RESEARCH ARTICLE

Ambrosiella roeperi: An Unreported Fungus Isolated from Ambrosia Beetles

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ABSTRACT

In this study, ambrosia beetles were collected using insect traps in Gunwi-gun, Daegu-si, South Korea, to investigate symbiotic fungi associated with beetles. Fungi were isolated from the collected beetles. Among the isolates, a strain obtained from *Ambrosiodmus rubricollis* was designated as ARI-24-A4. Cultural and molecular biological analyses confirmed that ARI-24-A4 belongs to *Ambrosiella*. Key morphological characteristics, including the structure of the conidiophores and the size of aleurioconidia (av. 12.9 μm × 12.5 μm), were examined to accurately identify the *Ambrosiella* species. A phylogenetic tree was constructed by combining the internal transcribed spacer (ITS) region, translation elongation factor 1-alpha (TEF1-α), and small subunit of nuclear ribosomal RNA (SSU) gene sequences to confirm the phylogenetic position. The strain was verified to share the same phylogenetic position as *Ambrosiella roeperi*. Therefore, ARI-24-A4 was confirmed to be *A. roeperi*, a species previously unreported in Korea. It has been recorded as a newly identified species in the country.

Keywords: Ambrosia beetles, *Ambrosiella roeperi*, Phylogeny, Symbiotic

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INTRODUCTION

Ambrosiella is a fungal genus defined by Harrington [1]. It belongs to the family Ceratocystidaceae within the order Microascales of the class Sordariomycetes. Many species are dispersed by insect vectors or through the frass of ambrosia beetles; some of them act as tree pathogens [2,3]. Currently, 20 *Ambrosiella* species are recorded in the Mycobank database. Among them, *A. xylebori* [4], *A. hartigii* [5], *A. beaveri* [6], and *A. roeperi* [7] are closely associated with the mesonotal pouch mycangia of the *Xylosandrus* complex. These mycangia are specialized beetle structures that store and transport symbiotic fungi into trees.

Ambrosia beetles (Coleoptera: Curculionidae: Scolytinae and Platypodinae) are major pests that bore tunnels into trees, consequently damaging the xylem and substantially harming forests and orchards [8]. Although they were historically regarded as forest pests [9], recent reports from Europe and the United States have shown that they can severely damage fruit trees such as apples, pears, and stone fruits. In Korea,

Xyleborinus saxesenii, *Xylosandrus germanus*, *Ambrosiodmus rubricollis*, and *Anisandrus apicalis* have been identified as major pests infesting apple trees [10]. They cultivate symbiotic fungi in nutrient-poor tree sections, which they use as a food source. As a result, this symbiotic relationship often causes the death of the host tree [11].

Ambrosiella species typically produce phialidic conidiophores characterized by the production of aleurioconidia located singly at the tips of aleurioconidiophores. These spores form short chains, and conidiophores have small collarettes at the apex [1,9]. The sexual state of *Ambrosiella* remains unknown, and genetic variations within the genus are limited [12]. Additionally, these species have been recovered or reported from five beetle genera [7].

In this study, symbiotic fungi were isolated to investigate the diversity and distribution of symbiotic fungi associated with the four major ambrosia beetle species known to affect apple trees in Korea. Among them, the fungal strain isolated from *A. rubricollis* was identified through morphological and molecular biological characteristics, and its phylogenetic position was determined.

MATERIALS AND METHODS

Fungal isolation from ambrosia beetles

Ambrosia beetles (*A. rubricollis*) were collected using a trap at the Apple Research Center in Gunwi-gun, Daegu-si, Korea (36°29′68.9″N, 128°46′56.1″E). Their bodies were surface-sterilized with 70% ethanol and then dried thoroughly for approximately 20 min. Afterward, the insects were dissected by separating the head and thorax from the abdomen. The segments were placed onto potato dextrose agar (PDA; Difco, Detroit, MI, USA) plates and incubated at 25℃ for 3 days. The grown mycelium was transferred to fresh PDA plates and incubated for 8 more days at 25℃. The pure isolated strain was designated as ARI-24-A4 and preserved in 20% glycerol stock at −80℃ for further analysis.

Morphological characterization

ARI-24-A4 was cultured on PDA at 25℃ for 8 days to assess its cultural and morphological characteristics. Following incubation, the diameter, color, and shape of the fungal colony were recorded. The morphological characteristics, including conidia and conidiophores, were observed through light microscopy (CX-43, Olympus, Japan).

