MYCOBIOLOGY
2019, VOL. 47, NO. 1, 76–86
https://doi.org/10.1080/12298093.2019.1572263

RESEARCH ARTICLE

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Genetic Diversity of the Pear Scab Fungus *Venturia nashicola* in Korea

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ABSTRACT

Scab disease caused by *Venturia nashicola* is of agroeconomic importance in cultivation of Asian pear. However, little is known about the degree of genetic diversity in the populations of this pathogen. In this study, we collected 55 isolates from pear scab lesions in 13 major cultivation areas in Korea and examined the diversity using sequences of internal transcribed spacer (ITS) region, β-tubulin (TUB2), and translation elongation factor-1α (TEF-1α) genes as molecular markers. Despite a low level of overall sequence variation, we found three distinctive subgroups from phylogenetic analysis of combined ITS, TUB2, and TEF-1α sequences. Among the three subgroups, subgroup 1 (60% of isolates collected) was predominant compared to subgroup 2 (23.6%) or subgroup 3 (16.4%) and was distributed throughout Korea. To understand the genetic diversity among the subgroups, RAPD analysis was performed. The isolates yielded highly diverse amplicon patterns and none of the defined subgroups within the dendrogram were supported by bootstrap values greater than 30%. Moreover, there is no significant correlation between the geographical distribution and the subgroups defined by molecular phylogeny. Our data suggest a low level of genetic diversification among the populations of *V. nashicola* in Korea.

ARTICLE HISTORY
Received 28 August 2018
Revised 17 December 2018
Accepted 24 December 2018

KEYWORDS
Genetic diversity; pear; scab; Venturia nashicola

1. Introduction

Pears (*Pyrus* L.) are among the most economically important fruit crops in temperate regions worldwide. Of over 30 diseases reported on pear trees in Korea [1], scab disease has been the most problematic for the growers [2–7]. Typical pear scab symptoms show dark or olive-colored spots on leaves (Figure 1(A,D,E)), petioles (Figure 1(B)), young fruits in early growing season (Figure 1(C)), and mature fruits in late growing season (Figure 1(F)), resulting in defoliation by early fall and deterioration of fruit quality.

Pear scab is caused by two pathogens, *Venturia nashicola* and *V. pirina* that display distinct host specificities. *Venturia nashicola* only infects Asian pears including Japanese pear and Chinese pear [8], whereas *V. pirina* is a pathogen of the European pear [9]. In addition, *V. inaequalis* causes apple scab, but is not pathogenic on pears [10–12]. Sivanesan (1977) has previously reported that *V. nashicola* and *V. pirina* are synonymous [13]. However, there have been several reports that these two pathogens are clearly distinguished by phylogenetic analysis [11,12,14,15], mycological characteristics, as well as pathogenicity [2].

To analyze the genetic variation in pathogenic fungi, several molecular markers such as the internal transcribed spacer (ITS) region, *β*-tubulin (TUB2), translation elongation factor-1α (TEF-1α), actin, calmodulin, and cytochrome oxidase I (COI), have been developed. The most routinely used among these markers is the internal transcribed spacer (ITS) sequences, spanning the rRNA regions [16]. For *Venturia* spp., sequence analysis of ITS region showed clear separation between *V. nashicola* and *V. pirina* which are derived from Asian pear and European pear, respectively [11].

However, the ITS sequence alone provides limited resolution in phylogenetic analysis of closely related species. Multigene sequence analysis has been known to enable resolution of variation at inter- and even intraspecies levels [8,17]. Zhao et al., reported that multigene phylogenetic analysis using a combined data set of ITS region, partial TUB2, and partial TEF-1α gene sequences showed clear separation of *V. nashicola* and *V. pirina* [12].

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Supplemental data for this article can be accessed here.
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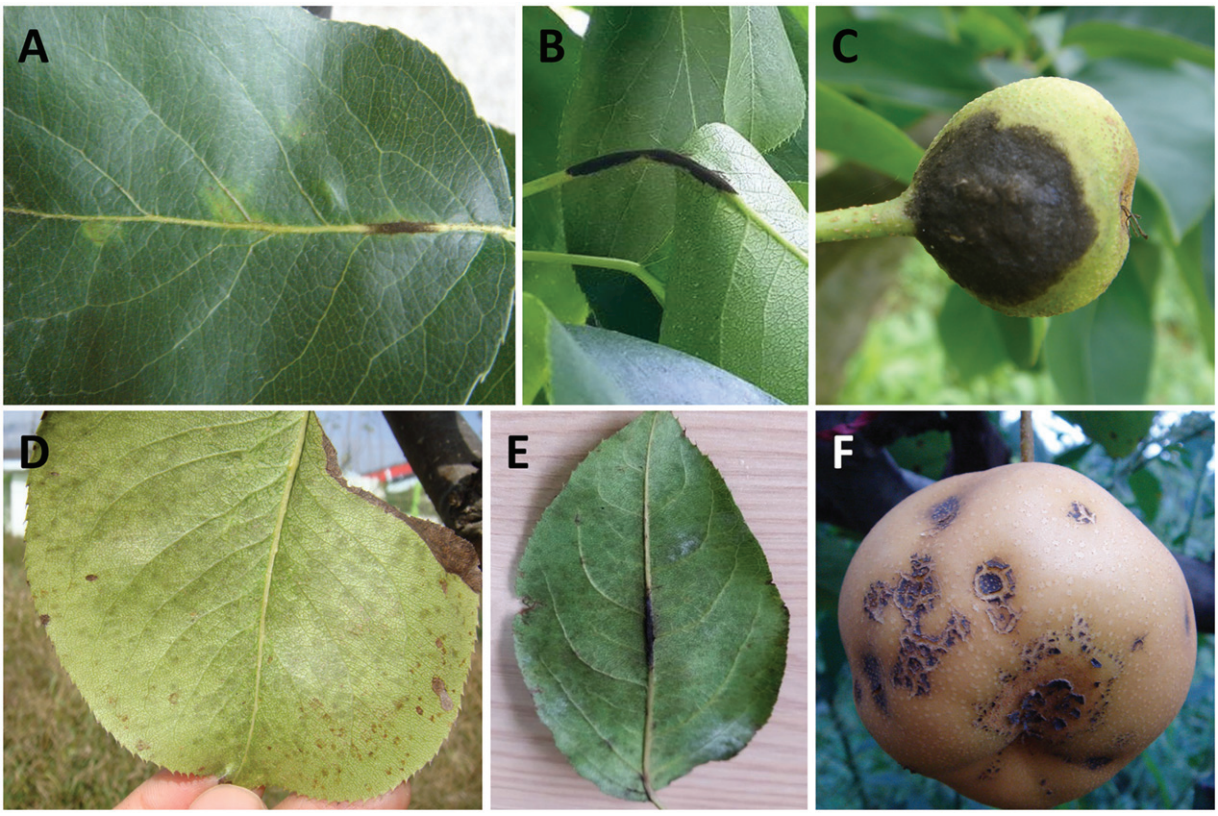

Figure 1. Typical symptoms of pear scab. Irregular dark sooty spots (A) on leaf vein, (B) petiole, and (C) young fruit in early growing season. Irregular mild sooty spot (D) on magnified leaf surface, (E) along the midrib and major lateral veins on the leaf surface, and (F) on mature fruit in late growing season.

