RESEARCH ARTICLE

First Record of *Raffaelea promiscua* Associated with Ambrosia Beetles in Korea

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ABSTRACT

In the present study, we isolated a fungus from an ambrosia beetle collected from a trap in an apple orchard in Gunwi-gun, Daegu, South Korea. The isolate was designated as ARI-25-A11. To classify the strain at a species level, both morphological and molecular analyses were conducted, and its phylogenetic position was determined. For cultural characterization, ARI-25-A11 was cultured on 2% malt extract agar at 25°C for 10 days, resulting in a colony diameter of 30.4–32.9 mm. White mycelium was observed at the center, and as the incubation period progressed, the center gradually darkened from olive to brown. Morphologically, the conidiophores were mononematous, septate, and hyaline, whereas the conidia were aseptate, predominantly oval, with an enlarged upper part and a tapered base, measuring an average of 5.9 × 3.5 µm. For molecular identification, the large subunit ribosomal RNA (LSU), small subunit ribosomal RNA (SSU), and β-tubulin (*β-tub*) genes were amplified and sequenced. The LSU and SSU sequences showed 100% identity with those of *Raffaelea promiscua* PL1001, and the *β-tub* gene sequence completely matched that of both *R. promiscua* PL1001 and CMW55899^T. Phylogenetic analysis identified ARI-25-A11 within the same clade as *R. promiscua*. Based on morphological and molecular data, ARI-25-A11 was identified as *R. promiscua*, representing the first report of this species among ambrosia beetles in Korea.

Keywords: Ambrosia beetles, *Raffaelea promiscua*, Symbiotic fungi, Taxonomy

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INTRODUCTION

Ambrosia beetles are small wood-boring insects belonging to the family Curculionidae, with approximately 3,500 species reported till date. They are classified into two subfamilies, Platypodinae and Scolytinae [1]. Most beetles maintain obligate mutualistic relationships with fungi, with female beetles primarily collecting fungal symbionts and storing them in specialized structures called mycangia, enabling the transmission of symbionts across generations [2]. Most species of ambrosia beetles harbor fungal symbionts belonging to the orders Ophiostomatales and Microascales, both within Ascomycota [3]. Among these, the genera *Afroraffaelea* [4], *Aureovirgo* [5], and *Raffaelea* [6], within Ophiostomatales, are considered the major fungal symbionts of ambrosia beetles.

The genus *Raffaelea*, belonging to the phylum Ascomycota and family Ophiostomataceae, was established by Arx & Hennebert [7] to accommodate *Raffaelea ambrosiae* isolated from beetles of the genus *Platypus*. Although most *Raffaelea* species are saprotrophic, some exhibit strong pathogenicity and cause serious damage to forests and agriculture. Notable examples include *R. quercivora*, the causative agent of Japanese oak wilt [8]; *R. quercus-mongolicae*, reported in Korea [9]; and *R. lauricola*, which is responsible for laurel wilt in the United States [10].

In the past, owing to the limitations of taxonomic techniques, the distinction between *Raffaelea* and *Ambrosiella* was unclear. These genera are distinguished primarily by their conidiogenous cell development. In *Raffaelea*, conidiogenesis occurs through sympodial branching, in which new cells are produced laterally. In contrast, *Ambrosiella* exhibits percurrent proliferation in which new cells are formed through the elongation of existing conidiogenous cells. However, these morphological differences are subtle and difficult to discern under a microscope, often leading to frequent misclassifications of the two genera [11]. Recently, molecular phylogenetic approaches have been used to clarify the taxonomic relationships among these fungi. Ribosomal DNA sequence analysis revealed that *Raffaelea* belongs to the order Ophiostomatales, whereas *Ambrosiella* belongs to the order Microascales, confirming that these two genera are not closely related phylogenetically [12]. Accordingly, the aim of the present study was to clarify the taxonomic identity of fungal isolates obtained from ambrosia beetles collected from apple orchards, using morphological characterization and rDNA-based molecular phylogenetic analyses.

