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Research Note

Characterization and Pathogenicity of *Alternaria vanuatuensis*, a New Record from *Allium* Plants in Korea and China

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Abstract *Alternaria* from different *Allium* plants was characterized by multilocus sequence analysis. Based on sequences of the β-tubulin (BT2b), the *Alternaria* allergen a1 (Alt a1), and the RNA polymerase II second largest subunit (RPB2) genes and phylogenetic data analysis, isolates were divided into two groups. The two groups were identical to representative isolates of *A. porri* (EGS48-147) and *A. vanuatuensis* (EGS45-018). The conidial characteristics and pathogenicity of *A. vanuatuensis* also well supported the molecular characteristics. This is the first record of *A. vanuatuensis* E. G. Simmons & C. F. Hill from Korea and China.

Keywords *Allium* plants, *Alternaria vanuatuensis*, China, Morphology, Phylogeny, Korea

The purple blotch of onion caused by *Alternaria porri* (Ellis) Cif. having been reported from almost every part of the world [1]. Pandotra [2] described this disease as a serious problem throughout onion-producing countries of the world. The name *A. porri* has been widely used for decades as an uncritical identification for any large-spored *Alternaria* found on a member of Alliaceae. The usage has frequently been erroneous; moreover, Simmons [3] recorded at least 4 other readily distinguishable taxa with large, long-beaked conidia from *Allium*. These taxa were *A. ascaloniae* E. G. Simmons & C. F. Hill, *A. iranica* E. G. Simmons & Y. Ghosta, *A. prasonis* E. G. Simmons, and *A. vanuatuensis* E. G. Simmons & C. F. Hill. *A. vanuatuensis* has been reported from many countries [3]. Recently, various molecular tools have been used to delimit fungal taxa that were previously described based on morphological and host range criteria. Amplification of the β-tubulin, histone 3, glyceraldehyde-3-phosphate dehydrogenase (*gpd*), *Alternaria* allergen a1 (Alt al), elongation factor 1-alpha (EF-1α), and RNA polymerase II (RPB2) genes has revealed a relatively high genetic diversity among species or interspecies of *Alternaria* and other fungal genera [4, 5].

The objectives of the present study were (1) to describe the newly recorded species, *A. vanuatuensis*, by using multilocus molecular data analysis and morphological differentiation isolated from Korea and China; and (2) to evaluate the pathogenicity of the newly recorded species on spring onion leaves in Korea.

Isolates of *Alternaria* obtained from *Allium ascaloniae*, *A. cepa*, *A. fistulosum*, and *Allium* sp. were used in this study (Table 1). The *Alternaria* isolates were obtained from parts of the plants showing leaf spot symptoms, by using the single spore isolation method. All of the isolates were deposited in the Culture Collection of Chungnam National University and reference isolates were obtained from EG Simmons (Mycological Services, Crawfordsville, IN, USA). All isolates were maintained on potato dextrose agar (PDA; Difco, Detroit, MI, USA) slants at 4°C, and also in 20% glycerol stock solution at −70°C.

Genomic DNA was extracted by the HiGene Genomic DNA Prep Kit (BIOFACT, Daejeon, Korea). Partial sequences of target regions (ITS4, *gpd*, BT2b, Alt a1, and RPB2 gene)

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Table 1. *Alternaria* isolates used in this study, their origin and GenBank accession numbers

