Mycobiology

Research Article

Phylogenetic Status of an Unrecorded Species of *Curvularia*, *C. spicifera*, Based on Current Classification System of *Curvularia* and *Bipolaris* Group Using Multi Loci

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Abstract A seed-borne fungus, *Curvularia* sp. EML-KWD01, was isolated from an indigenous wheat seed by standard blotter method. This fungus was characterized based on the morphological characteristics and molecular phylogenetic analysis. Phylogenetic status of the fungus was determined using sequences of three loci: rDNA internal transcribed spacer, large ribosomal subunit, and glyceraldehyde 3-phosphate dehydrogenase gene. Multi loci sequencing analysis revealed that this fungus was *Curvularia spicifera* within *Curvularia* group 2 of family Pleosporaceae.

Keywords *Curvularia* group, *Curvularia spicifera*, Pleosporaceae, Seed-borne fungi, Wheat

The genus *Curvularia* (family: Pleosporaceae, order: Pleosporales) known as hyphomycete fungus was established by Boedijn [1] and typified by *C. lunata* (Wakker) Boedijn. Currently, 30 *Curvularia* species have been reported [2]. *Curvularia* and *Bipolaris* are closely related to each other with same teleomorph (*Cochliobolus*) [3, 4]. Main differences among the genera of *Bipolaris*, *Curvularia*, *Drechslera*, and *Exserohilum* are their conidial shape, the presence and the shape of protruding hilum, and the conidial outline of the basal portion [5]. *Curvularia* species have dark mycelia and geniculate conidiophores with sympodial and distoseptate conidia [3, 6]. *Bipolaris* contains about 45 species. They are mostly subtropical and tropical plant parasites as well as human pathogens. *Bipolaris* is characterized by fusiform to ellipsoidal shaped conidia, central cells not much darker but broader than the distal ones, hilum not protuberant, and bipolar germination. However, some *Bipolaris* species have short and straight conidia with intermediate conidial characteristics. These *Bipolaris* species may look different from the generic type *B. maydis* that has large and gently curving conidia.

In recent years, molecular biology has provided fundamental tools to study genera with complex taxonomy. rDNA sequencing has been used as a main tool to identify fungi and discriminate related species [7, 8]. This has been especially useful for some *Curvularia* species. To assess the evolutionary relationships of *Cochliobolus*, *Pseudocochliobolus*, *Curvularia*, and *Bipolaris*, Berbee *et al.* [9] have conducted phylogenetic analyses of rDNA internal transcribed spacer (ITS) regions and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene sequences. Furthermore, molecular phylogenetic analysis based on multi loci has been increasingly used as a tool to determine new species [10, 11]. Recently, Manamgoda *et al.* [12] have re-evaluated the taxonomy of the genera *Bipolaris*, *Cochliobolus*, and *Curvularia* based on combined genetic analysis of rDNA ITS, 28S, GAPDH, and translation elongation factor 1-α genes and confirmed that these genera were divided into two monophyletic groups: *Bipolaris* and *Cochliobolus* species clustered in group 1 together with their respective type species *B. maydis* (Y. Nisik. & C. Miyake) Shoemaker. In contrast, *Curvularia* including species that were first named as *Bipolaris*, *Cochliobolus*, *Pseudocochliobolus* but later re-classified as *Curvularia* were clustered in group 2 with its generic type *C. lunata*. Some *Bipolaris* species were re-classified into genus *Curvularia*,

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including *C. australiensis*, *C. coisis*, *C. ellisii*, *C. graminicola*, *C. hawaiiensis*, *C. ovariicola*, *C. spicifera*, *C. ravenelli*, and *C. tripogonis*. The objectives of this study were to describe an unrecorded species of *C. spicifera* EML-KWD01 based on morphological characteristics, phylogenetic analysis, and taxonomic re-evaluation of relationships within *Curvularia* and *Bipolaris* groups using multi loci sequences.

MATERIALS AND METHODS

Fungal isolates. Seeds harvested from an indigenous wheat in 2012 were sampled from farms and markets. Seeds were surface-sterilized with 2% sodium hypochlorite (NaOCl) for 30 sec, washed with sterilized distilled water, plated directly on a moist blotter, and incubated at 27°C for 2~4 days to allow germination. After germination, seeds were stored in a freezer (–80°C) for 1 hr and then replaced in the same incubator. Recovered fungi were investigated with a stereo microscope, and fungal spores on the seed were inoculated to potato dextrose agar (PDA; Difco, Montreal, Canada) using a capillary tube. A seed-borne fungus, EML-KWD01, was purely isolated from the wheat seed, subcultured on PDA, and then preserved in 20% glycerol stock in a deep freezer (–80°C) at Environmental

