [13] from soil and *A. roeperi* ARI-24-A4 [14] from ambrosia beetles.

In the present study, ambrosia beetles that damaged apple trees in domestic orchards were collected using beetle traps. The fungi isolated from these beetles were identified based on morphological and molecular phylogenetic analyses. The results of these identifications are reported here.

MATERIALS AND METHODS

Fungal isolation from ambrosia beetles

Ambrosia beetles were collected using a beetle trap in an orchard at the Apple Research Center in Gunwi-gun, Daegu-si, Korea (36°29′68.9″N, 128°46′56.1″E). The beetle bodies were surface-sterilized with 70% ethanol and thoroughly dried for approximately 10 min. Subsequently, the insects were dissected by separating the head and thorax from the abdomen. Each segment was transferred onto potato dextrose agar (PDA; Difco, Detroit, MI, USA) plates and incubated at 25℃ for 3 d. The resulting mycelial growth was subcultured onto fresh PDA plates and incubated for an additional 8 d at the same temperature. The resulting pure culture was labeled as ARI-24-A5 and preserved in 20% glycerol at -80℃ for future use.

Morphological characterization

The isolated ARI-24-A5 was cultured on PDA medium at 25℃ for 8 d to examine its cultural and mycological characteristics. After cultivation, various features of the fungal colonies, such as diameter and color, were observed. The morphology and size of the conidia and conidiophores were examined and recorded using a light microscope (CX-43; Olympus, Japan).

Genomic DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing

For molecular analysis and to assess phylogenetic relationships, genomic DNA from strain ARI-24-A5 was extracted using the HiGene Genomic DNA Preparation Kit (Biofact, Daejeon, Korea) following the manufacturer's guidelines. Subsequently, partial sequences of the internal transcribed spacer (ITS) regions, small subunit of nuclear ribosomal RNA (SSU), translation elongation factor 1-alpha (TEF1-α), and RNA polymerase II subunit 1 (RPB1) genes were amplified and obtained using PCR. The ITS regions were amplified using the ITS1F/ITS4 [15,16] and ITS5/LR3 primers [16]. The SSU gene was amplified using the NS-1/NS-6 primers [16,17]. The TEF1-α gene was amplified using the EFCF1.5/EFCF6 primer pair [18]. The RPB1 gene was amplified using the RPB1-Af/RPB1-Cr primer pair [12,19,20]. The PCR amplification products were verified through electrophoresis on 1% agarose gels, followed by staining with ethidium bromide. The resulting PCR products were purified using EXOSAP-IT reagent (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's guidelines. Sequencing was performed by Solgent Co. Ltd. (Daejeon, Korea). Sequence analysis was performed using SeqMan Lasergene software (DNAStar Inc., Madison, Wisconsin, USA). The ITS regions, SSU, TEF1-α, and RPB1 gene sequences were deposited in GenBank with accession numbers LC835911 (ITS), LC835913 (SSU), LC835915 (TEF1-α), and LC848343 (RPB1), respectively.

Molecular phylogenetic analysis

The sequences of *Ambrosiella* spp. were retrieved from the National Center for Biotechnology Information (NCBI) database. These sequences were aligned using Clustal X 2.0, in MEGA 7 [21]. A phylogenetic tree was constructed based on concatenated nucleotide sequences of the ITS region, TEF1-α, and SSU genes. For the analysis, the nearest neighbor interchange method was employed using Kimura's two-parameter model [22], excluding gaps. A phylogenetic tree was constructed using the maximum likelihood (ML) method [23], and reliability was assessed using bootstrap values from 1,000 replicates.

RESULTS

Mycological characteristics of strain ARI-24-A5

After culturing on PDA at 25℃ for 8 d, the colony diameter reached 72.3–79.2 mm. On the front side, white mycelia were observed in the center, which transitioned from olive to white toward the edges. In addition, the surface was covered with abundant aerial mycelia. The back side was generally olive-gray in color (Fig. 1A).