Species	Isolate ID[a]	Host	Origin	GenBank accession Nos.				
				Alt a1	BT2	GPD	ITS	RPB
Alternaria porri	CNU093037	*Allium fistulosum* L.	China	JF331530	JF331559	JF331473	JF331442	JF331590
A. porri	CNU093038	*A. fistulosum* L.	China	JF331531	JF331559	JF331474	JF331443	JF331591
A. porri	CNU3480	*A. fistulosum* L.	Korea	JF331539	JF331567	JF331482	JF331451	JF331599
A. porri	CNU103013	*A. cepa*	Korea	JF331543	JF331571	JF331486	JF331455	JF331603
A. vanuatuensis	CNU093020	*A. fistulosum* L.	China	JF331545	JF500412	JF331488	JF331501	JF331605
A. vanuatuensis	CNU093033	*A. fistulosum* L.	China	JF331547	JF500414	JF331490	JF331503	JF331607
A. vanuatuensis	CNU3367	*A. fistulosum* L.	Korea	JF331549	JF500416	JF331492	JF331505	JF331609
A. vanuatuensis	CNU094020	*A. fistulosum* L.	Korea	JF331550	JF500417	JF331493	JF331506	JF331610
A. porri	EGS48-147	*A. cepa*	USA	JF331538	JF331566	JF331481	JF331450	JF331598
A. vanuatuensis	EGS45-018	*A. cepa*	New Zealand	JF331551	JF500418	JF331494	JF331507	JF331611
A. alternata	EGS34-016	*Arachis hypogea*	India	AY563301	JQ672039	AY278808	AF347031	JQ811951
A. alternantherae	EGS52-039	*Solanum melongena*	China	-	JQ672051	KC584096	KC584179	KC584374
A. ascaloniae	EGS46-052	*Allium ascalonicum*	New Zealand	JF500423	JF331572	JF331499	JF331512	JF331616
A. dauci	CNU3568	*Daucus carota*	Korea	JX213313	-	JF417695	JF417685	KC584392
A. iranica	EGS51-075	*Allium cepa*	Iran	JF331556	JF331440	JF331456	JF331513	JF331617
A. macrospora	CBS117228	*Gossypium barbadense*	USA	-	JQ672066	KC584124	KC584204	KC584410
A. panax	CNU085010	*Panax ginseng*	Korea	JX213305	JF417596	JF417650	JF417569	JF417677
A. prasonis	EGS52-006	*Allium porrum*	USA	JF331557	JF331441	JF331457	JF331514	JF331618
A. solani	CBS116651	*Solanum tuberosum*	USA	GQ180096	-	KC584139	KC584217	KC584430
A. tagetica	CBS479.81	*Tagetes erecta*	UK	AY563297	JQ672065	KC584143	KC584221	KC584434
A. tenuissima	EGS34-015	*Dianthus* sp.	UK	AY563302	JQ672040	AY278809	AF347032	JQ811961

ITS, internal transcribed spacer.

[a]CNU: Chungnam National University, Korea; EGS: Emory G Simmons, Mycological Services, Crawfordsville, IN 47933, USA; CBS: CBS-KNAW Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, the Netherlands.

were conducted for PCR amplification and were performed following a previously described method [6]. Successfully amplified DNA products were sequenced after PCR amplification and compared with the sequences of related *Alternaria* species available in the GenBank database by using BLAST search. Sequences generated from the materials used in the present study and sequences retrieved from GenBank were initially aligned by using the CLUSTAL X program [7]; the alignment was refined by using the PHYDIT program ver. 3.2 [8]. Maximum parsimony and maximum likelihood trees were reconstructed by using the MEGA5 program. The relative stability of the branches was assessed by conducting bootstrap analysis with 1,000 replications. *Alternaria prasonis* (EGS52-006) was used as outgroup.

For colony observation, six representative isolates from Korea and China and type strain isolates were grown on V8 juice agar at 23°C and 15°C temperatures. Colony, conidiophores and conidial characteristics were examined after 7 days of incubation. Isolates were mounted in lactophenol and measured under a light microscope (BX50; Olympus, Tokyo, Japan) and taken photographs with Artray Artcam 300MI digital camera (Artray Co. Ltd., Tokyo, Japan).

Pathogenicity tests were conducted with selected isolates on detached leaves of spring onion (*Allium fistulosum* L., cv. Chosundaepa). The detached leaves were collected from fully expanded leaves of 5-mon-old plants. Colonies with spores were flushed with sterile distilled water and the concentration of spore suspension was adjusted to 1×10^5 spores/mL for spraying. Surface sterilized detached spring onion leaves were inoculated with 20 μL of conidial suspension and incubated in covered plastic boxes (to maintain high humidity) at 25°C for 3~5 days. After incubation, the resulting lesions were recorded and the lesion results were averaged.

PCR amplification of the internal transcribed spacer (ITS) region and *gpd* gene of the isolates generated 553~554 and 565~566 bp fragments, respectively. Parsimonious trees generated by the ITS region and *gpd* gene were unable to differentiate *A. vanuatuensis* from *A. porri*. The phylogenetic analysis of the sequences of BT2b, Alt a1 and RPB2 for all isolates clearly differentiated these two fungal groups from each other. Maximum likelihood analysis based on combined sequence of BT2b, Alt a1 and RPB2 revealed that isolates of *A. porri* including the representative strain EGS48-147 formed monophyletic clade supported by high bootstrap value (93%) and *A. vanuatuensis* including the ex-type EGS45-018 formed monophyletic clade supported by a bootstrap value of 90% (Fig. 1). In conclusion, *A. vanuatuensis* group of Korean and Chinese isolates including the ex-type is differed from the related *A. porri* isolates. Also *A. vanuatuensis* differed from other related species.