Random amplified polymorphic DNA (RAPD) analysis has been extensively applied to detect genetic difference among races, formae speciales, or population of plant pathogenic fungi [18–22]. The RAPD method allows rapid and simple detection of genetic variation, compared to other molecular genotyping methods, such as restriction fragment length polymorphisms or amplified fragment length polymorphisms.

In Korea, more than 80% of cultivated pear is cv. "Niitaka". Since cv. "Niitaka" is highly susceptible to *V. nashicola*, an extensive breeding program has been conducted to develop disease resistant cultivar to replace it [6,23,24]. Understanding the genetic variation of *V. nashicola* should support and compliment the development of resistant cultivars for effective disease control.

The objectives of this study was to investigate the genetic diversity and population structure of *V. nashicola* isolates from Asian pear in Korea based on ITS, TUB2, and TEF-1α gene sequences and by RAPD analysis.

2. Materials and methods

2.1. Collection of isolates and culture condition

In order to investigate the genetic diversity of the Korean *V. nashicola* population, fungal isolates were collected from scab lesions of pear trees cultivated in 13 major pear growing areas over 2013–2015 (Table 1). As an authentic isolates, KCTC6484 from Korean Collection for Type Cultures (KCTC) and four isolates from Japan, MAFF615002, MAFFF615003, MAFF615023, and MAFF615029, which have been previously used for crossing [25], were included in our analysis (Table 1).

All the isolates were cultured on potato dextrose agar (PDA) at 20°C in the dark for 60 d and then used for the experiments. For DNA extraction, fungal isolates were grown in 50 ml of potato dextrose broth at 20°C for 60 d.

2.2. DNA extraction and PCR amplification

Genomic DNA was extracted using the DNeasy Plant Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration of the extracted genomic DNA was estimated by spectrophotometry (Nanodrop; Thermo Scientific, Waltham, MA). The extracted genomic DNA was kept at -20°C for further experiments.

The partial 18S rRNA-ITS (internal transcribed spacer)1-5.8S rRNA-ITS2-28S rRNA region, was amplified with universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) as described by White et al. [16]. PCR was performed using

Table 1. Isolates of *Venturia nashicola*, *V. pirina,* and *V. inaequalis* used in this study.