MATERIALS AND METHODS

Isolation of fungi associated with ambrosia beetles

Ambrosia beetles were trapped in an apple orchard located at the Apple Research Center in Gunwi-gun, Daegu-si, Republic of Korea (36°29′68.9″N, 128°46′56.1″E). After collection, the beetles were surface-sterilized using 70% ethanol and allowed to dry in the air for approximately 10 min. Sterilized specimens were transferred onto potato dextrose agar (PDA; Difco, Detroit, MI, USA) and incubated at 25°C for 3 days. The fungal growth was transferred to fresh PDA plates and incubated again under the same conditions for 10 days to obtain pure cultures. One isolate, designated ARI-25-A11, was preserved in 20% glycerol at −80°C for long-term storage. The specimen was deposited in the Korean Agricultural Culture Collection (KACC) and assigned the accession number KACC 411081.

Morphological characterization

The isolate ARI-25-A11 was cultured on PDA and 2% malt extract agar (MEA; Oxoid, Basingstoke, UK) at 25°C for 10 days to assess its cultural and morphological characteristics. After incubation, various colony traits, such as the diameter and pigmentation, were examined. The structure and dimensions of the conidiophores and conidia were observed and documented using a light microscope (CX-43; Olympus, Japan).

Genomic DNA extraction, PCR amplification, and sequencing

Genomic DNA was extracted from ARI-25-A11 to compare its sequence with those of related strains in the National Center for Biotechnology Information (NCBI) database and determine its phylogenetic position. Partial sequences of the large subunit ribosomal RNA (LSU), small subunit ribosomal RNA (SSU), and β-tubulin (*β-tub*) genes were obtained through PCR amplification. The LSU genes were amplified using the LR0R/LR5 primer pair [13], the SSU genes were amplified using the NS1/NS4 primers [14], and the *β-tub* genes were amplified using the Bt2a/Bt2b primers [15].

The PCR amplicons were confirmed via electrophoresis on 1% agarose gels and visualized through ethidium bromide staining. The amplified DNA was purified using EXOSAP-IT (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Sequencing was performed by Solgent Co., Ltd. (Daejeon, Korea), and the resulting reads were assembled and analyzed using the SeqMan module within the Lasergene software suite (DNAStar Inc., Madison, WI, USA). The finalized sequences were deposited in GenBank under the accession numbers LC878894 (LSU), LC878895 (SSU), and LC878896 (*β-tub*).

Molecular phylogenetic analysis

Nucleotide sequences of *Raffaelea* species were retrieved from the NCBI database (Table 1). Sequence alignment was performed using Clustal X 2.0, within the MEGA 7 software suite [16]. Concatenated LSU, SSU, and *β-tub* gene sequences were used to construct a phylogenetic tree. Phylogenetic analysis was conducted using the nearest neighbor interchange algorithm under Kimura's two-parameter substitution model [17], with gap sites excluded from the dataset. The tree was generated using the maximum likelihood method [18], and node support was evaluated based on 1,000 bootstrap replicates.

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

Species	Strain	Isolation sources	GenBank accession numbers		
			LSU	SSU	*β-tub*
Claviceps fusiformis	ATCC 26019	Poaceae	U17402	DQ522539	AF263569
Raffaelea albimanens	CBS 271.70^{T}	*Crossotarsus externedentatus*	NG_064077	NG_062680	MT644111
Raffaelea ambrosiae	CBS 185.64^{T}	*Platypus cylindrus*	NG_074464	NG_070903	MT644094
Raffaelea arxii	CBS 273.70^{T}	*Xyleborus volvulus*	NG_074465	NG_062649	MW066753
Raffaelea canadensis	CBS 805.70^{T}	*Gnathotrichus sulcatus*	MH871751	AY858658	EU977466
Raffaelea cyclorhipidii	Hulcr 7168^{T}	*Cyclorhipidion ohnoi*	KX267104	KX267130	KX267115
Raffaelea fusca	90p2	*Xyleborus glabratus*	KR018415	KR018399	KR018441
Raffaelea promiscua	CMW 55899^{T}	*Xyleborinus saxesenii*	MW028144	N/A	MW066750
Raffaelea promiscua	**ARI-25-A11**	**Ambrosia beetle**	**LC878894**	**LC878895**	**LC878896**
Raffaelea santoroi	CBS 399.67^{T}	*Monarthrum mutatus*	NG_064067	EU984261	EU977476
Raffaelea subalba	C2401	*Xyleborus glabratus*	EU177443	KJ909304	KJ909305
Raffaelea subfusca	195	*Xyleborus bispinatus*	MG673963	MG674029	MG674055
Raffaelea sulcati	CBS 806.70	*Gnathotrichus sulcatus*	NG_064084	NG_062681	EU977477
Raffaelea tritirachium	CBS 726.69	*Monarthrum mali*	MH871169	NG_063092	EU977478