When incubated on PDA, malt extract agar (MEA), and V8 juice agar at 25°C for 7 days, *A. vanuatuensis* from Korea and China formed single-spored colonies with similar

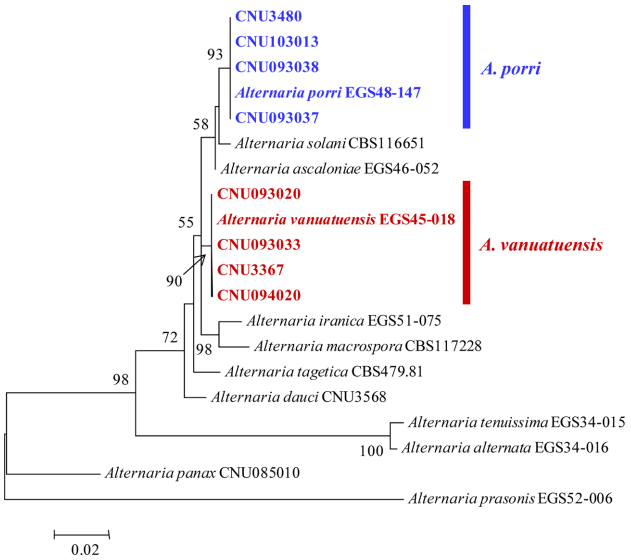

Fig. 1. Maximum likelihood tree inferred from the combined dataset of the BT2b, Alt a1, and RPB2 gene sequences of *Alternaria vanuatuensis*, *Alternaria porri*, and other related species. Numbers represent bootstrap values obtained after a bootstrap test with 1,000 replications. Bar indicates the number of nucleotide substitutions.

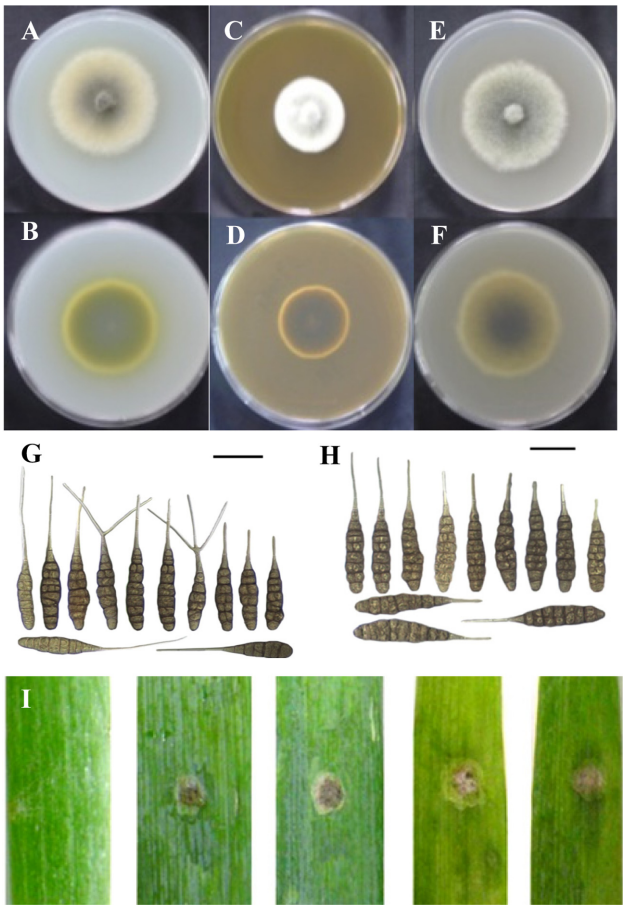

Fig. 2. Colony characteristics of *Alternaria vanuatuensis* formed on potato dextrose agar (A, B), malt extract agar (C, D), and V8 juice agar (E, F) after incubation at 25°C for 7 days. Conidia of *A. vanuatuensis* formed on V8 juice agar after incubation for 7 days at 23°C (G) and 15°C (H) (scale bars: G, H = 40 μm). Leaf spots caused by *A. vanuatuensis* of artificially inoculated onto detached leaves of spring onion after 3 days at 25°C; left, uninoculated control (I).

characteristics to those of *A. porri*. On PDA, colonies of *A. vanuatuensis* were vinaceous buff or pale yellow. The colony texture was generally cottony to woolly, and yellow pigments were clearly visible in the agar medium beneath the mycelial mat. Isolates typically produced colonies about 70 mm in diameter after 7 days. On MEA, colonies were smoky grey to white. The colony texture was generally woolly to cottony, and diffusible pigments were not visible. Isolates typically produced colonies measuring 50~70 mm in diameter after 7 days. On V8 juice agar, colonies were pale mouse gray to pale greenish gray/smoky gray. The colony texture was felty to woolly. Isolates typically produced colonies measuring approximately 70 mm in diameter after 7 days (Fig. 2).