Isolate	Host plant	Origin	Year	ITS	β-tubulin	TEF-1α	References
Venturia nashicola							
NJGC1340	Japanese pear	Naju, Korea	2013	MH725922	MH725982	MH726042	–[b]
NJBH1325	Japanese pear	Naju, Korea	2013	MH725923	MH725983	MH726043	–
14VNNJ11	Japanese pear	Naju, Korea	2014	MH725924	MH725984	MH726044	–
14VNNJ18	Japanese pear	Naju, Korea	2014	MH725925	MH725985	MH726045	–
14VNNJ19	Japanese pear	Naju, Korea	2014	MH725926	MH725986	MH726046	–
14VNNJ26	Japanese pear	Naju, Korea	2014	MH725927	MH725987	MH726047	–
14VNNJ27	Japanese pear	Naju, Korea	2014	MH725928	MH725988	MH726048	–
NJJS1	Japanese pear	Naju, Korea	2015	MH725929	MH725989	MH726049	–
NJGC1	Japanese pear	Naju, Korea	2015	MH725930	MH725990	MH726050	–
NJAP-1	Japanese pear	Naju, Korea	2015	MH725931	MH725991	MH726051	–
NJSJ-1	Japanese pear	Naju, Korea	2015	MH725932	MH725992	MH726052	–
NJHC-1	Japanese pear	Naju, Korea	2015	MH725933	MH725993	MH726053	–
NJCB-1	Japanese pear	Naju, Korea	2015	MH725934	MH725994	MH726054	–
NJBR-1	Japanese pear	Naju, Korea	2015	MH725935	MH725995	MH726055	–
14VNHD1-11	Japanese pear	Hadong, Korea	2014	MH725936	MH725996	MH726056	–
14VNHD2-3	Japanese pear	Hadong, Korea	2014	MH725937	MH725997	MH726057	–
14VNHD3-08	Japanese pear	Hadong, Korea	2014	MH725938	MH725998	MH726058	–
14VNHD3-31	Japanese pear	Hadong, Korea	2014	MH725939	MH725999	MH726059	–
15VNGNHD1-2	Japanese pear	Hadong, Korea	2015	MH725940	MH726000	MH726060	–
15VNGNHD2-7	Japanese pear	Hadong, Korea	2015	MH725941	MH726001	MH726061	–
15VNGNHD3-7	Japanese pear	Hadong, Korea	2015	MH725942	MH726002	MH726062	–
15VNGNHD3-11	Japanese pear	Hadong, Korea	2015	MH725943	MH726003	MH726063	–
HSPB1	Japanese pear	Hwasung, Korea	2015	MH725944	MH726004	MH726064	–
HSPB2	Japanese pear	Hwasung, Korea	2015	MH725945	MH726005	MH726065	–
HSHJ1	Japanese pear	Hwasung, Korea	2015	MH725946	MH726006	MH726066	–
HSJS1	Japanese pear	Hwasung, Korea	2015	MH725947	MH726007	MH726067	–
HSSK1	Japanese pear	Hwasung, Korea	2015	MH725948	MH726008	MH726068	–
15VNHS1-1	Japanese pear	Hwasung, Korea	2015	MH725949	MH726009	MH726069	–
15VNHS6-9	Japanese pear	Hwasung, Korea	2015	MH725950	MH726010	MH726070	–
14VNMU01	Japanese pear	Cheongju, Korea	2014	MH725951	MH726011	MH726071	–
14VNMU30	Japanese pear	Cheongju, Korea	2014	MH725952	MH726012	MH726072	–
15VNCHJNS-1	Japanese pear	Cheongju, Korea	2015	MH725953	MH726013	MH726073	–
15VNCHJNS-8	Japanese pear	Cheongju, Korea	2015	MH725954	MH726014	MH726074	–
14VNOC07	Japanese pear	Okcheon, Korea	2014	MH725955	MH726015	MH726075	–
14VNOC40	Japanese pear	Okcheon, Korea	2014	MH725956	MH726016	MH726076	–
15VNOCOC-1	Japanese pear	Okcheon, Korea	2014	MH725957	MH726017	MH726077	–
15VNOCAN-8	Japanese pear	Okcheon, Korea	2014	MH725958	MH726018	MH726078	–
14VNSJ11	Japanese pear	Sangju, Korea	2014	MH725959	MH726019	MH726079	–
14VNSJ37	Japanese pear	Sangju, Korea	2014	MH725960	MH726020	MH726080	–
15SAJ-A-6	Japanese pear	Sangju, Korea	2015	MH725961	MH726021	MH726081	–
15SAJ-P-4	Japanese pear	Sangju, Korea	2015	MH725962	MH726022	MH726082	–
15VNJNSC1-2	Japanese pear	Suncheon, Korea	2015	MH725963	MH726023	MH726083	–
15VNJNSC3-19	Japanese pear	Suncheon, Korea	2015	MH725964	MH726024	MH726084	–
14VNULS01	Japanese pear	Ulsan, Korea	2014	MH725965	MH726025	MH726085	–
14VNULS27	Japanese pear	Ulsan, Korea	2014	MH725966	MH726026	MH726086	–
15VNPTAJH-2	Japanese pear	Pyeongtaek, Korea	2015	MH725967	MH726027	MH726087	–
15VNPTAJH-5	Japanese pear	Pyeongtaek, Korea	2015	MH725968	MH726028	MH726088	–
14VNNS05	Japanese pear	Nonsan, Korea	2014	MH725969	MH726029	MH726089	–
14VNNS44	Japanese pear	Nonsan, Korea	2014	MH725970	MH726030	MH726090	–
15VNESSS-4	Japanese pear	Eumseong, Korea	2015	MH725971	MH726031	MH726091	–
15VNESSS-9	Japanese pear	Eumseong, Korea	2015	MH725972	MH726032	MH726092	–
15VNYDYG-1	Japanese pear	Yeongdong, Korea	2015	MH725973	MH726033	MH726093	–
15VNYDYG-5	Japanese pear	Yeongdong, Korea	2015	MH725974	MH726034	MH726094	–
14VNWJ1-02	Japanese pear	Wonju, Korea	2014	MH725975	MH726035	MH726095	–
14VNWJ2-41	Japanese pear	Wonju, Korea	2014	MH725976	MH726036	MH726096	–
KCTC6484	Japanese pear	Korea	-[a]	MH725917	MH725977	MH726037	[4]
MAFF615002	Japanese pear	Ibaraki, Japan	1979	MH725918	MH725978	MH726038	[4]
MAFF615003	Japanese pear	Shizuoka, Japan	1980	MH725919	MH725979	MH726039	[4]
MAFF615023	Japanese pear	Kouchi, Japan	1981	MH725920	MH725980	MH726040	[4]
MAFF615029	Japanese pear	Fukuoka, Japan	1993	MH725921	MH725981	MH726041	[4]
JS-115	Japanese pear	Ohita, Japan	1980	HQ434387	HQ434431	HQ434343	[12,33]
Yasato2-1-1	Japanese pear	Ibaraki, Japan	1992	HQ434394	HQ434438	HQ434350	[12,33]
Mamenashi12A No.1-1	Wild pear	Tottori, Japan	1991	HQ434386	HQ434430	HQ434342	[12,33]
Mamenashi12A No.1-3	Wild pear	Tottori, Japan	1991	HQ434388	HQ434432	HQ434344	[12,33]
Mamenashi12A No.1-4	Wild pear	Tottori, Japan	1991	HQ434389	HQ434433	HQ434345	[12,33]
Mamenashi12B No.1-1	Wild pear	Tottori, Japan	1991	HQ434390	HQ434436	HQ434348	[12,33]
Mamenashi12B No.1-2	Wild pear	Tottori, Japan	1991	HQ434392	HQ434434	HQ434346	[12,33]
Mamenashi12B No.53-1	Wild pear	Tottori, Japan	1992	HQ434391	HQ434435	HQ434347	[12,33]
OYO-1	Wild pear	Tochigi, Japan	1999	HQ434393	HQ434437	HQ434349	[12,34]
V. pirina							
38995	European pear	Israel	–	HQ434425	HQ434469	HQ434381	[12,35]
38996	European pear	Israel	–	HQ434424	HQ434468	HQ434380	[12,35]
V. inaequalis							
HIR-2	Apple	Aomori, Japan	1995	HQ434423	HQ434467	HQ434379	[12]
Ibaraki 1	Apple	Ibaraki, Japan	1998	HQ434422	HQ434378	HQ434378	[12]

[a]Collection year unknown.
[b]In this study.

AccuPower PCR Premix (Bioneer, Daejeon, Korea). After adding 1 µl of template DNA (100 ng/µl), 1 µl of each ITS1 and ITS4 primer to the reaction solution, the final volume was adjusted to 20 µl with distilled water. PCR amplification was carried out using Thermal Cycler (Takara Bio Inc., Shiga, Japan) as follows: initial denaturation at 94°C for 3 min, followed by 30 cycles of 50 s at 94°C, 50 s at 55°C and 2 min at 72°C, and then finally 10 min at 72°C.

The partial TUB2 sequence was amplified with primer Btub14F (5′-AACCAAATTGGTGCTGCCTTCTG-3′) and BtubR (5′-TGGAGGACATCTTAAGACCACG-3′) as described by Kwak et al. [26]. The partial TEF-1α sequence was amplified with primer VNEFI-f (5′-ACTTGATCTACAAGTGCGGTG-3′) and VNEFI-r (5′-AGGAGTCTCACTTCCAGAG-3′) as described by Zhao et al. [12].

TUB2and TEF-1α samples were prepared in the same way as ITS samples. The PCR of TUB2 and TEF-1α was carried out as follows: initial denaturation of 3 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 55°C and 2 min at 72°C, and then finally 5 min at 72°C.

2.3. Nucleotide sequencing

The amplified PCR products were purified using AccuPrep™ PCR purification kit (Bioneer) according to the manufacturer's instructions and then subjected to sequencing analysis at Cosmogenetech (Daejeon, Korea).

2.4. Sequence alignments and phylogenetic analysis

The generated sequences were edited and assembled using Codoncode program (CodonCode Corporation, Dedham, MA). All the sequences generated in this study were initially verified by Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) in National Center for Biotechnology Information (NCBI).