Table 1. Sequences of isolates used in this study with GenBank accession numbers

Species	Strain No.[a]	Locality	Host	GenBank accession No.			References
				ITS	GAPDH	LSU	
Alternaria alternata	EGS 34.0160	India	*Arachis hypogaea*	AF071346	AF081400	-	[9]
Bipolaris chloridis	CBS 242.77	Australia	*Chloris gayana*	JN192372	JN600961	-	[4]
B. crustacea	8225-1	Unknown	Unknown	AF163070	-	AF163977	[8]
B. cynodontis	ICMP 6128	New Zealand	*Cynodon dactylon*	JX256412	JX276427	JX256380	[12]
B. maydis	C5 (141-1-2)	Unknown	Unknown	AF071325	AF081380	-	[9]
B. microlaenae	CBS 280.91	Australia	*Microlaenae stipoidis*	JN601032	JN600974	JN600996	[12]
B. oryzae	MFLUCC 10-0694	Thailand	*Oryza sativa*	JX256413	JX276428	JX256381	[12]
B. papendorfii	9084c	Unknown	Unknown	AF163075	-	AF163980	Unknown
B. peregianensis	BRIP 12790	Australia	*Cynodon dactylon*	JN601034	JN600977	JN601000	[4]
B. sorokiniana	ICMP 6233	Solomon Islands	*Lolium perenne*	JX256418	-	JX256386	[12]
	A20	Unknown	Unknown	AF071329	AF081385	-	[9]
B. spicifera	BRIP12529	Unknown	Unknown	AF163076	-	AF163985	Unknown
B. victoriae	CBS 174.57	USA	*Avena sativa*	JN601027	-	JN600983	[4]
B. zeae	BRIP 11512	Australia	*Zea mays*	KJ415538	KJ415408	KJ415493	[13]
Cochliobolus australiensis	CBS 172.57	Vietnam	*Oryza sativa*	JN601026	JN601036	JN600981	[4]
Co. ovariicola	BRIP 15882	Unknown	Unknown	JN601031	JN600971	JN600992	[4]
Curvularia asianensis	MFLUCC 10-0687	Unknown	Unknown	JX256422	JX276435	-	Unknown
	MFLUCC 10-0711	Thailand	*Panicum* sp.	JX256424	JX276436	JX256391	[12]
C. australiensis	IMI 53994	Australia	Unknown	KC424595	KC747744	KC445296	[11]
C. gladioli	ICMP 6160	New Zealand	*Gladiolus* sp.	JX256426	JX276438	JX256393	[12]
C. graminicola	BRIP 23186a	Australia	Unknown	JN192376	JN600964	JN600986	[12]
C. hawaiiensis	BRIP 10971	Australia	*Chloris gayana*	JN601030	JN600967	JN600989	[12]
C. inaequalis	C8-2	Unknown	Unknown	JQ585673	-	-	Unknown
	C8-1	Unknown	Unknown	JQ585672	-	-	Unknown
C. lunata	CBS 730.96	USA	Human lung biopsy	JX256429	JX276441	JX256396	[12]
	CBS 157.34	Indonesia	Unknown	JX256430	JX276442	JX256397	[12]
C. ovariicola	CBS 470.90	Australia	*Eragrostis interrupta*	JN192384	JN600976	JN600998	[4]
C. ravenelii	BRIP 13165	Australia	*Sporobolus fertilis*	JN192386	JN600978	JN601001	[4]
C. spicifera	**EML-KWD01**	**Korea**	***Triticum aestivum***	**KT351794**	**KT351793**	**KT351795**	**This study**
	CBS 274.52	Spain	Soil	JN192387	JN600979	JX256400	[4]
	BRIP 10940a	Australia	*Triticum aestivum*	KC424603	KC747753	KC445305	[13]
C. trifolii	ICMP 6149	New Zealand	*Setaria glauca*	JX256434	JX276457	JX256402	[12]
	CPC 2995	Australia	*Leucospermum* sp.	JN712459	-	JN712523	Unknown
C. tsudea	BRIP 10970a	Australia	*Chloris gayana*	KC424605	KC747755	KC445307	[11]
	ATCC 44764	Japan	*Chloris gayana*	KC424596	KC747745	KC445297	[11]
C. tuberculata	CBS 146.63	India	*Zea mays*	JX256433	JX276445	JX256401	[12]

ITS, internal transcribed spacer; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LSU, large subunit.
[a]CBS (Centraalbureau Voor Schimmelcultures, Utrecht, Netherlands), ATCC (American Type Culture Collection, Manassas, Virginia), BRIP (Plant Pathology Herbarium, Brisbane, Queensland, Australia), IMI (CABI Bioscience Genetic Resource Collection, CABI Bioscience UK Centre, Egham, UK), MFLUCC (Mae Fah Luang University Culture Collection, Chiang Rai, Thailand), EML (Environmental Microbiology Lab Fungal Herbarium, Chonnam National University, Gwangju, South Korea).