Genomic DNA extraction, PCR amplification, and sequencing

Fungal mycelia were cultured on PDA plates at 25℃ for 5 days and scraped off using a sterile blade to extract genomic DNA. DNA was extracted using a HiGene genomic DNA prep kit (Biofact, Daejeon, Korea) in accordance with the manufacturer's instructions. Polymerase chain reaction (PCR) was performed targeting the internal transcribed spacer (ITS) regions, the small subunit of nuclear ribosomal

RNA (SSU), and the partial sequences of the translation elongation factor 1-alpha (TEF1-α) gene. The ITS regions were amplified using the ITS1F/ITS4 primers [13,14]. The SSU gene was amplified using the NS-1/NS-6 primers [14,15]. The TEF1-α gene was amplified using the EFCF1.5/EFCF6 primer pair [16]. The PCR products were verified on 1% agarose gels and stained with ethidium bromide. The amplified PCR products were purified using an EXOSAP-IT kit (Thermo Fisher Scientific, Waltham, MA, USA) in accordance with the manufacturer's instructions, and the purified DNA fragments were sequenced by Macrogen Co., Ltd. (Daejeon, Korea). Sequence data were analyzed using SeqMan Lasergene software (DNAStar Inc., Madison, Wisconsin, USA). The ITS regions, SSU, and TEF1-α gene sequences were deposited in GenBank under accession numbers LC835910, LC835912, and LC835914, respectively.

Molecular phylogenetic analysis

The sequences of *Ambrosiella* species were obtained from the National Center for Biotechnology Information (NCBI) database (Table 1). These sequences were aligned using Clustal X 2.0 in MEGA 7 (https://www.megasoftware.net/) [17]. The concatenated nucleotide sequences that included the ITS region and partial sequences of the TEF1-α and SSU genes were phylogenetically analyzed using the maximum-likelihood (ML) [18] method. In ML analysis, the nearest neighbor interchange heuristic search method and Kimura's two-parameter model were used [19], and gaps were excluded from the analysis. The reliability of the ML analysis was indicated by bootstrap values derived from 1,000 replicates.

Table 1. List of species included in the phylogenetic analyses and 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 beaveri	CBS 121750	*Cnestus mutilatus*	KF669875	KT318380	KR673882
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	**ARI-24-A4**	***Ambrosiodmus rubricollis***	**LC835910**	**LC835914**	**LC835912**
Ambrosiella roeperi	CBS 135864^{T}	*Xylosandrus crassiusculus*	KF669871	KT318384	KR673886
Ambrosiella xylebori	CBS 110.61^{T}	*Xylosandrus compactus*	KF669874	KT318385	KR673887
Catunica adiposa	CBS 183.86	N/A	JN604448	HM569644	KR673891

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

RESULTS

Mycological characteristics of *A. roeperi* (ARI-24-A4)

When cultured on PDA at 25℃ for 8 days, the diameter of colony growth was 65.2–70.2 mm. The front side developed white conidia at the center, appearing predominantly white but changing to olivaceous toward the edges (Fig. 1A). On the back side, the overall color was brown, but it lightened to a pale brown toward the edges (Fig. 1B). As the culture period extended, dark-brown droplets exuded from the mycelium, and a sour smell was released.

Aleurioconidiophores are hyaline, smooth, and cylindrical, with conidia and a collarette forming at the terminal ends of conidiophores (Figs. 1C and 1D). Aleurioconidia are hyaline with visible cell organelles inside. Their cell walls are thick, and the conidia are nearly cylindrical, with an average size of 12.9 µm × 12.5 µm (n = 50). When aleurioconidia detach from aleurioconidiophores, most of them carry along a conidiophore cell (Fig. 1F), and the same collarette type can be observed. While most aleurioconidia appear spherical, collarettes are cylindrical, and the point of attachment to the conidiophore cell is blunt; therefore, these characteristics clearly distinguish the two forms (Figs. 1G and 1H).