The sequences verified by BLAST searches and the sequences retrieved from GenBank (http://www.ncbi.nlm.gov) were initially aligned using Clustal W (MEGA ver. 7.0) [27]. Two *Venturia* species, *V. inaequalis* and *V. pirina,* which are pathogens of European pear and apples, respectively, were used as outgroups (Table 1). Phylogenetic analysis was conducted using the maximum likelihood (ML) method and neighbor joining (NJ) in MEGA version 7.0 software [27]. Reliability of the inferred tree was tested by 1000 bootstrap replications of the ML and NJ tree to verify the reliability of the branches. Tamura-Nei model of the ML method and Kimura two-parameter model for the NJ method were used for the analyses.

Table 2. Nucleotide sequences of 10-mer oligonucleotide primers used for RAPD analysis of *Venturia nashicola* isolates.

Primers[a]	Nucleotide sequence (5′→3′)
OPA-15	TTCCGAACCC
OPB-17	AGGGAACGAG
OPC-04	CCGCATCTAC
OPD-16	AGGGCGTAAG
OPF-01	ACGGATCCTG
OPI-14	TGACGGCGGT
OPJ-10	AAGCCCGAGG
OPJ-12	GTCCCGTGGT
OPJ-20	AAGCGGCCTC
OPO-12	CAGTGCTGTG

[a]Primer sequences were provided by Operon Technologies.

2.5. RAPD and statistical analysis

Ten 10-mer primers were used for RAPD analysis (Table 2). PCR was performed using AccuPower PCR Premix (Bioneer). After adding 1 µl of template DNA (100 ng/µl) and 1 µl of primer (10 pmol/µl) to the reaction mixture, the final volume of the reaction was adjusted to 25 µl with distilled water.

The PCR was performed as follows: initial cycles of 5 min at 94°C, followed by 40 cycles of 30 s at 94°C, 1 min at 37°C and 1 min at 72°C, and then finally 7 min at 72°C. The PCR products were analyzed by electrophoresis in 1.5% agarose gels and detected by staining with Dyne LoadingSTAR (DYNE BIO., Seongnam, Korea).

RAPD profiles were scored visually with a binary scoring system (1 for presence and 0 for absence at each band position). Only the major amplification products were scored, and this was based on the assumption that products of the same size and electrophoretic mobility in different isolates were identical. Data obtained from the 10 primers were pooled together. Based on this similarity matrix, a phenogram was constructed via the unweighted pair group methods using arithmetic means (UPGMA) analysis in NTSYS-pc program ver.2.2 (Exeter Software, Setauket, NY). Bootstrapping with 2000 replicates was carried out to assess the robustness of clusters in the phenograms using WINBOOT program [28] and NTSYS-PC.

3. Results

3.1. Identification of fungal isolates

Identification of the collected isolates was carried out based on the morphological characteristics as reported by Tanaka and Yamamoto [8] and Ishii and Yanase [2]. The isolates produced dark colonies on PDA (Figure 2(A)). The conidia produced on the

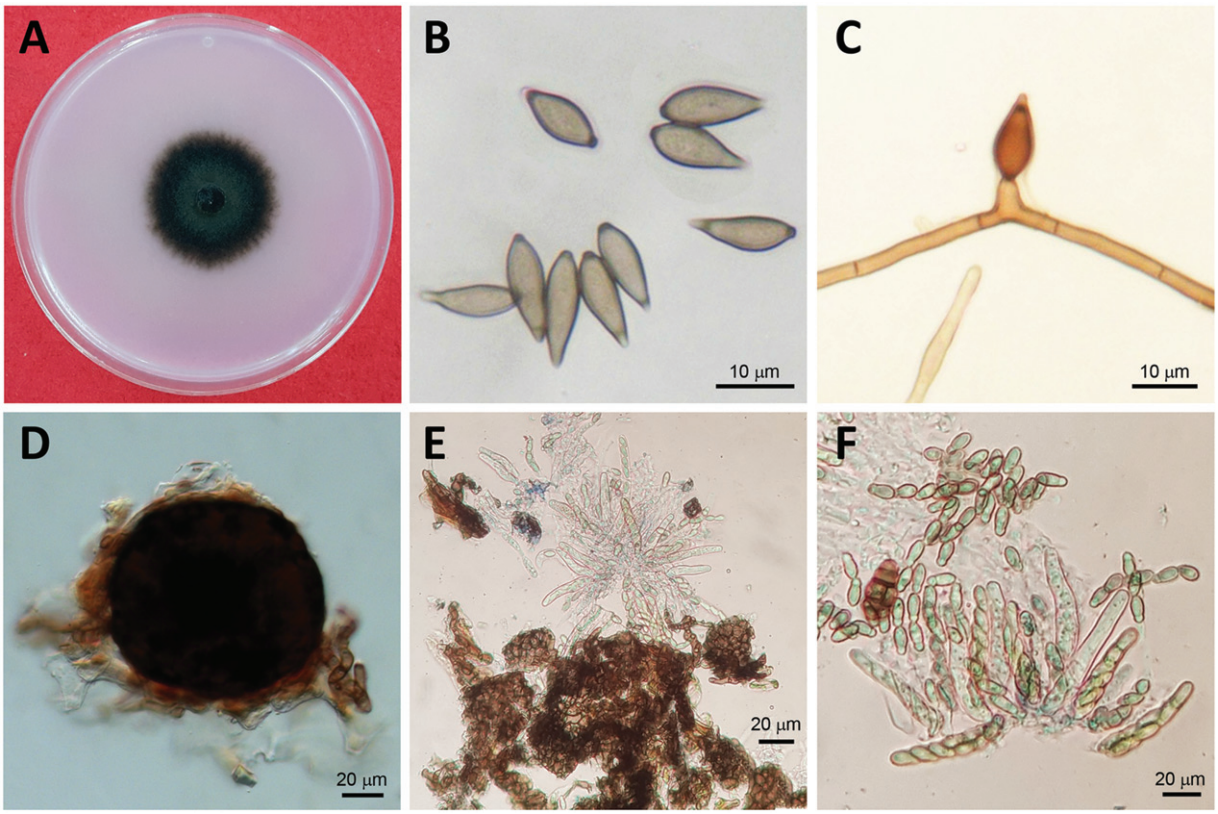

Figure 2. Mycological characteristics of *Venturia nashicola*. (A) 60 d-old colony on PDA at 20 °C in the dark; (B) Conidia; (C) Conidiophore; (D) Pseudothecia, (E) Asci; (F) Ascospores.