The aleurioconidiophores were hyaline, smooth, ovoid, and monilioid, with branching structures (Fig. 1B). Conidia and collarettes were formed at the terminal ends of the conidiophores (Figs. 1C and D). Aleurioconidia are hyaline, ranging from spherical to subglobose in shape, with an average size of

11.1 × 10.8 μm (n = 50), were located at the ends of the aleurioconidiophores. When the aleurioconidia detached from the terminal end, they typically carried one or two conidiophores (Fig. 1E). In most cases, aleurioconidia detach from multiple conidiophore cells during chain formation (Fig. 1F).

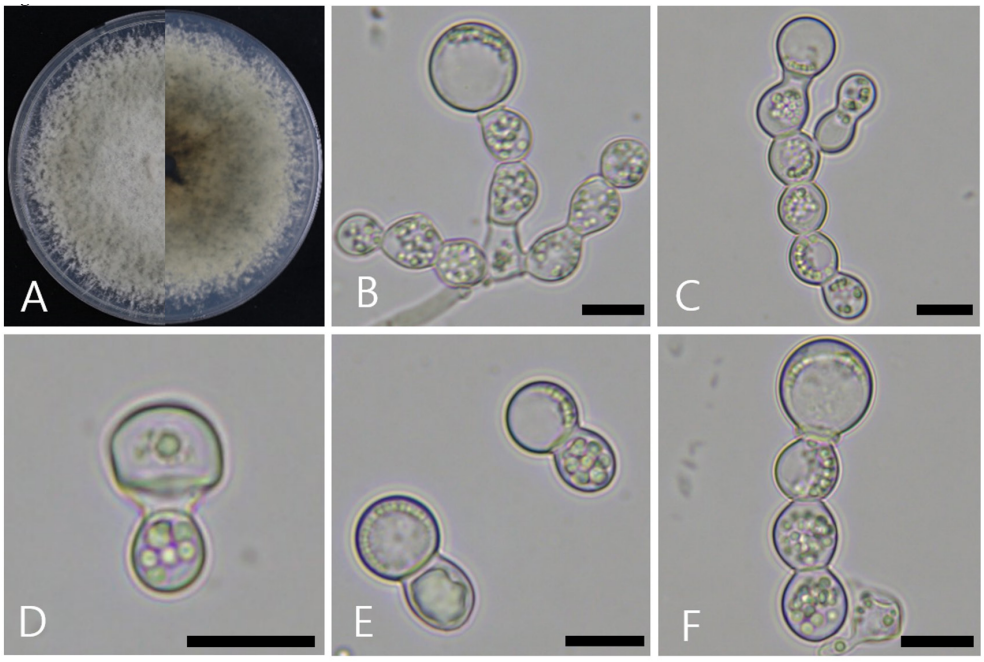

Fig. 1. Cultural and morphological characteristics of *Ambrosiella catenulata*. A: Front and reverse view of the colony grown on potato dextrose agar (PDA) for 8 days at 25℃. B, C: Aleurioconidiophores with terminal aleurioconidia. D: Aleurioconidiophores with collarettes. E: Aleurioconidiophores with aleurioconidia. F: Aleurioconidiophores with aleurioconidia and forms a monilioid chain. Scale bars: B–F = 10 μm.

Phylogenetic analysis

To analyze the molecular and phylogenetic relationships of ARI-24-A5, four molecular markers (ITS region, TEF1-α, SSU, and RPB1) were used. The lengths of the sequences obtained were 1,078, 1,119, 1,318, and 762 bp, respectively. A Basic Local Alignment Search Tool (BLAST) search was conducted to compare these sequences with those of other strains in the NCBI database. For the ITS region, ARI-24-A5 showed the highest similarity (100%) to *Ambrosiella catenulata* W186q, followed by 99.8% similarity with *A. xylebori* Hulcr5114 and *A. cleistominuta* C3843. For TEF1-α, ARI-24-A5 exhibited 100% similarity with *A. catenulata* W186q, 97.2% with *A. xylebori* CBS 110.61, and 96.3% with *A. grosmanniae* 1002HHS1. For SSU, ARI-24-A5 displayed 100.0% similarity with *A. catenulata* C3913 and 99.9% similarity with *A. batrae*, *A. xylebori*, and *A. cleistominuta*. Finally, RPB1 of ARI-24-A5 showed 100% similarity with three strains of *A. catenulata* (12B1, 12B2, and W186q), 99.1% similarity with *A. xylebori* JH12105, 96.5% similarity with *A. grosmanniae* JH12109, and 95.2% similarity with *A. beaveri* W204qT. This analysis indicated that ARI-24-A5 matched perfectly (100%) with *A. catenulata* across all four genetic markers (ITS, TEF1-α, SSU, and RPB1). To analyze the phylogenetic relationships of ARI-24-A5, a multilocus sequence