LSU: large subunit ribosomal RNA; SSU: small subunit ribosomal RNA; *β-tub*: β-tubulin; N/A: Not available.
^{T}ex-type.
The isolated strain is shown in bold.

RESULTS

Mycological characteristics of ARI-25-A11

When cultured on PDA at 25°C for 10 days, the colony diameter reached 31.5–33.5 mm (av. 32.4 mm). On the front side, the center appeared dark olive in color, with the color gradually fading toward the edges to appear white. Initially, the mycelia grew into the medium; however, over time, a small amount of aerial mycelia was also observed. The reverse side of the colony was entirely white, with a dark brown band (Fig. 1A, B). Under the same conditions on 2% MEA, the fungus exhibited slower growth, with colony diameters reaching 30.4–32.9 mm (av. 31.6 mm). The colonies were flat and circular, initially appearing white, but gradually turned olive and then dark brown from the center as incubation progressed. Most of the hyphae were submerged in the medium (Fig. 1C, D).

The conidiophores were mononematous, septate, and hyaline. They were mostly unbranched but occasionally showed branching (Fig. 1E). Typically upright and straight, the structures become narrower toward the apex and were sometimes simplified as conidiogenous cells. Conidiogenous cells were integrated, hyaline, and exhibited a blastic mode of development. They were cylindrical or peg-like in shape and narrow toward the apex (Fig. 1F, G). The conidia were hyaline, aseptate, and formed blastically. Moreover, they were round, oval, or oblong with an enlarged upper portion and a tapered base. Conidia were often clustered near the conidiogenous cells (Fig. 1H). Further, their size range was 5.2–6.6 × 2.8–4.2 μm (av. 5.9 × 3.5 μm, n = 50) (Fig. 1I).