Table 3. Comparison of mycological characteristics of the isolates collected in this study and *Venturia nashicola* isolates described previously.

Characteristics		Isolate from this study	*Venturia nashicola*[a]
Conidia	Shape	Dark brown, long oval or fusiform	Dark brown, long oval or fusiform
	Size (μm)	8.2–18.8 (12.9)×5.1–7.8 (6.5)	7.5–22.5(14.4)×5.0–7.5(6.0)
Conidiophores	Shape	Brown, clustered, erect	Brown, clustered, erect, simple
	Height (μm)	10–15	10–20
Pseudothecia	Shape	Wall dark colored, globoid	Wall dark colored, brownish cells, depressed , conic, globoid
	Size (μm)	55–122 × 59–140	50.0–150 × 53–138
Asci	Shape	Hyaline, club-shaped	Hyaline, clavate or long ovate
	Size (μm)	38–63 × 5–10	35–60 × 5–10
Ascospore	Shape	Hyaline or light brown, two-celled, shoesole-shaped	Hyaline or light brown, two-celled, shoesole-shaped
	Size (μm)	12.0–15.0 × 3.0–4.5	10–15 × 3.8–6.3

[a]Tanaka and Yamamoto [8].

plates were brown and unicellular, long oval or fusiform measuring 8.2 to 18.8 μm × 5.1 to 7.8 μm (Figure 2(B)). Conidiophores were also brown in color, clustered, and erect measuring 10 to 15 μm (Figure 2(C)). Pseudothecia was dark brown and globoid with brown cells measuring 50 to 150 μm × 59 to 140 μm (Figure 2(D)). Asci were hyaline and club-shaped measuring 38 to 63 μm × 5 to 10 μm (Figure 2(E)) and ascospores were two-celled, hyaline, or light brown and shoesole-shaped, measuring 12 to 15 μm × 3 to 4.5 μm (Figure 2(F)). These morphological characteristics are consistent with those previously described for *V. nashicola* [2,8] (Table 3).

The ITS (NCBI accession numbers: MH725922-MH725921), TUB2 (MH725982-MH725981), and TEF-1α (MH726042-MH726041) sequences matched with *V. nashicola* reference sequences [12] with over 99% identity for all the isolates that we examined in this study.

3.2. Variation of ITS region sequences

Three ITS2 sequence types could be assigned to the 60 isolates sequenced in this study and nine sequences of *N. nashicola* isolates retrieved from GenBank (Table 1). The consensus nucleotide sequences of ITS2 are shown in Figure 3(A). The three ITS2 types are designated subgroup 1 to 3; ITS2-subgroup 1 had G and C; ITS2-subgroup 2 had A and C; and ITS2-subgroup 3 had G and T at the 46th and the 143rd positions of the ITS2 region sequences (Supplementary Table S1). Phylogenetic analysis based on ITS sequences showed that *V. nashicola*

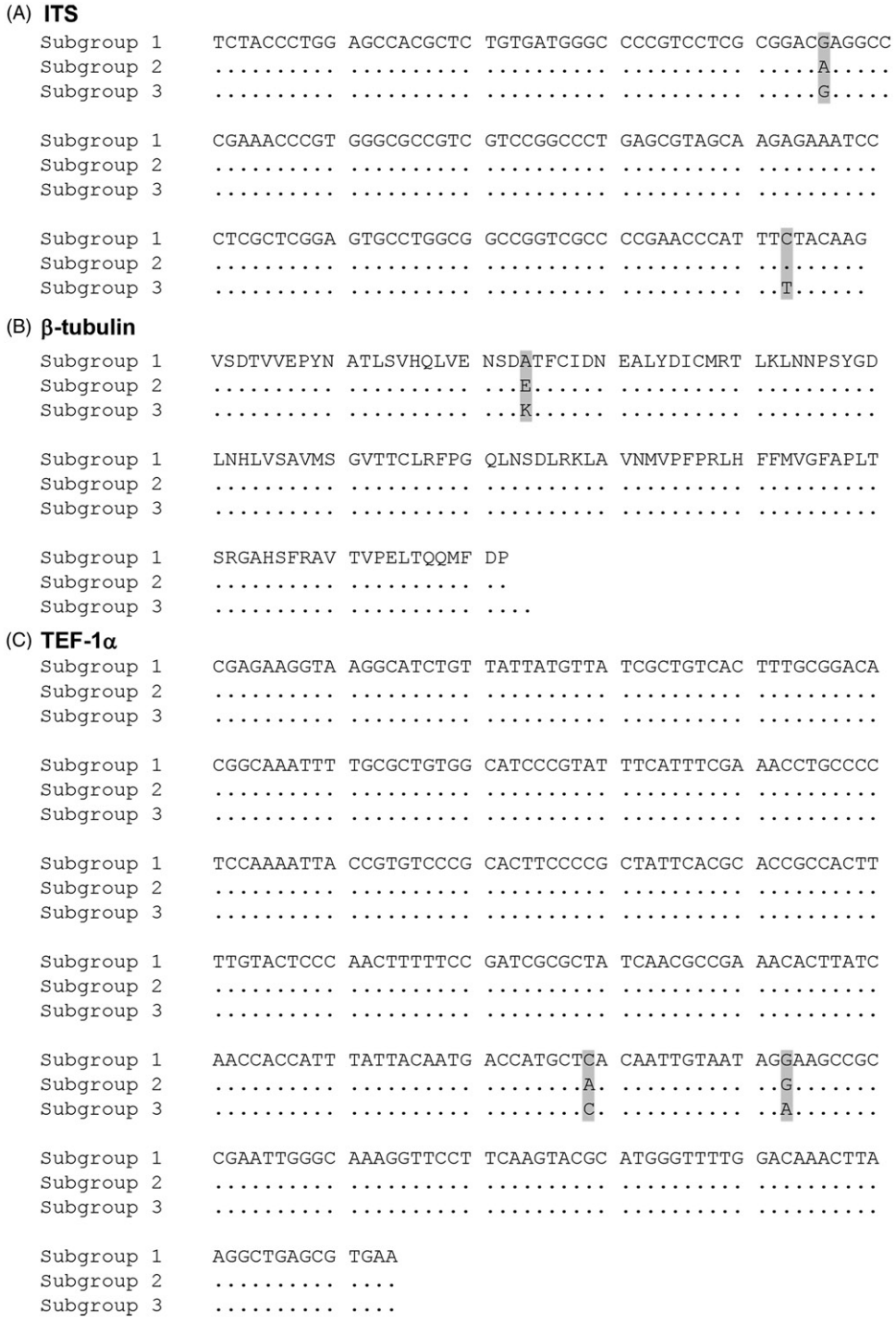

Figure 3. Comparison of representative sequences of subgroups 1 to 3. (A) ITS2 region. (B) Partial protein sequences of β-tubulin. (C) Translation elongation factor-1α.

isolates were classified into three subgroups (subgroup 1, 2 and 3), clearly distinct from *V. pirina* and *V. inaequalis* isolates (Supplementary Figure S1).