Microbiology Laboratory (EML) culture collection, Chonnam National University, Gwangju, Korea. The strain was also deposited as ex-type (KOSPFGC0919) at culture collection of National Institute of Biological Resources (NIBR), Incheon, Korea.

DNA extraction and PCR. Isolates were cultured on PDA overlaid with cellophane at 27°C for 3~5 days. Total genomic DNA was extracted with HiGene Genomic DNA Prep Kit for fungi (Biofact Co., Daejeon, Korea). Ribosomal DNA ITS and 28S regions were amplified with universal primer pairs ITS1/ITS4 and LROR/LR5F [14] in a 20 µL reaction mixture using AccuPower PCR Premix (Bioneer Co., Daejeon, Korea) containing Taq DNA polymerase, dNTPs, buffer, and tracking dye. PCR reaction was carried out with the following parameters: 2 min at 95°C for initial denaturation, followed by 30 cycles of 1 min at 94°C for denaturation, 30 sec at 54°C for annealing, and 1 min at 72°C for extension, with 10 min at 72°C for terminal extension. To amplify GAPDH gene, primers gpd1 and gpd2 [12] were used. PCR reaction was conducted using the following conditions; 2 min at 96°C for initial denaturation, followed by 35 cycles of 1 min at 96°C for denaturation, 1 min at 52°C for annealing, and 45 sec at 72°C for extension, with 10 min at 72°C for terminal extension [12]. PCR products were purified using AccuPrep PCR Purification Kit (Bioneer Co.), according to the manufacturer's instructions. Sequencing was performed on an ABI 3700 Automated DNA sequencer (Applied Biosystems Inc., Foster City, CA, USA).

Phylogenetic analysis. Sequences from this study and those from GenBank database (Table 1) were aligned to each other using ClustalX v. 1.83 [15] and edited with BioEdit v. 5.0.9.1 [16]. Phylogenetic analyses were performed using MEGA 6 [17], and neighbor-joining (NJ) tree was constructed using Kimura's two-parameter method. A maximum parsimony tree was constructed for combined datasets of rDNA ITS, 28S, and GAPDH gene sequences of EML-