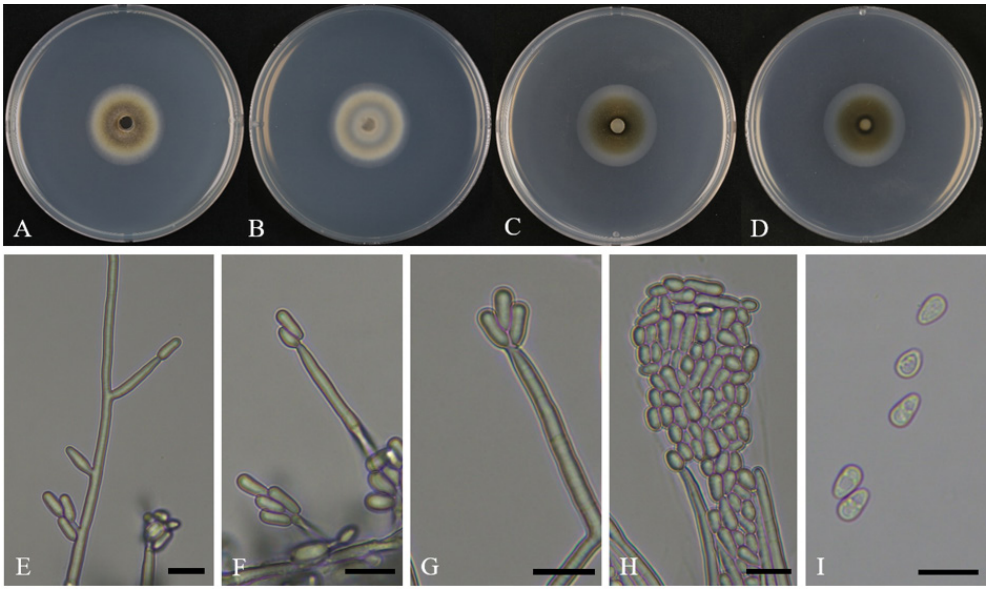

Fig. 1. Cultural and morphological characteristics of ARI-25-A11 (*Raffaelea promiscua*). A, B: Front and reverse sides of the colony grown on potato dextrose agar (PDA) for 10 days at 25°C. C, D: Front and reverse sides of the colony grown on 2% malt extract agar (MEA) for 10 days at 25°C. E: Branched conidiophores. F–H: Conidiogenous cells producing conidia. I: Conidia. Scale bars: E–I, 10 μm.

Phylogenetic and DNA sequence analysis

Three molecular markers (LSU, SSU, and *β-tub*) were employed to investigate the molecular and phylogenetic relationships of ARI-25-A11. The lengths of the obtained sequences were 840 bp, 1,022 bp, and 564 bp. These sequences were compared with those of other fungal strains in the NCBI database using a Basic Local Alignment Search Tool (BLAST) search. For the LSU gene, ARI-25-A11 showed the highest similarity, 100.0%, with *Raffaelea promiscua* PL1001 and *R. cyclorhipidii* LWP286, followed by 97.2% similarity with *R. canadensis* CMW 25536 and 94.8% similarity with *R. subalba*. For the SSU gene, ARI-25-A11 showed 100.0% similarity with *R. promiscua* PL1001 and *R. canadensis* C3169, whereas the similarities with *R. cyclorhipidia* C2711, *R. quercina* CBS 147555, and *R. ambrosiae* CBS 185.64 were 99.4%, 99.1%, and 94.8%, respectively. For the *β-tub* gene, ARI-25-A11 exhibited 100.0% similarity with both *R. promiscua* PL1001 and *R. promiscua* CMW55899^{T}, while showing a lower similarity of 90.2% with *R. canadensis* CBS 168.66.

Based on a comparison with sequences registered in the NCBI database, ARI-25-A11 showed high similarity to *R. promiscua*, *R. cyclorhipidii*, and *R. canadensis*, when assessing the LSU and SSU genes, and was found to be most closely related to *R. promiscua* when assessing the *β-tub* gene. Notably, all three gene sequences of ARI-25-A11 were nearly identical to those of *R. promiscua* PL1001, indicating a close genetic relationship. In addition, to determine the phylogenetic position, concatenated sequences of the LSU, SSU, and *β-tub* genes were used to construct a dataset of 2,426 bp in length. *Claviceps fusiformis* ATCC 26019 was used as an outgroup for the phylogenetic tree construction. The resulting phylogenetic tree revealed that ARI-25-A11 forms a distinct clade with *R. promiscua* (Fig. 2).