3.3. Variation of β-tubulin sequences

Three TUB2 amino acid sequence types were detected from the 60 isolates examined in this study. The consensus protein sequences of TUB2 are shown in Figure 3(B). We detected the three types of variation in the 198th codon defining subgroups 1 to 3: subgroup 1 had alanine (A, GCG); subgroup 2 had glutamine (E, GAG); and subgroup 3 had lysine (K, AAG) (Supplementary Table S1). All the 55 isolates collected in this study and two standard isolates, MAFF615003 and MAFF615029, belonged to subgroup 1. The standard isolates KCTC6484 and MAFF615002 belonged to subgroup 2, whereas MAFF615023 was classified as subgroup 3 (Supplementary Table S1). Phylogenetic analysis showed that *V. nashicola* isolates were classified into two branches, clearly distinguished from *V. pirina* and *V. inaequalis* isolates (Supplementary Figure S2).

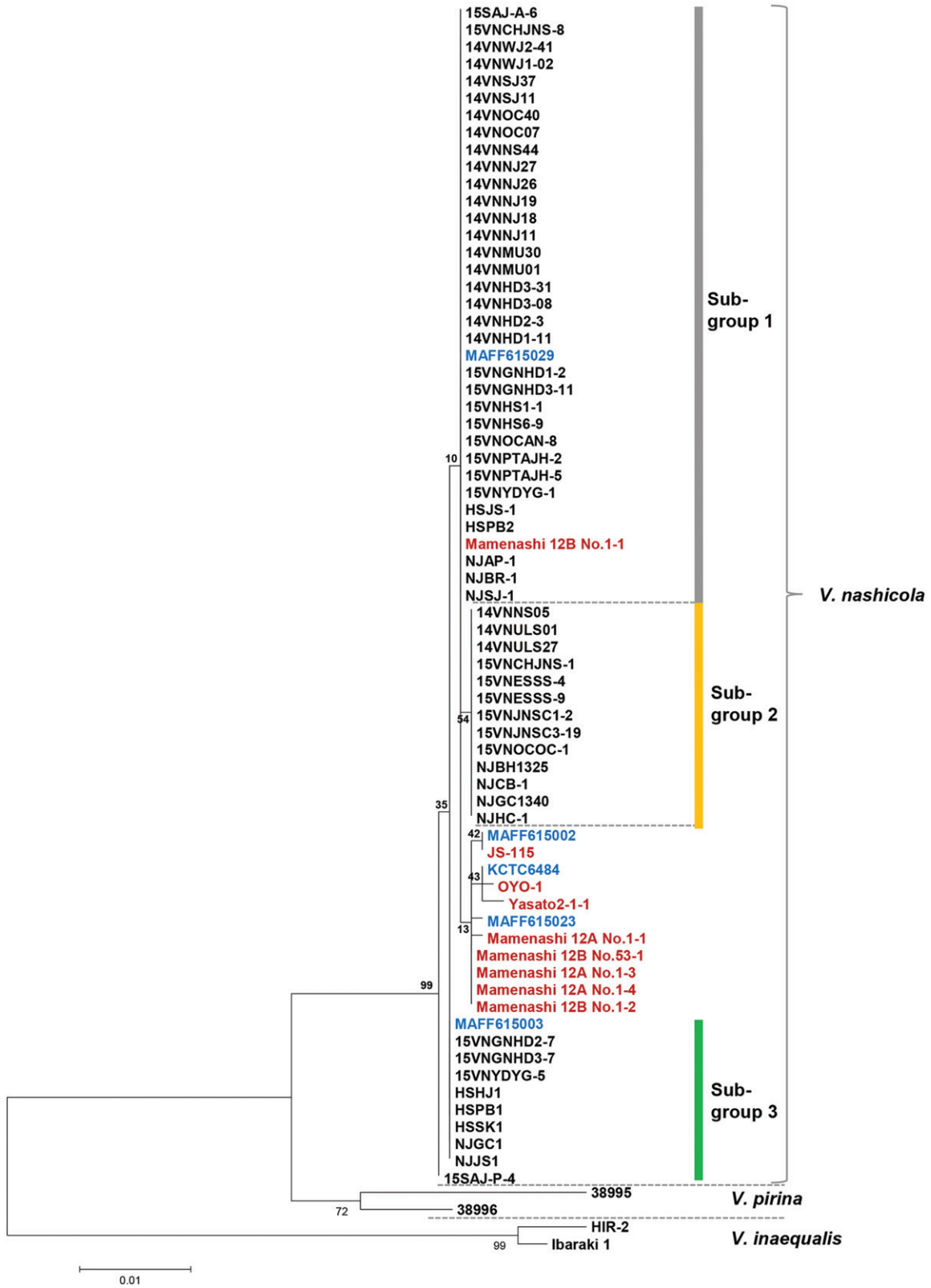

Figure 4. Maximum likelihood tree generated by a combined alignment of ITS region including 5.8S rRNA, partial β-tubulin gene and partial translation elongation factor 1-α gene sequences from *Venturia nashicola*. All *V. nashicola* sequences were newly generated in this study, except those marked in red, which were retrieved from GenBank. Standard *V. nashicola* isolates from Korea (KCTC, Korean culture collection) and Japan (MAFF) are indicated in blue. Isolates of *V. pirina* and *V. inaequalis* were used as outgroups. The numbers at nodes represent the percentage of their occurrence in 1000 bootstrap replicates.