Conidia of *A. vanuatuensis* and *A. porri* formed on V8A at 23°C were observed and found similar in body length and number of septa but varied in beak length (Table 2). The conidia were either ellipsoid or obclavate. They were solitary or in chains of 2~3 through the agency of secondary conidiophores. The ratio of beak and body length of *A. vanuatuensis* was shorter than that of *A. porri* at 23°C. The

Table 2. Morphological characteristics of *A. porri* and *A. vanuatuensis* formed on V8 juice agar at 23°C and 15°C

Species	Isolates No.	Conidial length (μm)						Septa		Origin
		23°C			15°C			23°C	15°C	
		Body	Beak	Width	Body	Beak	Width			
Alternaria porri	EGS48-147	78.4	87.4	18.1	87.0	52.0	18.7	6~11	5~13	USA
	CNU103013	85.6	96.0	19.7	87.0	55.7	24.5	5~14	5~10	Korea
	CNU093038	79.4	89.0	18.0	96.0	61.0	20.2	4~10	5~13	China
	Average	81.1	90.8	18.6	90.0	56.2	21.1	4~11	5~13	
A. vanuatuensis	EGS45-018	89.2	61.4	18.4	94.0	39.7	21.4	6~10	5~12	New Zealand
	CNU094020	88.0	59.1	19.9	99.0	22.2	23.2	6~10	5~10	Korea
	CNU093033	74.8	54.7	18.0	87.0	24.9	21.4	6~11	5~12	China
	Average	84.0	58.4	18.8	93.3	28.9	22.0	6~11	5~12	

Table 3. Pathogenicity of *Alternaria vanuatuensis* and *Alternaria porri* on leaves of spring onion (*Allium fistulosum* L.) treated with 20 μL spore suspension (1×10^5/mL) at 25°C for 3 days

Species	Isolates No.	Disease severity[a]
A. porri	EGS48-147	+++
A. porri	CNU093038	+++
A. porri	CNU103013	+++
A. vanuatuensis	EGS45-018	++
A. vanuatuensis	CNU094020	+
A. vanuatuensis	CNU093020	+

[a]+, disease symptom $< 5 \times 5$ mm^2; ++, disease symptom, 5×5 mm^2~15×15 mm^2; +++, disease symptom $> 15 \times 15$ mm^2.

average beak length of *A. vanuatuensis* of EGS45-018, CNU094020 and CNU093033 were 61. 4, 59.1, and 54.7, respectively which were shorter than the average beak length of *A. porri* (the length of *A. porri* of EGS48-147, CNU103013, and CNU093038 were 87.4, 96.0, and 89.0 μm, respectively) (Table 2).

In pathogenicity, isolates of *A. vanuatuensis* were less pathogenic than isolates of *A. porri* and caused lesions of below 10 mm^2 in size. The isolates of *A. porri* caused relatively higher disease severity and the size of lesion was often > 20 mm^2 (Table 3). Both the Korean and Chinese isolates showed the similar results.

Taxonomic controversy has existed in *Alternaria* because of variable morphological characterization [9, 10]. Quayyum *et al.* [11] emphasized that a comprehensive taxonomic and phylogenetic analysis of this species is dependent on the use of a larger number of isolates and additional morphological characters. In the present study, *Alternaria* isolates obtained from *Allium* plants were divided into two groups based on sequence analyses of the BT2b, Alt a1, and RPB2 genes. Additionally, it was observed that the partial sequences of the ITS regions and the *gpd* genes from *A. porri* and *A. vanuatuensis* were identical to each other. Our results are in accordance with those of Simmons [3], who differentiated 4 different species of *Alternaria* from *Allium* plants including *A. vanuatuensis* and *A. porri*. The RPB2 sequences differentiated these species. Park *et al.* [4] also re-examined the relationship between *A. radicina* and *A. carotiincultae*, which were previously considered as a synonym [5]. The species were divided into 2 distinct lineages based on the EF-1α, β-tubulin, and Alt a1 gene sequences; moreover. In the present study, our phylogenetic analysis revealed the same trend between *A. vanuatuensis* and *A. porri*.

The colony morphology of *A. vanuatuensis* was similar to that of *A. porri*. *A. vanuatuensis* produced diffusible pigments on PDA and V8 juice agar, but not on MEA. However, examination of the conidial morphology revealed that *A. vanuatuensis* produced a significantly shorter beak than did *A. porri*. Moreover, the results of pathogenicity suggested that *A. porri* causes a higher disease severity than *A. vanuatuensis* on *Allium* plants.

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