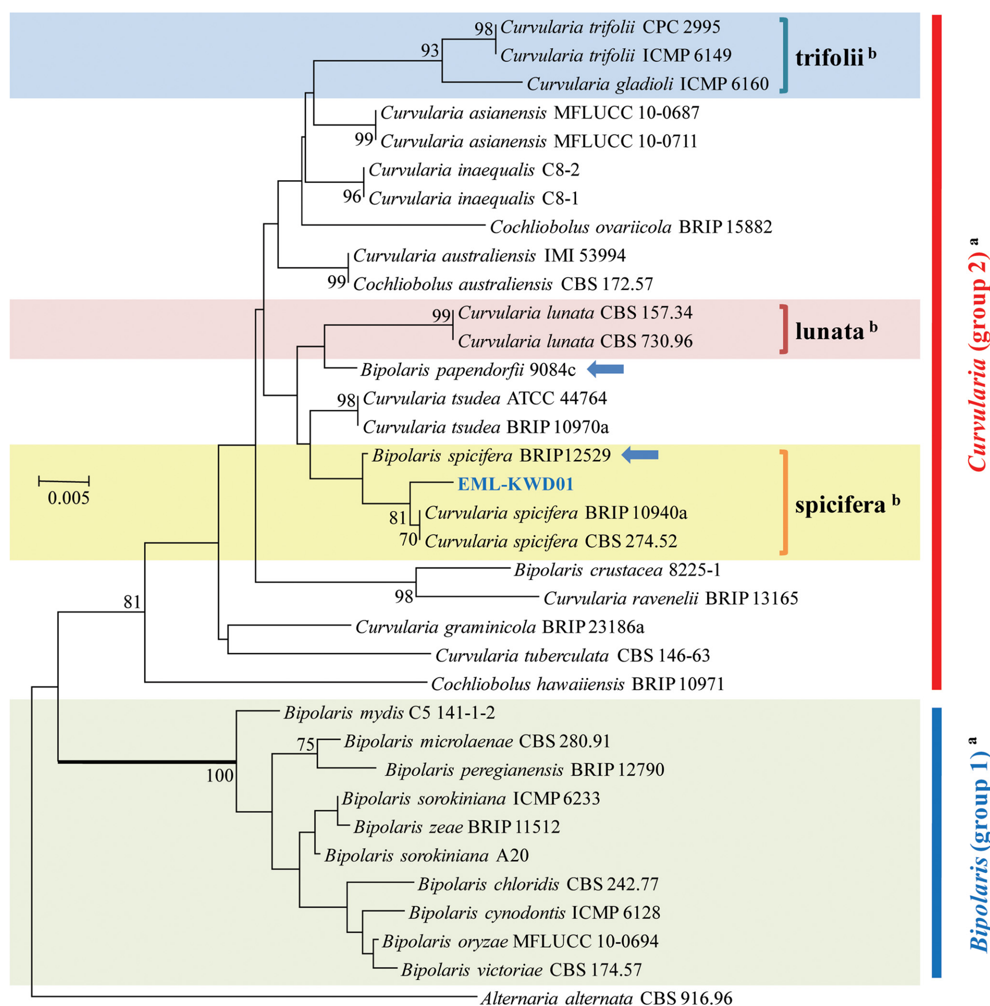

Fig. 1. Neighbor-joining tree inferred from nuclear ribosomal rDNA internal transcribed spacer sequences of EML-KWD01 isolate and related taxa. *Alternaria alternata* was used as outgroup. Bootstrap support values ≥ 50% are indicated at nodes. Arrows represent that the genus name of *Bipolaris* should be changed to *Curvularia*. [a]Classification by Manamgoda *et al.* (2012) [12]. [b]Classification by Madrid *et al.* (2014) [18].

KWD01 isolate using the MEGA 6 program. *Alternaria alternata* was used as outgroup.

Morphological studies. For microscopic examination and determination of the growth rate, EML-KWD01 isolate was grown on PDA, oatmeal agar (OA; oatmeal 30 g/L, agar 15 g/L), malt extract agar (MEA; Difco), or V-8 juice agar (V-8; Campbell, Camden, NJ, USA) at 18°C, 25°C, 32°C, and 37°C in the dark for 7 days. Morphological characteristics of fungal structures were determined under a light microscope (DFC290; Leica, Wetzlar, Germany) after preparing lactophenol slide mounts. Fine structures of the fungus were observed using scanning electron microscopy. Fungal samples were cultured on PDA medium in the dark at 27°C for 7 days. Samples were fixed in 2.5% paraformaldehyde-glutaraldehyde buffer with 0.05 M phosphate (pH 7.2; Junsei, Tokyo, Japan) for 2 hr and washed in carcodylate buffer (Junsei). Cellular membranes were preserved by fixing the samples in 1% osmium tetroxide (diluted in carcodylate buffer; Electron Microscopy Sciences, Hatfield, PA, USA) for 1 hr, washed again in carcodylate buffer, dehydrated in graded ethanol (Emsure, Darmstadt, Germany) and isoamyl acetate (Junsei), and dried under a fume hood. Finally, these samples were covered with gold in a sputter coater and observed using a Hitachi S4700 field emission scanning electron microscope (Hitachi, Tokyo, Japan) at Korea Basic Science Institute, Gwangju, Korea.

Mycelial growth rate measurement. The diameter of growing colonies were measured every 2 days. Measurements of all colonies were made in two directions at right angles to each other for both controls. Temporal increase in the radius was plotted against time. Linear regression was calculated in order to estimate the growth rate in mm/day for each species under each set of environmental conditions [19]. All experiments were repeated three times.

RESULTS

Phylogenetic status. A phylogenetic tree was inferred using NJ method. Combined loci (ITS/28S/GAPDH) alignment was calculated for 36 strains (Table 1). Phylogenetic tree was constructed using the maximum parsimony method. A total of three most parsimonious trees were generated by a heuristic search using the combined dataset of 856 characters from the three loci. Basic Local Alignment Search Tool (BLAST) analysis of ITS sequences indicated that the EML-KWD01 isolate resembled *C. spicifera* R12 (accession No. KC315931) the most, displaying a 99.6% (523/525 bp) homology (Fig. 1). The GAPDH gene sequences of EML-KWD01 and that of *C. spicifera* CBS 274.52 (accession No. JN600979) shared 99.0% (514/519 bp)