3.4. Variation of TEF-1α sequences

The 60 isolates from this study and nine *N. nashicola* sequences retrieved from GenBank were classified into three types according to variation of TEF-1α sequence at positions 229 and 243 (Table 1). The consensus nucleotide sequences of TEF-1α are shown in Figure 3(C). The three TEF-1α types were designated subgroups 1 to 3; subgroup 1 had

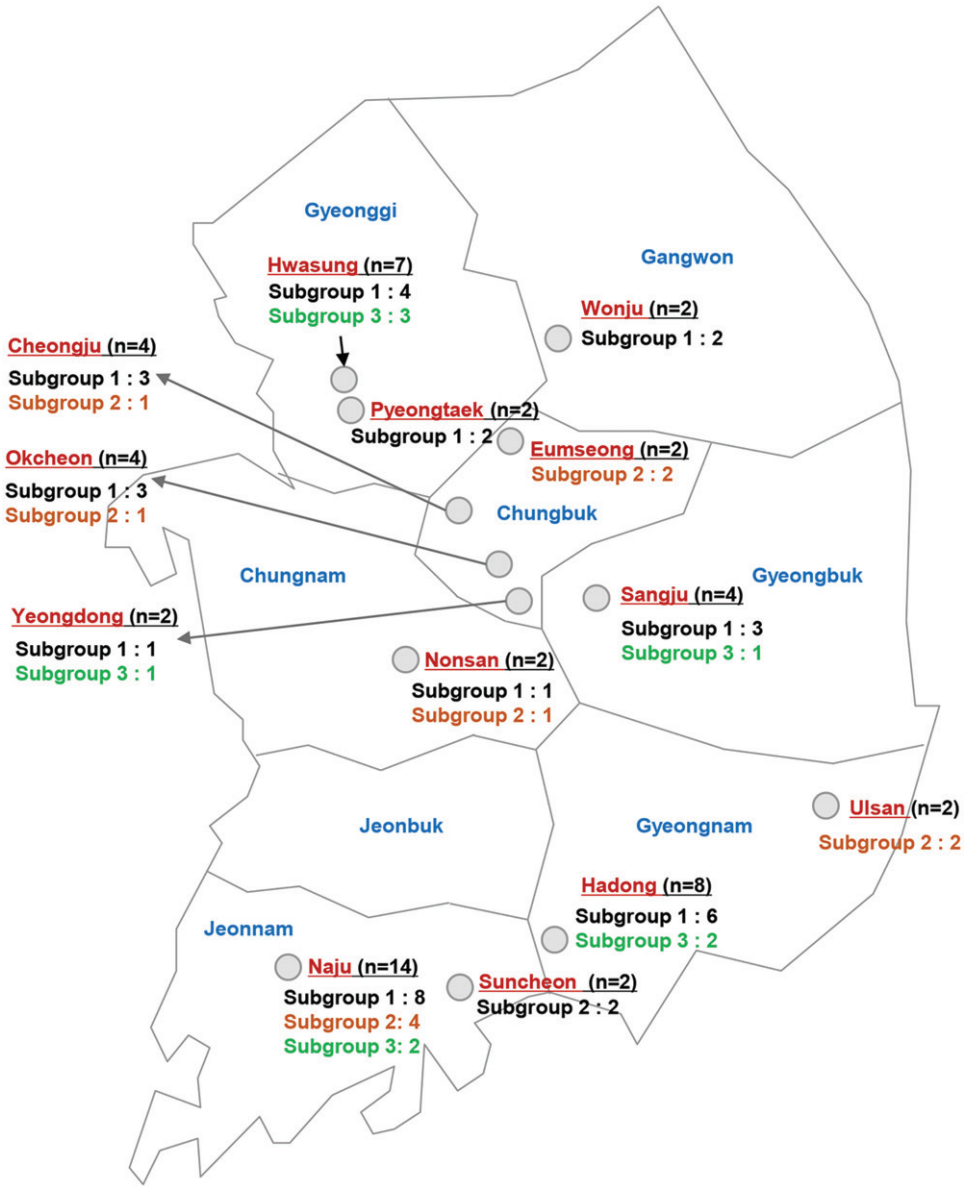

Figure 5. Collection map of *Venturia nashicola* isolates from Japanese pear in Korea over 2013–2015 and distribution of subgroups 1 to 3. *n*=number of isolates.

C and A; subgroup 2 had C and G; and subgroup 3 had A and G in 229th and 243rd positions, respectively (Supplementary Table S1). Phylogenetic analysis showed that *V. nashicola* isolates were grouped into two branches, clearly distinct from *V. pirina* and *V. inaequalis* isolates (Supplementary Figure S3). All the *V. nashicola* isolates were clustered together, except isolates KCTC6484, 15SAJ-P-4, Yasato2-1-1, and OYO-1. These four isolates were clustered separately (Supplementary Figure S3).

3.5. Phylogenetic analysis

The combined sequence of the ITS region, TUB2, and TEF-1α genes used in this study is composed of 1137 nucleotides, containing 1129 conserved nucleotides and 8 variable positions. ML analyses were carried out to generate phylogenetic trees of the isolates. One of the ML trees is shown in Figure 5. A NJ tree is included as Supplementary Figure S4 for comparison with the ML tree.

Three clusters were detected among the *V. nashicola* isolates, but with weakly supporting bootstrap values. We designated subgroups 1 to 3 (Figure 4), which turned out to have the identical composition as the ITS2-based subgroups 1 to 3. The geographical distribution of subgroups 1 to 3 is marked in the 13 major pear-cultivating regions in Korea (Figure 5). Despite low bootstrap values supporting the branches, we designated these subgroups 1 to 3 (Figure 4) to detect spatiotemporal differences. However, we could not find a correlation between subgroups and sampling locations or sampling years (data not shown).

3.6. RAPD analysis

We examined 60 isolates including 55 isolates collected in this study and five standard *V. nashicola*

