Characterization and Pathogenicity of \textit{Alternaria vanuatuensis}, a New Record from \textit{Allium} Plants in Korea and China

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\textbf{Abstract} \textit{Alternaria} from different \textit{Allium} plants was characterized by multilocus sequence analysis. Based on sequences of the $\beta$-tubulin (BT2b), the \textit{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 \textit{A. porri} (EGS48-147) and \textit{A. vanuatuensis} (EGS45-018). The conidial characteristics and pathogenicity of \textit{A. vanuatuensis} also well supported the molecular characteristics. This is the first record of \textit{A. vanuatuensis} E. G. Simmons & C. F. Hill from Korea and China.

\textbf{Keywords} \textit{Allium} plants, \textit{Alternaria vanuatuensis}, China, Morphology, Phylogeny, Korea

The purple blotch of onion caused by \textit{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 \textit{A. porri} has been widely used for decades as an uncritical identification for any large-spored \textit{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 \textit{Allium}. These taxa were \textit{A. ascaloniae} E. G. Simmons & C. F. Hill, \textit{A. iranica} E. G. Simmons & Y. Ghosta, \textit{A. prasonis} E. G. Simmons, and \textit{A. vanuatuensis} E. G. Simmons & C. F. Hill. \textit{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 $\beta$-tubulin, histone 3, glyceraldehyde-3-phosphate dehydrogenase (gpd), \textit{Alternaria} allergen a1 (Alt a1), elongation factor 1-alpha (EF-1$\alpha$), and RNA polymerase II (RPB2) genes has revealed a relatively high genetic diversity among species or interspecies of \textit{Alternaria} and other fungal genera [4, 5].

The objectives of the present study were (1) to describe the newly recorded species, \textit{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 \textit{Alternaria} obtained from \textit{Allium ascaloniae}, \textit{A. cepa}, \textit{A. fistulosum}, and \textit{Allium} sp. were used in this study (Table 1). The \textit{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)
Alternaria vanuatuensis from Korea and China

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

| Species | Isolate ID | Host | Origin | GenBank accession Nos. |
|---------|------------|------|--------|-----------------------|
| Alternaria porri | CNU093037 | Allium fistulosum L. | China | JF331530 JF331559 JF331473 JF331442 JF331590 |
| A. porri | CNU093038 | Allium fistulosum L. | Korea | JF331531 JF331539 JF331482 JF331451 JF331599 |
| A. porri | CNU3480 | Allium fistulosum L. | Korea | JF331543 JF331571 JF331486 JF331455 JF331603 |
| A. vanuatuensis | CNU093020 | Allium fistulosum L. | Korea | JF331545 JF331488 JF331501 JF331605 |
| A. vanuatuensis | CNU093033 | Allium fistulosum L. | China | JF331547 JF331404 JF331490 JF331503 JF331607 |
| A. vanuatuensis | CNU3367 | Allium fistulosum L. | Korea | JF331549 JF331416 JF331492 JF331505 JF331609 |
| A. vanuatuensis | CNU094020 | Allium fistulosum L. | Korea | JF331550 JF331417 JF331506 JF331610 |
| A. porri | EGS48-147 | A. cepa | USA | JF331538 JF331566 JF331481 JF331450 JF331598 |
| A. vanuatuensis | EGS45-018 | A. cepa | New Zealand | JF331551 JF330048 JF331494 JF331507 JF331611 |
| A. alternata | EGS34-016 | Arachis hypogea | India | AY563301 IQ672039 AY278808 AF347031 JQ811951 |
| A. alternanthera | EGS52-039 | Solanum melongena | China | - | KC584096 KC584179 KC584374 |
| A. ascalonae | 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 | CBS17228 | Gossypium barbadense | USA | - | JQ672066 KC584124 KC584204 KC584410 |
| A. panax | CN085010 | Panax ginseng | Korea | JX213305 | JF417596 JF417650 JF417679 KC584392 |
| A. prasinos | 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. tenuisima | EGS34-015 | Dianthus sp. | UK | AY563302 JQ672040 AY278809 AF347032 JQ811961 |

ITS, internal transcribed spacer.
* CNU: Chungnam National University, Korea; EGS: Emory G Simmons, Mycological Services, Crawfordsville, IN 47933, USA; CBS: CBS-KNAW Fungal Biodiversity Centre, Uppsalaalan 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 prasinos (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. Chosundaengae). 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 × 10^6 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
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.

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

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

**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).
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. carotininctae*, which were previously considered as a synonym [5]. The species were divided into 2 distinct lineages based on the EF-1a, 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.

**ACKNOWLEDGEMENTS**

This work was supported by a grant from National Institute of Biological Resources (NIBR), funded by the Ministry of Environment, and in part by the Bio-industry Technology Development program (111095-3) for IPET, Ministry of Agriculture, Food, and Rural Affairs, Republic of Korea.

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