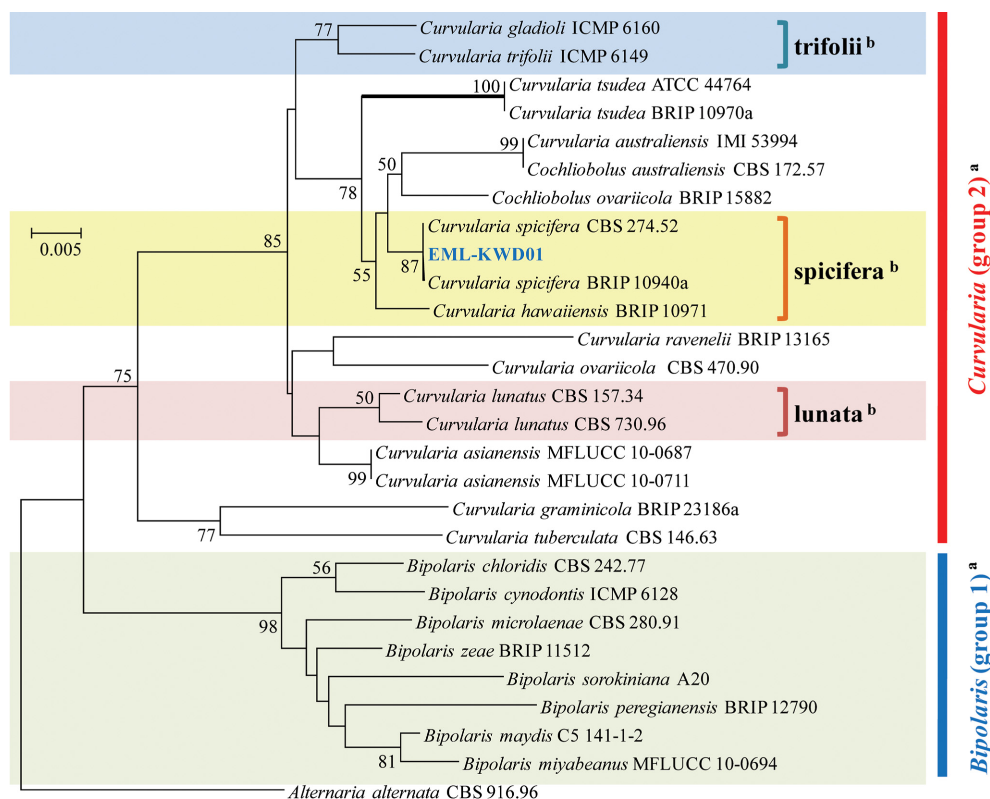

Fig. 2. Neighbor-joining tree inferred from glyceraldehyde 3-phosphate dehydrogenase gene sequences of EML-KWD01 isolate and related taxa. *Alternaria alternata* was used as outgroup. Bootstrap support values ≥ 50% are indicated at nodes. [a]Classification by Manamgoda *et al.* (2012) [12]. [b]Classification by Madrid *et al.* (2014) [18].

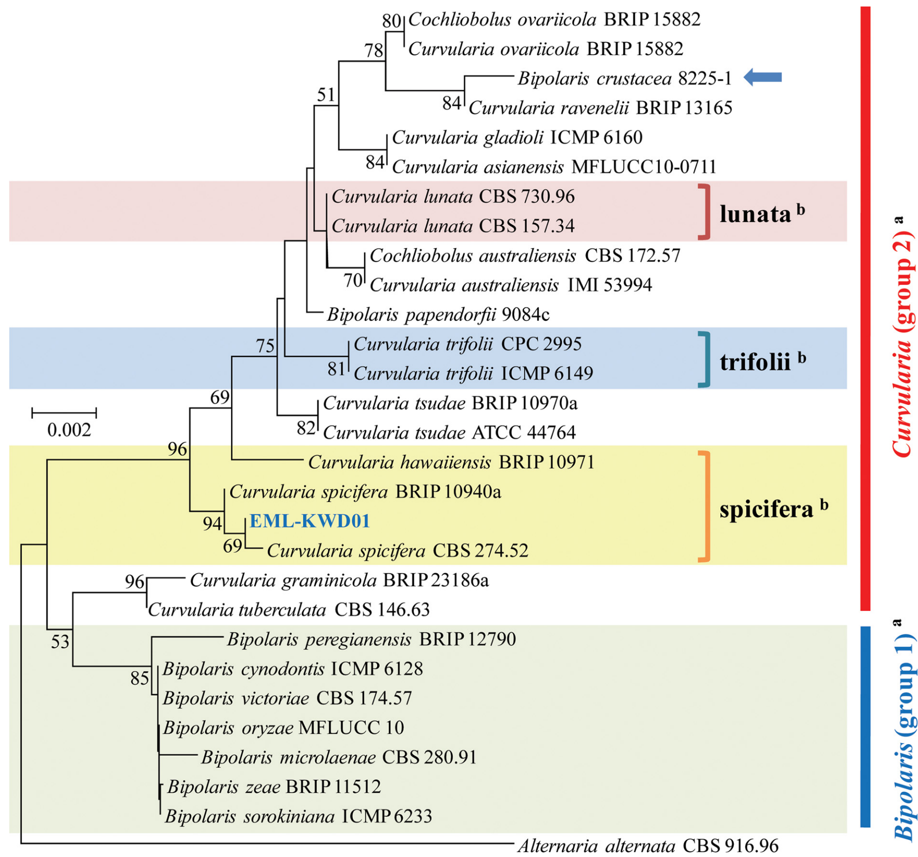

Fig. 3. Neighbor-joining tree inferred from large subunit rDNA sequences of EML-KWD01 isolate and related taxa. *Alternaria alternata* was used as outgroup. Bootstrap support values ≥ 50% are indicated at nodes. The arrow represents that the genus name of *Bipolaris* should be changed to *Curvularia*. [a]Classification by Manamgoda *et al.* (2012) [12]. [b]Classification by Madrid *et al.* (2014) [18].