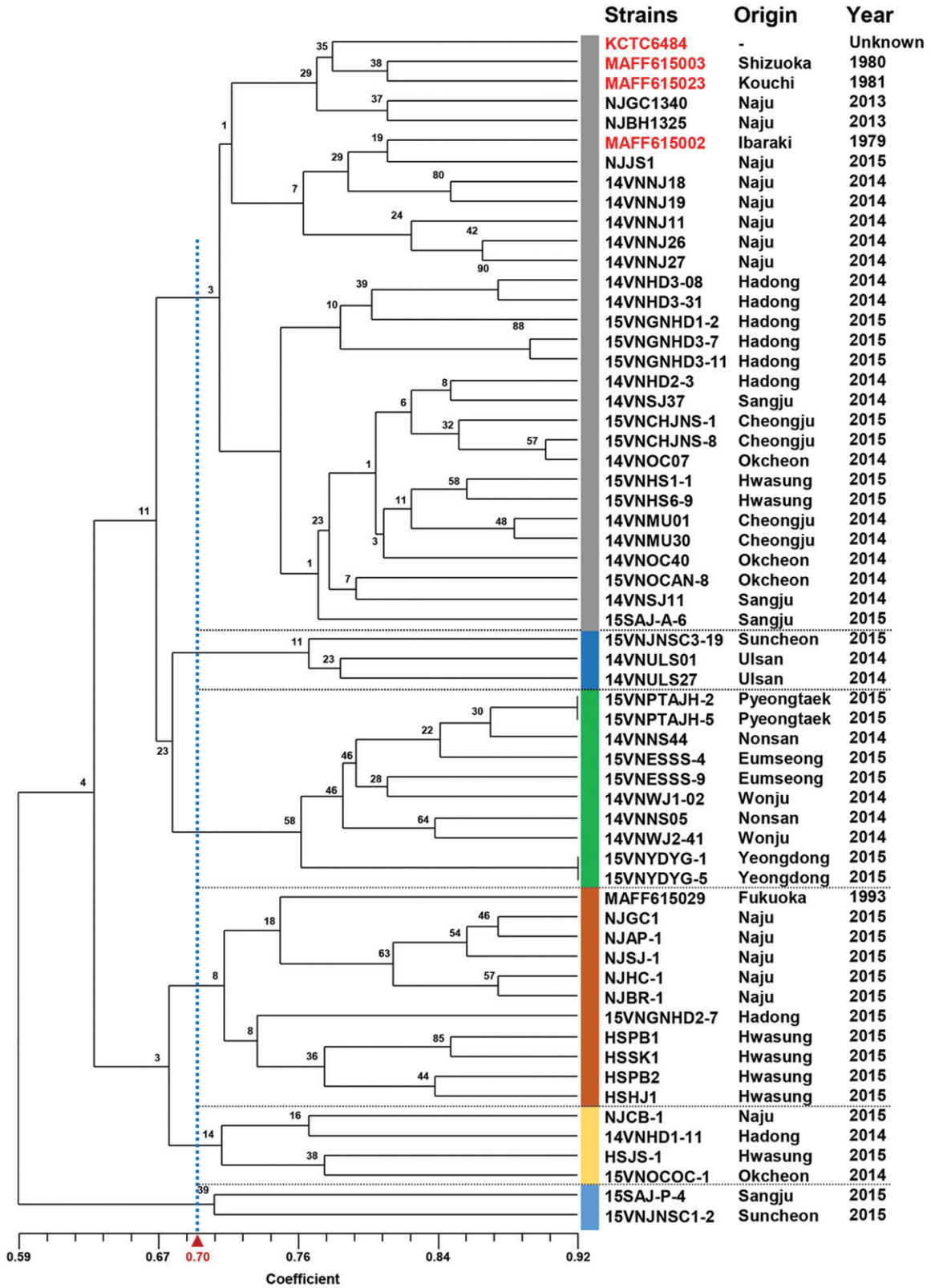

Figure 6. UPGMA dendrogram of 55 *Venturia nashicola* isolates collected in this study and five standard *V. nashicola* isolates based on RAPD polymorphism. Data from 10 different 10-mer primers were combined and used to construct a dendrogram. Origin and year of collection was indicated on the right for individual isolates. Standard *V. nashicola* isolates of Korea and Japan are indicated in red.

isolates (Table 1). In total, 108 polymorphic bands were detected by PCR amplification using 10 random primers. The isolates tested exhibited highly dissimilar band patterns. A dendrogram by UPGMA is shown in Figure 6. Greater than 59% similarity was observed based on 108 polymorphic bands detected. Six clusters were arbitrarily defined based on 70% similarity in the dendrogram (Figure 6). However, none of these clusters are supported by boostrapping (<58% support). We could not find

any correlation between the clusters and geographical (sampling location) or temporal (sampling year) distribution (data not shown).

4. Discussion

Phylogenetic analyses are pertinent for resolving fungal taxa and understanding the evolution of fungal populations, especially as an ever-increasing number of fungi are being discovered and their genomes sequenced [29]. In *Venturia* spp., four molecular markers including ITS, TUB2, TEF-1α, and endopolygalacturonase genes have been used for molecular phylogenetic analysis [12,15]. Zhao et al. [12,15] reported that multigene phylogenetic analysis separates the two species, *V. nashicola* from *V. pirina*, which were previously considered to be identical, into distinct evolutionary lineages, indicating the power of multigene phylogenetic analysis.

In this study, we examined the 55 *V. nashicola* isolates collected from 13 major pear cultivation areas in Korea to elucidate genetic differences among the isolates. Alignment of combined ITS-5.8S to ITS2 region, TUB2 and TEF-1α sequences revealed that 1129 bp out of 1137 bp were conserved between the 55 *V. nashicola* isolates and five standard isolates analyzed. These sequence data suggest that little overall genetic difference among the *V. nashicola* isolates, despite geographical distance between isolates and a temporal separation of over 30 years between the standard isolates and those isolates collected in this study.

Although there was only little sequence variation among the isolates, we have identified three amino acid substitutions in analysis of the TUB2 sequence at the 198th codon: GCG (A, alanine), GAG (E, glutamate), and AAG (K, lysine) (Figure 3). Moreover, all the 55 Korean *V. nashicola* isolates that were collected in this study code for an alanine at the 198th codon of TUB2. In a previous study, Kwak et al. (2017) reported that mutation of E198A may indicate whether the isolates are resistant to benzimidazole fungicides [26]. It is already known that benzimidazole fungicides bind to fungal TUB2 and inhibit microtubule formation [30,31]. Considering our results, most of *V. nashicola* isolates from the 13 major pear cultivation areas in Korea are likely to be resistant to benzimidazole fungicides.

Unlike almost identical sequences obtained from sequencing the ITS region, TUB2, and TEF-1α genes, RAPD results showed high variation of band patterns. However, because bootstrap values did not support the clusters, all the isolates may be clonal. This result suggests low genetic diversity in the populations of *V. nashicola* in Korea. The reasons behind the low genetic diversification in the *V. nashicola* population are thought to be as follows: (1) a single pear cultivar, "Niitaka", constitutes 83% of all pears grown in Korea, (2) most of the *V. nashicola* isolates used in the analysis were collected from "Niitaka" trees, (3) a few commercial nurseries limited to specific regions distribute pear seedlings nationwide, (4) the main pear production areas have been growing pear for over 60 years.

Through continuous monitoring of *V. nashicola* populations from diverse pear cultivars in different geographic areas, it is expected that a more precise analysis of diversity and detection of race differentiation events would be possible. This would in turn assist development of resistant cultivars and establishment of effective control strategies against pear scab in the future.

Disclosure statement

No potential conflict of interest was reported by the authors. It has not been published elsewhere and it has not been submitted simultaneously for publication elsewhere.

Funding

This study was carried out with the support of the Cooperative Research Program for Agricultural Science and Technology Development [PJ01019702, PJ011879], Rural Development Administration, Republic of Korea.

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