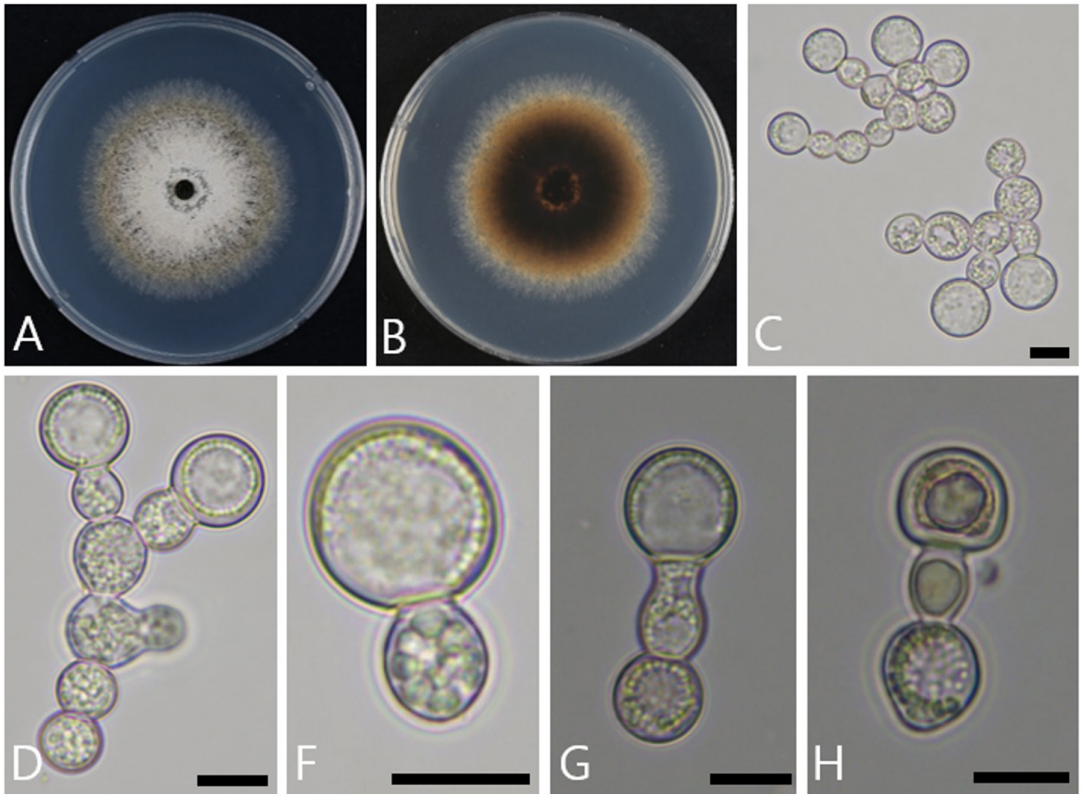

Fig. 1. Cultural and morphological characteristics of *Ambrosiella roperi*. A, B: Front and reverse view of the colony grown on potato dextrose agar (PDA) for 8 days at 25℃. C, D: Aleurioconidiophores with terminal aleurioconidia. F: Aleurioconidia. G: Aleurioconidiophores with aleurioconidia. H: Aleurioconidiophores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

Comparison of mycological characteristics

The cultural and morphological characteristics of ARI-24-A4 were consistent with those of *A. roeperi*. However, ARI-24-A4 clearly differed from its closely related species *A. grosmanniae*. In terms of cultural characteristics, the growth rate and colony color clearly varied. When cultured on PDA for 8 days, ARI-24-A4 reached a diameter of 65.2–70.4 mm; by comparison, *A. grosmanniae* grew faster, reaching 67.0–85.0 mm in diameter. In terms of color, ARI-24-A4 displayed a white to olive coloration, whereas *A. grosmanniae* was gray to dark gray. Therefore, they were clearly distinct from each other. In terms of morphological characteristics, ARI-24-A4 had significantly larger conidia (12.9 μm × 12.5 μm) than *A. grosmanniae* (7.5–12.0 μm × 7.5–12.0 μm). These differences confirmed that ARI-24-A4 is distinct from *A. grosmanniae* (Table 2).

Table 2. Comparison of the morphological characteristics of the ARI-24-A4 strain and the reference species *Ambrosiella roeperi* and *A. grosmanniae*