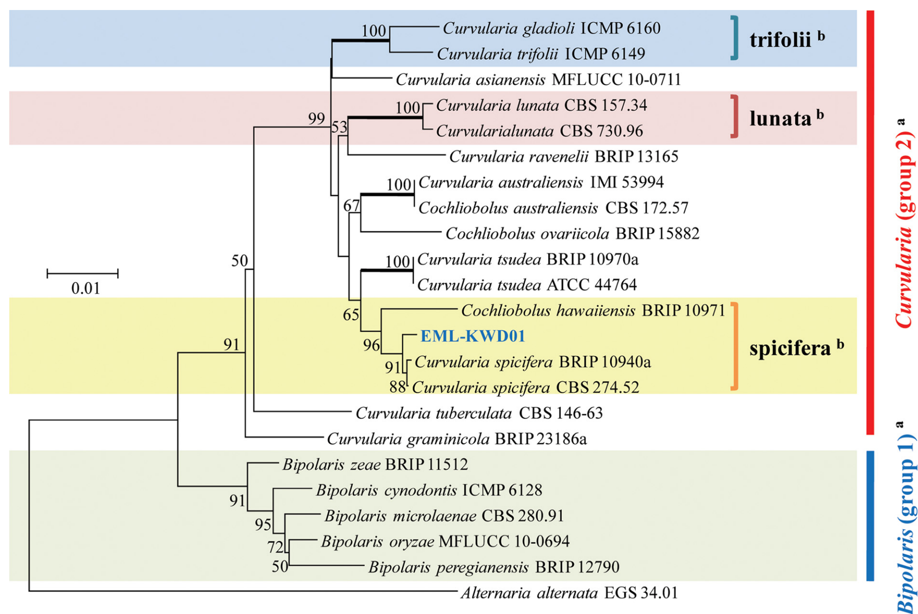

Fig. 4. A phylogenetic tree showing the relationship of EML-KWD01 isolate with known species. The tree was generated from parsimony analysis based on the combination of rDNA internal transcribed spacer, 28S, and glyceraldehyde 3-phosphate dehydrogenase gene sequences using MEGA6. *Alternaria alternata* was used as outgroup. Bootstrap support values ≥ 50% are indicated at nodes. [a]Classification system by Manamgoda *et al.* (2012) [12]. [b]Classification system by Madrid *et al.* (2014) [18].

identities. The rDNA 28S sequence of EML-KWD01 shared 98.6% (820/832 bp) and 98.6% (821/832 bp) sequence identities with *C. spicifera* BRIP 10940a (accession No. KC445305) and *C. spicifera* CBS 274.52 (accession No. JX256400), respectively (Figs. 2 and 3). The tree generated from combined dataset of ITS, 28S, and GAPDH gene had a consistency index of 0.632 and a retention index of 0.814 (Fig. 4). Most nodes in the combined analysis showed increased clade support as measured by bootstrap analysis. The phylogenetic tree also showed that the unknown isolate EML-KWD01 belonged to family Pleosporaceae. Combining phylogenetic analyses of the ITS, 28S, and GAPDH gene sequences and morphological features, we were able to classify this species as *C. spicifera* belonging to *Curvularia* group 2 of family Pleosporaceae (Fig. 4).

Taxonomy. *Curvularia spicifera* (Bainier) Boedijn, Bull. Soc. Mycol. Fr. 24: 81 (1909) [MB#278597] (Table 2, Figs. 5 and 6)

≡ *Drechslera spicifera* (Bainier) Arx, The genera of fungi sporulating in pure culture: 222 (1970) [MB#313402]

≡ *Bipolaris spicifera* (Bainier) Subram, Hyphomycetes: an account of Indian species, except Cercosporae: 756 (1971) [MB#309557]

Etymology: This species was isolated from wheat seed collected from an agricultural farm in Korea.