analysis (MLSA) was conducted using ITS, TEF1-α, and SSU, based on the process of Mayers et al. [24], with nucleotide sequence data retrieved from the NCBI database for *Ambrosiella* species (Table 1). The phylogenetic tree was constructed using the ML method and confirmed that ARI-24-A5 shares the same phylogenetic position as *A. catenulata* and is clearly distinguishable from other species (Fig. 2). Based on comprehensive phylogenetic analyses, ARI-24-A5 was conclusively identified as identical to *A. catenulata* at the species level.

Table 1. The following is a list of species included in the phylogenetic analyses, along with their corresponding GenBank accession numbers

Species	Strain	Associated ambrosia beetle	GenBank accession numbers		
			ITS	TEF1-α	SSU
Ambrosiella batrae	CBS 139735^{T}	*Anisandrus sayi*	KR611322	KT290320	KR673881
Ambrosiella catenulata	C3913	Ambrosia beetle	MG950184	MG944394	MG950189
Ambrosiella cleistominuta	C3843	*Anisandrus maiche*	KX909940	KX925304	KX925309
Ambrosiella grosmanniae	CBS 137359^{T}	*Xylosandrus germanus*	KR611324	KT318382	KR673884
Ambrosiella hartigii	CBS 404.82	*Anisandrus dispar*	KF669873	KT318383	KR673885
Ambrosiella nakashimae	CBS 139739^{T}	*Xylosandrus amputatus*	KR611323	KT318381	KR673883
Ambrosiella remansi	M290	*Remansus mutabilis*	KX342068	KX342072	KX354426
Ambrosiella roeperi	CBS 135864^{T}	*Xylosandrus crassiusculus*	KF669871	KT318384	KR673886
Ambrosiella xylebori	CBS 110.61^{T}	*Xylosandrus compactus*	KF669874	KT318385	KR673887
Ambrosiella catenulata	**ARI-24-A5**	**Ambrosia beetle**	**LC835911**	**LC835915**	**LC835913**

ITS: internal transcribed spacer regions; TEF1-α: translation elongation factor 1-α; SSU: small subunit of nuclear ribosomal RNA.
^{T} ex-type. The isolated strain is shown in bold.