Characteristics		*A. roeperi*[a] (ARI-24-A4)	*A. roeperi*[b]	*A. grosmanniae*[b,c]
Colony	Color	Front side: White conidia found in the center, and changes in color to olivaceous toward the periphery Back side: Brown overall, becoming lighter toward the edges	Olivaceous to gray, dark gray, to dark-brown superficial	Gray to dark gray with an abundant aerial mycelium
	Shape	Colonies on PDA attaining 65.2–70.4 mm diam after 8 days at 25℃	Colonies on PDA attaining 60.5–70.0 mm diam after 8 days at 25℃	Colonies on PDA attaining 67.0–85.0 mm diam after 8 days at 25℃
Aleurio conidiophores	Color	Hyaline	Hyaline to subhyaline	Hyaline
	Shape	Smooth and cylindrical, with organelles observed within the cells; a single spore or a collarette at the terminal of the conidiophore	Smooth, cylindrical, monilioid, branched, with or without a collarette on the top cell of conidiophores	Smooth, ellipsoidal with or without collarettes on the top cell of conidiophores
Aleurio conidia	Color	Hyaline	Hyaline	Hyaline to subhyaline
	Shape	Thick-walled, smooth, with organelles observed, almost spherical in shape	Thick-walled, globose to subglobose	Thick-walled, globose single and terminal on aleu rioconidiophores
	Size (μm)	(9–)12.9(–16) × (9–)12.5(–16)	(7–)9.0–13.0(–16) × (5–)8.0–12.0(–14)	7.5–12.0 × 7.5–12.0

[a] Fungal strain studied in this paper; [b] Source of description [23]; [c] Sources of description [22].

Phylogenetic analysis

The partial nucleotide sequences of the ITS regions, TEF1-α, and SSU genes to analyze the molecular and phylogenetic relationships of ARI-24-A4, and their lengths were 591, 1,095, and 1,299 bp, respectively. BLAST searches were conducted to compare these sequences with those registered in the NCBI database. For the ITS region, ARI-24-A4 exhibited the highest similarity of 100.0% with *A. roeperi* C2448, 97.5% similarity with *A. catenulata* W20-44-06, 96.3% with *A. grosmanniae* CBS 137359, and 95.7% with *A. xylebori* CBS 110.61. Based on the TEF1-α gene sequence, ARI-24-A4 showed a 100.0% similarity with *A. roeperi* CBS 135864, 95.2% with *A. xylebori* AFTOL-ID 1285, 94.8% with *A. grosmanniae* 1002HHS1, and 93.7% with *A. xylebori* C1650. For the ITS region, the species with the highest similarity of 100.0% was *A. roeperi*. For the SSU gene sequence, ARI-24-A4 exhibited 99.9% similarity with *A. batrae* CBS 139735, 99.8% with *A. xylebori* CBS 110.61, 99.8% with *A. roeperi* CBS 135864, and 99.8% with *A. catenulata* C3913.

For the ITS region and TEF1-α gene sequences, *A. roeperi* was identified as the most closely related species with a 100.0% similarity. However, for the SSU gene sequence, no clear distinction was observed as the similarities were 99.9–99.8% for *A. batrae*, *A. xylebori*, and *A. roeperi*. Therefore, multilocus sequence analysis was performed by concatenating the ITS, TEF1-α, and SSU sequences, and a phylogenetic tree was constructed using the ML method to determine the phylogenetic position of ARI-24-A4. The phylogenetic tree revealed that ARI-24-A4 formed a clade with *A. roeperi*, indicating a species-level relationship with *A. roeperi* (Fig. 2). Additionally, the phylogenetic tree clearly distinguished ARI-24-A4 from *A. grosmanniae*, *A. batrae*, and *A. xylebori*, which exhibited a high SSU sequence similarity. The topology of the ML tree indicated that *A. grosmanniae* could be the second close phylogenetic relative of the isolate. The mycological characteristics and phylogenetic position of ARI-24-A4 confirmed that it is indeed identical to *A. roeperi* at the species level. This study is the first to report this fungal species in Korea.