Description: The colony diameter on PDA was approximately 4~6 mm at 25°C 5 days after inoculation. The colony color was pale and olivaceous gray. The reverse of the colony was olivaceous. The conidiophores were simple, erect or ascendent, pigmented, geniculate from sympodial elongations, branched, variable length, with a width of 4.7~7.3 μm (average, 5.5 μm). Conidia were produced singly through pores, and frequently produced on several two side pores of tapering conidiophores. Conidia were not curved, oblong to cylindrical with 2~3 (predominantly 3-

Table 2. Morphological characteristics comparison between isolate EML-KWD01 and references

Character	Present isolate	Sivanesan [20][a]	Koo *et al.* [21][b]
Colony			
Color	Pale olivaceous gray, reverse olivaceous	NA	NA
Conidia			
Shape	Straight, oblong to cylindrical	Straight, oblong or cylindrical	Straight, oblong or cylindrical
Color	Brown to dark brown	Golden brown	Brown
Size (μm)	15~23 × 7~9	20~40 × 9~14	20~31 × 7~12.5
Hilum (width, μm)	0~2 to 3	2~3	2~3
Septum	2- to 3-distoseptate (predominantly 3-septate)	3-distoseptate	3-distoseptate
Conidiophores			
Shape	Flexuous	Flexuous	Flexuous
Color	Mid to dark brown	Mid to dark brown	Mid to dark brown
Length	Up to 100 μm long or more	Up to 300 μm long or more	105~705 μm
Width (μm)	4.7~7.3 (average, 5.5)	4~9	4.3~5.7

NA, not available.

[a]From the description of *Bipolaris spicifera* by Sivanesan (1987) [20].

[b]From the description of *Bipolaris spicifera* by Koo *et al.* (2003) [21].

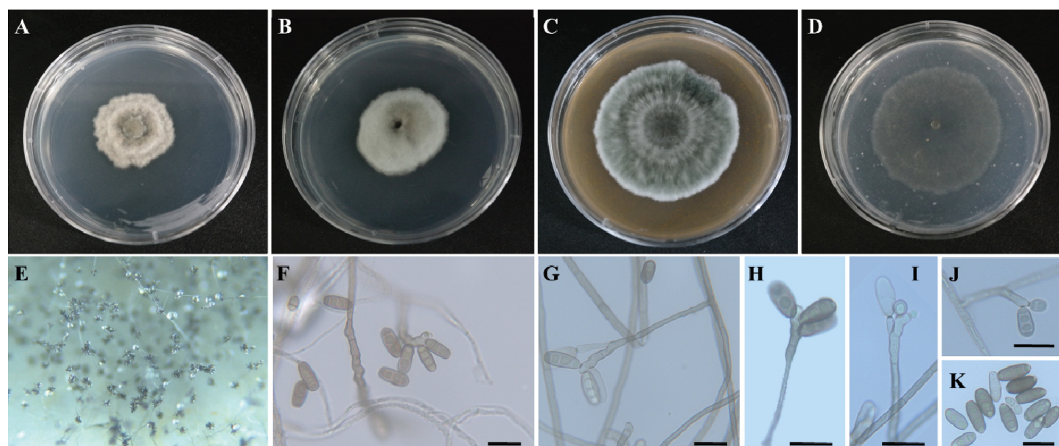

Fig. 5. Morphology of EML-KWD01 isolated from wheat seeds. A, Colonies grown on malt extract agar; B, Potato dextrose agar; C, V-8; and D, Oatmeal agar media at 25°C for 7 days; E~J, Conidia with 2~3 transverse (predominantly 3-septate) septa on geniculate conidiophores; K, Oblong to cylindrical-shaped conidia without or with hilum (scale bars: F~K = 20 μm).

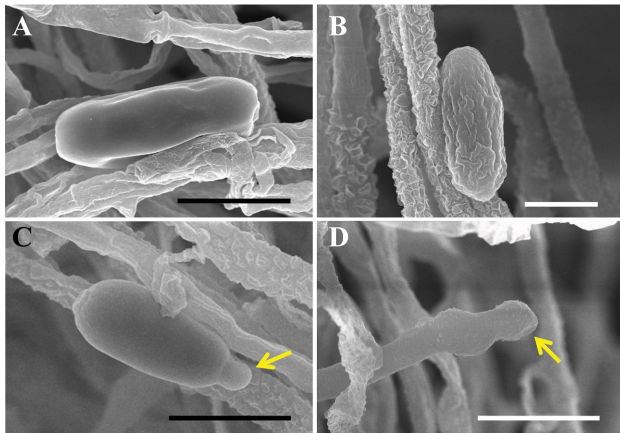

Fig. 6. Scanning electron micrograph of conidia and conidiophores of *Curvularia spicifera* EML-KWD01. A, B, Conidia with smooth or wrinkled surface; C, Germinating conidium (yellow arrow); D, Conidiophore with geniculate pore (yellow arrow) (scale bars: A, C, D = 10 μm, B = 5 μm).