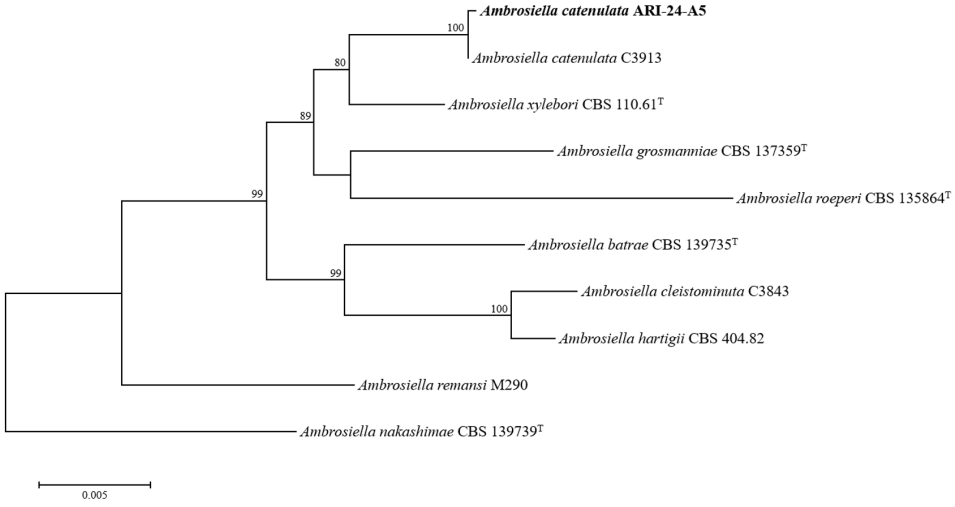

Fig. 2. Maximum-likelihood phylogenetic tree of ARI-24-A5 based on the combined sequences (ITS + TEF1-α + SSU), showing the phylogenetic position of the ARI-24-A5 strain among *Ambrosiella* species. Bootstrap values (based on 1,000 replications) greater than 70% are indicated at the branch points. The isolated strain is highlighted in bold. *Ambrosiella nakashimae* (CBS 139739^{T}) was used as the outgroup. Bar = 0.005 substitutions per nucleotide position. 'T' indicates the type strain. ITS: internal transcribed spacer regions; TEF1-α: translation elongation factor 1-α; SSU: small subunit of nuclear ribosomal RNA.

Comparison of mycological characteristics

The culture and morphological characteristics of ARI-24-A5 were consistent with those of *A. catenulata*. *A. catenulata* is morphologically very similar to *A. roeperi*, and the ARI-24-A5 strain showed similar trends to *A. roeperi* in terms of the shape and size of its aleurioconidia. The size of the aleurioconidia in ARI-24-A5 was (7–)11.1(–16) × (7–)10.8(–13) μm, which was closely similar to that of the aleurioconidia in *A. roeperi*, which was (7–)11.0(–16) × (5–)10.0(–14) μm. In addition, both species have spherical aleurioconidia. However, when conidia dislodge, two or more conidiophore cells typically remain attached to the dislodged aleurioconidia in *A. catenulata*, often forming a chain-like structure, whereas in *A. roeperi*, typically only one cell remains. Furthermore, the sporodochia of *A. catenulata* were spherical, whereas those of *A. roeperi* appeared diffuse and cushion-like (Table 2).

Table 2. Comparison of the morphological characteristics of strain ARI-24-A5 with those of the reference species *Ambrosiella catenulata* and *A. roeperi*

Characteristics		*A. catenulata*[a] (ARI-24-A5)	*A. catenulata*[b]	*A. roeperi*[b]
Colony	Color	Overall light gray color is exhibited, with olive coloration observed at the edges. Additionally, abundant aerial mycelium is formed	Olivaceous to gray, surface covered with abundant aerial mycelium	Olivaceous to gray, dark gray, to dark-brown superficial
	Shape	Colonies on PDA attaining 72.0–79.0 mm diam after 8 days at 25℃	Colonies on PDA attaining 52.0–59.0 mm diam after 8 days at 25℃	Colonies on PDA attaining 60.5–70.0 mm diam after 8 days at 25℃
Aleurioconidiophores	Color	Hyaline	Hyaline	Hyaline to subhyaline
	Shape	Smooth and ellipsoidal, forming monilioid chains, with the part attached to the spores observed as thickened septa	Smooth, ellipsoidal, monilioid, branched, sometimes the collarette appearing as thickened septa	Smooth, cylindrical, monilioid, branched, with or without a collarette on the top cell of conidiophores
Aleurioconidia	Color	Hyaline	Hyaline	Hyaline
	Shape	Thick-walled, smooth, with organelles observed, almost spherical in shape, When detaching, it mostly contains one or more conidiophore cells and forms a chain-like	Globose to subglobose, single and terminal on aleur ioconidiophores, when detaching, mostly carrying two (rarely one) or more conidiophore cells	Thick-walled, globose to subglobose, if detachable, it mostly carries a single conidiophore cell, which is cushion-like in shape
	Size (μm)	(7–)11.1(–16) × (7–)10.8(–13)	(8–)9–13(–16) × (7 .5–)9–12(–13)	(7–)9–13(–16) × (5–)8–12(–14)

[a] Fungal strain studied in this paper; [b] Source of description [31].