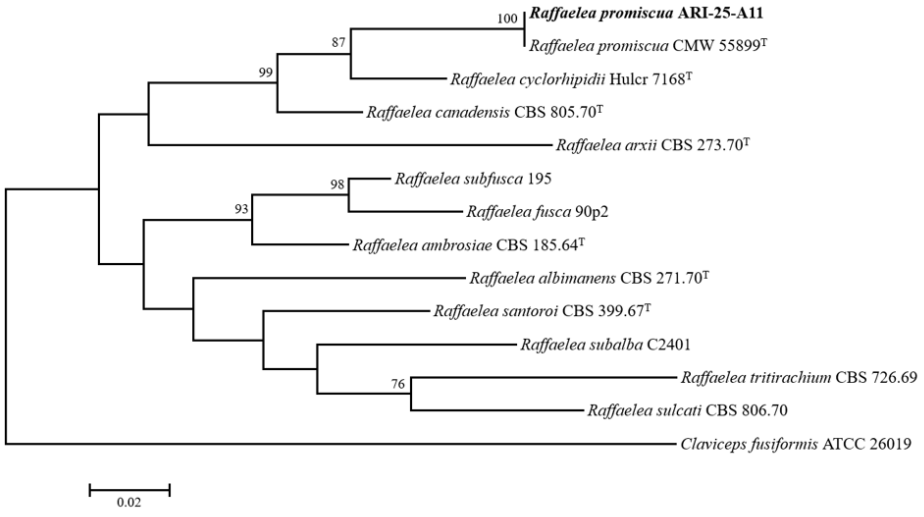

Fig. 2. Maximum-likelihood phylogenetic tree illustrating the placement of strain ARI-25-A11 within the genus *Raffaelea*, based on concatenated sequences of large subunit ribosomal RNA (LSU), small subunit ribosomal RNA (SSU), and β-tubulin (*β-tub*) genes. Bootstrap values greater than 70% (from 1,000 replicates) are shown at the corresponding branches. The isolate obtained in this study is highlighted in bold. *Claviceps fusiformis* (ATCC 26019) was used as the outgroup. The scale bar represents 0.02 nucleotide substitutions per site. ^{T} denotes the type strain.

Morphological comparison with related taxa

Based on the molecular marker sequences and phylogenetic analyses, ARI-25-A11 was identified as a close relative of *Raffaelea promiscua* and *R. cyclorhipidii*. The conidia of ARI-25-A11 were found to be predominantly oblong with swollen upper parts, measuring an average of 5.9 × 3.5 μm, making it morphologically similar to *R. promiscua*. In contrast, *R. cyclorhipidii* produced larger, more elongated ellipsoidal conidia with an average size of 7.3 × 3.5 μm, clearly distinguishing it from ARI-25-A11. In culture, ARI-25-A11 formed smooth colonies with aerial hyphae, whereas *R. cyclorhipidii* produced colonies with tough and wrinkled surfaces, highlighting a notable difference in colony morphology (Table 2).

Table 2. Comparison of the morphological characteristics of strain ARI-24-A11 with those of the reference species *Raffaelea promiscua* and *R. cyclorhipidii*

Characteristics		*R. promiscua* [a] (ARI-25-A11)	*R. promiscua* [b]	*R. cyclorhipidii* [c]
Colony	Color, Shape	Flat and circular, the colonies gradually changed from white to dark olive over time, with most hyphae submerged within the medium.	Flat, circular colony with smooth margins; mostly submerged hyphae with some aerial growth. Yeast-like growth at the inoculation site; color changed from white to olive.	Colonies have a tough, wrinkled surface with a subhyaline reverse; initially cream-colored, turning olivaceous, golden brown, or blackish with age
	Size (mm)	Colonies on 2% MEA attaining 31.6 mm diam after 10 days at 25°C	Colonies on 2% MEA attaining 34.0 mm diam after 10 days at 25°C	Colonies on MEA attaining 47.5 mm diam after 9 days at 25°C
Conidiophores	Color	Hyaline	Hyaline	N/A
	Shape	Mononematous, septate, mostly unbranched, upright, straight, and tapering toward the apex, sometimes reduced to conidiogenous cells.	Mononematous, macronematous, straight to undulate, tapering apically, occasionally branched.	N/A
Conidiophores cell	Color	Hyaline	Hyaline	Hyaline
	Shape	Integrated, and blastic, cylindrical or peg-like in shape, and taper toward the apex.	Integrated, cylindrical or peg-like; tapering toward apex; blastic	Conidia form mainly at the apex of the conidiogenous cells, occasionally sessile and lateral
Conidia	Color	Hyaline	Hyaline	Hyaline
	Shape	Hyaline, aseptate, blastic, and vary in shaperound, oval, or oblong with a swollen upper part and tapered base; often clustered near conidiogenous cells.	Aseptate, mostly oblong with swollen upper part, rounded apex, tapering base, truncated base, yeast-like budding observed	Produced singly, aseptate, elliptical to elongate, sometimes truncate at the base; capable of budding
	Size (μm)	5.9 × 3.5	5.1 × 2.5	7.3 × 3.5

MEA: malt extract agar; N/A: Not available.
[a]Fungal strain studied in this paper; [b]Source of description [2]; [c]Source of description [27].