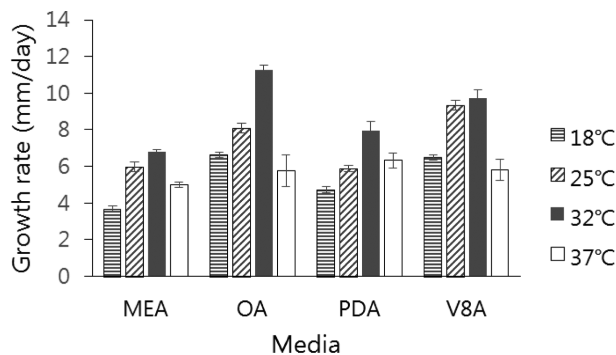

Fig. 7. Effect of temperature and media on the growth of *Curvularia spicifera* EML-KWD01. Mycelial growth was evaluated on four kinds of media (malt extract agar [MEA], oatmeal agar [OA], potato dextrose agar [PDA], and V-8 agar [V8A]) after 7 days at 18°C, 25°C, 32°C, and 37°C.

septate) transverse septa, hilum found (but not commonly), and measured 6.51~9.17 μm wide × 9.23~21.17 μm long.

Mycelial growth. The isolate grew across a wide range of temperatures with various growth rates on MEA, OA, PDA, and V-8 media. The average colony sizes of EML-KWD01 isolate after 7 days of growth on MEA, OA, PDA, and V-8 were 6.8, 11.2, 7.9, and 9.7 mm, respectively. Optimal growth was observed on OA medium at 32°C. Slower growth was observed at 18°C and 37°C (Fig. 7).

DISCUSSION

Most species of *Curvularia* are found in subtropical to tropical regions, although a few are found in temperate zones. They are identified as major causal pathogens of sugarcane and grasses [6, 20-22]. Some *Curvularia* species are also important to different industries owing to their ability of producing secondary metabolites, including substances with antimicrobial properties, enzymes, and precursors used for steroid production [13, 23-25]. *Curvularia spicifera* (≡ *Bipolaris spicifera*) is known as a common allergen to humans. It can cause opportunistic infection in immunocompromised people such as acquired immune deficiency syndrome patients [18, 26, 27]. In Korea, this species has been detected from an imported grass seed without detailed descriptions [21]. However, it has not been detected from indigenous wheat seed.

Recently, ITS region has been used to identify fungi to discriminate species [28, 29]. However, phylogenetic studies based on the ITS region have limited utility in identifying species, especially among members in the order of Pleosporales. In a recent phylogenetic study using multigene analysis, Manamgoda *et al.* [12] clarified the taxonomy of *Curvularia*. We used three loci to infer the phylogenetic relationships among species in the genus of *Curvularia* based on previous phylogenetic studies.

Although genus *Curvularia* can be easily distinguished from *Bipolaris* and *Drechslera* spp., there has been some difficulty to distinguish *Curvularia* and *Bipolaris* due to their conidial (wall) shape, size, and septation. *Bipolaris* and *Cochliobolus* species are not monophyletically clustered in Group 1 along with their type species. However, *Curvularia* species including species of *Bipolaris*, *Cochliobolus*, and *Curvularia* are clustered in Group 2 with its generic type [12]. Berbee *et al.* [9] have presented a phylogenetic analysis of *Bipolaris* and *Curvularia* species using combined sequences of ITS rDNA, large subunit (LSU) rDNA, and GAPDH gene.

In this study, rDNA ITS, and GAPDH genes provided sufficient phylogenetic information for the separation of *Cochliobolus* species into two groups. Our molecular phylogenetic data based on the sequence analyses of three loci of rDNA ITS, 28S, and GAPDH gene showed that the phylogenies were clustered into two main groups and that the isolate EML-KWD01 was an unrecorded *Curvularia* species, *C. spicifera* belonging to *Curvularia* group 2 within the Pleosporaceae family (Figs. 1~4). Our results are in consistent with those obtained by Berbee *et al.* [9] and Manamgoda *et al.* [12] after using combined datasets of ITS, LSU (28S) rDNA, and GAPDH gene. As shown in Figs. 1 and 3, some *Curvularia* species were within the *Bipolaris* group. At the same time, some *Bipolaris* species were clustered in the *Curvularia* group 2. Our results revealed that the genus name of strains including *Bipolaris crustacea* 8225-1, *Bipolaris papendorfii* 9084c, and *Bipolaris spicifera* BRIP12529 in the phylogenetic trees should be changed to *Curvularia* species. Therefore, the status of some *Bipolaris* strains within the *Curvularia* group should be transferred to *Curvularia* group 2 based on the recent classification system. In Korea, Koo *et al.* [21] were the first ones who reported the species of *Bipolaris spicifera*. However, this old species name should be corrected to *Curvularia spicifera* based on recent classification system.

However, studies with more *Curvularia* and *Bipolaris* isolates in comparison with type species based on multi loci sequence analyses are needed in the future.

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