DISCUSSION

This study aimed to analyze symbiotic fungi isolated from ambrosia beetles collected from apple trees in Korea, with a focus on the accurate identification of fungal species associated with these beetles. The fungus isolated from ambrosia beetles was identified as *A. catenulata*.

Species of the genus *Ambrosiella* Brader ex Arx & Hennebert [25] have been shown to form symbiotic relationships with ambrosia beetles, establishing dense fungal gardens known as "ambrosia", which serve as the beetles exclusive food source [26–28]. These fungi are typically found in beetles with large and complex mesothoracic mycangia, which are specialized structures for storing and transporting fungi [12]. In contrast, beetles with smaller mycangia may host various species of the genus *Raffaelea* as well as other fungi. Specific species within the genus *Raffaelea* form symbiotic relationships with multiple beetle species [29,30]. A key morphological feature of *Ambrosiella* spp. is the formation of aleurioconidia, which are often

attached to one or more conidiophore cells, or positioned at the tip of an independent aleurioconidiophore. Aleurioconidia may exist as single units or, rarely, as short chains and may also form a collarette at the apex of the conidiophore [31].

To distinguish *Ambrosiella* spp., Mayers et al. [24] used ITS regions, TEF1-α, and SSU gene sequences to differentiate species such as *A. beaveri*, *A. ferruginea*, *A. hartigii*, *A. roeperi*, and *A. xylebori* [12]. However, these markers are insufficient for differentiating *A. nakashimae* from *A. beaveri*. In 2017, Lin et al. [31] proposed *A. catenulata* as a new species based on ITS, TEF1-α, and RPB1 gene sequences. However, these markers failed to differentiate *A. nakashimae* from *A. beaveri*. This finding is consistent with the results of Mayers et al. [24] and Lin et al. [31], who reported that their phylogenetic analysis aligned with the phylogenetic tree presented by Mayers et al. [24].

For this analysis, four molecular markers (ITS, TEF1-α, SSU, and RPB1) previously applied in related studies were used to determine the molecular phylogeny of strain ARI-24-A5. A BLAST search confirmed that this strain is *A. catenulata*. Its closest relative was identified as *A. xylebori*, which showed morphological similarities to *A. roeperi*, although significant morphological differences were observed in the sporodochia structure. To construct the phylogenetic tree, the ITS, TEF1-α, and SSU gene sequences were analyzed following the method used by Mayers. The RPB1 sequences used in the method of Lin et al. [31] were excluded because of the lack of sequence data for most *Ambrosiella* specimens. Both phylogenetic trees yielded consistent results, allowing the use of a single method.

The comprehensive findings of this study revealed that ARI-24-A5 shares the same phylogenetic position as *A. catenulata* C3913 and is distinct from other *Ambrosiella* spp.. This represents the first report of *A. catenulata* in Korea. In addition, *R. quercus-mongolicae* has been reported as a symbiotic fungus of the ambrosia beetle (*P. koryoensis*) that damages oak trees in Korea [1]. Although reports on symbiotic fungi associated with ambrosia beetles in Korean apple orchards remain limited, in the United States, symbiotic fungi such as *Ambrosiella xylebori* and *A. cleistominuta* have been isolated from ambrosia beetles that damage apple orchards in New York [2,32]. This indicates that the ambrosia beetles that damage apple trees in Korea may also be associated with symbiotic fungi other than *A. catenulata*, highlighting their potential for discovering additional symbiotic fungi. Understanding these symbiotic relationships is essential to devise sustainable pest management strategies. Future research should focus on thoroughly investigating the ecological and economic impacts of *A. catenulata* in Korean apple orchards and exploring the influence of environmental factors on its distribution.

CONFLICTS OF INTEREST

The authors declare that they have no potential conflicts of interest.

ACKNOWLEDGMENTS

This study was supported by the "Cooperative Research Program for Agriculture Science and

Technology Development (Project No. PJ01718404)" funded by the Rural Development Administration, Republic of Korea.

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