DISCUSSION

A comprehensive taxonomic revision of fungal symbionts associated with ambrosia beetles has not been undertaken since Batra [19]. At that time, most known symbionts were classified into anamorphic genera *Ambrosiella* or *Raffaelea* based on the belief that these fungi reproduced asexually [20]. Their simplified morphological features made an accurate classification difficult prior to the advent of DNA sequencing technology [21]. Originally, *Ambrosiella* and *Raffaelea* were classified based on the proliferation patterns of their conidiogenous cells (annellidic proliferation vs sympodial proliferation). However, subsequent studies revealed no substantial differences in proliferation modes between the two genera, and this morphological

criterion was deemed taxonomically unreliable [10].

The taxonomy of *Raffaelea* has been systematically revised using molecular phylogenetic techniques. Dreaden et al. [6] conducted the first comprehensive multigene phylogenetic analysis of the genus based on LSU, SSU, and *β-tub* gene sequences. They classified *Raffaelea brunnea*, *R. lauricola*, *R. scolytodis*, *R. arxii*, *R. gnathotrichi*, *R. fusca*, *R. subfusca*, *R. ellipticospora*, *R. ambrosiae*, *R. canadensis*, *R. albimanens*, *R. subalba*, *R. tritirachium*, *R. santoroi*, and *R. sulcati* as *Raffaelea* sensu stricto, whereas other species were considered to have uncertain taxonomic positions.

A previously unidentified strain, PL1004, was initially identified as *R. canadensis* by Eskalen and McDonald [22]. However, Dreaden et al. [6] confirmed that this strain is non-pathogenic and genetically distinct from *R. canadensis* and thus considered it a novel species. In 2021, fungi isolated from an ambrosia beetle were confirmed to be identical to PL1004 and officially described as *R. promiscua* [2].

In this study, the strain ARI-25-A11 was subjected to phylogenetic analysis based on LSU, SSU, and *β-tub* gene sequences. Sequence analysis revealed that ARI-25-A11 was identical to PL1004 across all examined loci, and a phylogenetic analysis placed it in the same clade as PL1004. Therefore, strain ARI-25-A11 was considered conspecific with PL1004.

Nel et al. [2] reported that strain CMW 55899, genetically identical to PL1004, is a new species and named it *R. promiscua*, based on internal transcribed spacer (ITS), LSU, and *β-tub* gene analyses. In our study, we attempted to sequence the ITS region but were unable to obtain high-quality sequences. This result aligns with previous findings that ITS amplification and interpretation are challenging in *Raffaelea* [23,24] and that many *Raffaelea* species lack ITS sequences in the NCBI database.

Accordingly, SSU was used instead of ITS for species identification. However, because Nel et al. [2] did not include the SSU gene when describing *R. promiscua* as a new species, none of the available *R. promiscua* strains in the NCBI database had all three genes (LSU, SSU, and *β-tub*). Therefore, in our analysis, the SSU gene sequence of *R. promiscua* CMW 55899^{T} was treated as a gap (missing data), and maximum likelihood analysis was carried out.

In 2022, a taxonomic revision of the order Ophiostomatales, including the genus *Raffaelea*, was conducted based on four gene regions ITS, LSU, *TEF1-α*, and *RPB2* [25]. However, this revision was considered overly inclusive, and many *Raffaelea* species lack sequence data for *TEF1-α* and *RPB2*, making accurate comparisons difficult. Therefore, in this study, we determined that using LSU, SSU, and *β-tub* gene sequences remains the most effective approach for identifying species within the genus *Raffaelea*. Our phylogenetic analysis further supports the validity of this method by enabling effective classification based on these markers. However, a consistent set of molecular markers for species identification has not been established, posing challenges when attempting to compare multiple strains. In particular, sequencing the ITS region remains problematic for many *Raffaelea* isolates. For example, for *R. cyclorhipidii* and *R. subfusca*, both previously reported in Korea, publicly available ITS sequences are lacking [26]. If more isolates of such species could be obtained in the future, it would be intriguing to investigate the intraspecific and intragenomic ITS variations among *Raffaelea* species distributed in Korea.

CONFLICT OF INTERESTS

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

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