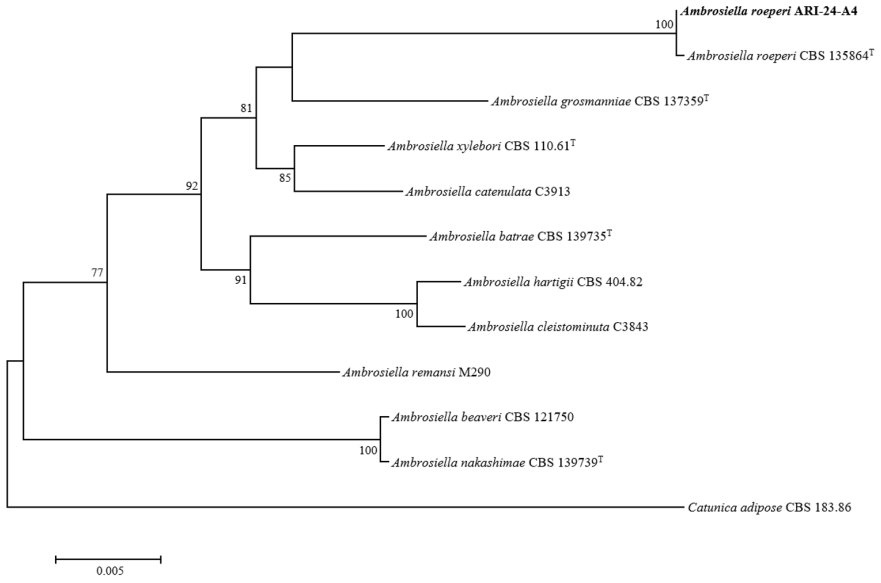

Fig. 2. Maximum-likelihood phylogenetic tree of ARI-24-A4 based on the combined sequences (ITS+TEF1-α+SSU), showing the phylogenetic position of the ARI-24-A4 strain among *Ambrosiella* species. Bootstrap values (based on 1,000 replications) greater than 70% are shown at branch points. The isolated strain is shown in bold. *Catunica adiposa* CBS 183.86 was used as an outgroup. Bar, 0.005 substitutions per nucleotide position. 'T' indicates the type strain. ITS: internal transcribed spacer; TEF1-α: translation elongation factor 1-alpha; SSU: small subunit of nuclear ribosomal RNA.

DISCUSSION

Ambrosiella species have been reported as symbiotic fungi associated with ambrosia beetles [20] by participating in the life cycle of beetles. These fungi have been found in ambrosia beetles with relatively large and complex mycangia (specialized structures for storing fungi) and have been reported from five beetle genera [7]. Studies on the mycangia of ambrosia beetles have shown that gland cells secrete substances into or near the mycangium, thereby supporting the growth of fungal symbionts [21]. furthermore, spores overflowing from the mycangium inoculate the galleries during construction. Female

Xylosandrus, *Anisandrus*, and *Cnestus* species within the tribe *Xyleborini* have large mesonotal mycangia, which are internal structures closely associated with *Ambrosiella* fungi. *A. hartigii*, *A. beaveri*, and *A. roeperi* are species associated with the mesonotal pouch mycangia of the *Xylosandrus* complex and known as key symbiotic fungi of ambrosia beetles. *A. ferruginea* and *A. trypodendri* are related to the prothoracic pleural mycangia of *Trypodendron* species [1,20].

In this study, ARI-24-A4 was discovered in *A. rubricollis*. BLAST searches using the sequences of the ITS regions, TEF1-α, and SSU genes confirmed that it represents a novel *A. roeperi* strain, and its close neighbor is *A. grosmanniae*. In previous studies, several molecular markers were used to identify *Ambrosiella* species. In 2014, Harrington utilized the large-subunit rDNA (LSU) single-gene sequence to identify five species: *A. beaveri*, *A. ferruginea*, *A. hartigii*, *A. roeperi*, and *A. xylebori* [7]. The phylogenetic positions of these species were confirmed by the concatenated sequences of the TEF1-α and SSU genes [22]. Later, the concatenated sequences of the ITS regions, TEF1-α, and RNA polymerase II subunit 1 (RPB1) genes were used to classify the novel species *A. catenulata* [23]. Despite the morphological differences between *A. nakashimae* and *A. beaveri*, this study revealed that their molecular markers were insufficient to distinguish between them. In our study, we used a different combination of three molecular markers, namely, the ITS regions, TEF1-α, and SSU genes, which were available for a larger number of *Ambrosiella* species than the RPB1 sequences. The results showed that ARI-24-A4 occupied a phylogenetic position alongside *A. roeperi* CBS 135864^{T}, and both strains were clearly different from other *Ambrosiella* species. The combination of the molecular markers used in this study was sufficient to distinguish *A. roeperi* and various other *Ambrosiella* species; however, similar to the case of the molecular markers in previous studies, such a combination did not provide a clear differentiation between *A. beaveri* and *A. nakashimae*. Therefore, additional specific genetic markers are needed to accurately differentiate *Ambrosiella* species.

ARI-24-A4, which was isolated from *A. rubricollis* in Korea, is an unrecorded species in Korea. Morphological and molecular analyses confirmed that it is the same as *A. roeperi*, suggesting that *Ambrosiella* species may be associated with *Ambrosiodmus* beetles. Thus, this study expands our understanding of the distribution of *Ambrosiella* and emphasizes the need for further investigations into the correlation, distribution, and biological roles of symbiotic fungi and ambrosia beetles.

CONFLICT OF INTERESTS

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

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