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Specific and Sensitive Detection of *Venturia nashicola*, the Scab Fungus of Asian Pears, by Nested PCR

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The fungus *Venturia nashicola* is the causal agent of scab on Asian pears. For the rapid and reliable identification as well as sensitive detection of *V. nashicola*, a PCR-based technique was developed. DNA fingerprints of three closely related species, *V. nashicola*, *V. pirina*, and *V. inaequalis*, were obtained by random amplified polymorphic DNA (RAPD) analysis. Two RAPD markers specific to *V. nashicola* were identified by PCR, after which two pairs of sequence characterized amplified region (SCAR) primers were designed from the nucleotide sequences of the markers. The SCAR primer pairs, designated as D12F/D12R and E11F/E11R, amplified 535-bp and 525-bp DNA fragments, respectively, only from genomic DNA of *V. nashicola*. The specificity of the primer sets was tested on strains representing three species of *Venturia* and 20 fungal plant pathogens. The nested PCR primer pair specific to *V. nashicola* was developed based on the sequence of the species-specific 525-bp DNA fragment amplified by primer set E11F/E11R. The internal primer pair Na11F/Na11R amplified a 235-bp fragment from *V. nashicola*, but not from any other fungal species tested. The nested PCR assay was sensitive enough to detect the specific fragment in 50 fg of *V. nashicola* DNA.

Keywords : Asian pear, nested PCR, pear scab, SCAR, *Venturia nashicola*

Pear scab is one of the most serious diseases affecting both Asian (Japanese pear, *Pyrus pyrifolia* var. *culta*; Chinese pear, *P. ussuriensis*) and European (*P. communis*) pears worldwide. *Venturia nashicola* is the causal agent of Asian pear scab (Tanaka and Yamamoto, 1964), whereas it is not pathogenic to European pear. Meanwhile, the causal fungus of European pear scab is *V. pirina* (Shabi, 1990). Because these two fungal species are genetically similar with minute phenotypic differences, some researchers considered that *V.*

nashicola might be a synonym of *V. pirina* (Shabi et al., 1973). To determine whether or not *V. nashicola* could be considered a distinct species, morphological examination, mating experiments, and pathogenicity tests were performed and the results revealed that *V. nashicola* is a species distinct from *V. pirina* (Ishii and Yanase, 2000). Another genetically related *Venturia* spp., *V. inaequalis*, was shown to be the causal agent of apple scab. Sequence analysis of the internal transcribed space (ITS) regions of rDNA revealed that the phylogenetic tree position of *V. inaequalis* is near the monophyletic group consisting of *V. nashicola* and *V. pirina* (Schnabel et al., 1999).

Detection and monitoring of scab pathogen are necessary for the implementation of efficient control strategies. In addition, as Asian pear scab is a quarantined disease in many countries, methods for the rapid and sensitive detection of *V. nashicola* are required. Recently, molecular methods based on the analysis of DNA sequences, such as ITS regions of the nuclear ribosomal RNA gene, have been widely applied to the identification and detection of plant pathogenic fungi. A species-specific PCR primers designed within the ITS1 and ITS2 regions have been developed to detect plant pathogenic fungi (Asano et al., 2010; Chen et al., 2006; Grote et al., 2002; Ma and Michailides, 2002; Ni et al., 2012). Further, PCR-based random amplified polymorphic DNA (RAPD) is a frequently applied molecular approach for the identification of inter- and intra-species specific molecular markers (Williams et al., 1990). However, as RAPD markers are difficult to reproduce, they should be converted into sequence characterized amplification region (SCAR) markers. Many SCAR primers were designed from the nucleotide sequences of the RAPD markers and have been used for the identification of economically important fungal pathogens (Chiocchetti et al., 2001; Grosch et al., 2006; López-Mondéjar et al., 2012; Rigotti et al., 2002; Li et al., 2008; Vandemark et al., 2000).

The aim of this work was to develop species-specific SCAR primers that can detect and differentiate *V. nashicola* from genetically similar fungal species, especially from *V. pirina* and *V. inaequalis*, based on the RAPD markers.

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Materials and Methods

Fungal strains. Fungal strains used in this study are listed in Table 1. Among them, nine isolates of *V. nashicola* were

collected from different regions in Korea.

Genomic DNA extraction. Fungal strains were grown on potato dextrose agar (PDA) medium at 25 °C. Genomic

Table 1. List of fungal strains used in this study

No.	Fungal species	Strain ^a	Origin	PCR assay ^b
1	<i>Venturia nashicola</i>	KCTC 6484	Unknown	++
2	<i>V. nashicola</i>	MAFF 615002	Ibaraki, Japan	++
3	<i>V. nashicola</i>	MAFF 615003	Shizuoka, Japan	++
4	<i>V. nashicola</i>	MAFF 615023	Kouchi, Japan	++
5	<i>V. nashicola</i>	MAFF 615029	Fukuoka, Japan	++
6	<i>V. pirina</i>	MAFF 615009	Akita, Japan	--
7	<i>V. pirina</i>	P1 (this lab.)	New Zealand	--
8	<i>V. pirina</i>	P2 (this lab.)	New Zealand	--
9	<i>V. inaequalis</i>	KCTC 6483	Unknown	--
10	<i>V. inaequalis</i>	KACC 40301	Korea	--
11	<i>V. inaequalis</i>	KACC 40302	Korea	--
12	<i>V. nashicola</i>	ABM-2	Ulju, Korea	++
13	<i>V. nashicola</i>	NJJ-1	Jinju, Korea	++
14	<i>V. nashicola</i>	NHD-1	Hadong, Korea	++
15	<i>V. nashicola</i>	NGV-43	Naju, Korea	++
16	<i>V. nashicola</i>	NBS-1	Boseong, Korea	++
17	<i>V. nashicola</i>	NHC-1	Hongcheon, Korea	++
18	<i>V. nashicola</i>	ABM-1	Naju, Korea	++
19	<i>V. nashicola</i>	NUJB-1	Uijeongbu, Korea	++
20	<i>V. nashicola</i>	NYP-1	Yangpyeong, Korea	++
21	<i>Diplodia</i> sp.	NJC-2 (this lab.)	Korea	--
22	<i>Monilinia</i> sp.	PKD-1 (this lab.)	Korea	--
23	<i>Alternaria alternanta</i>	ALD-1(this lab.)	Korea	--
24	<i>Pestalotiopsis</i> sp.	YBH-5 (this lab.)	Korea	--
25	<i>Colletotrichum</i> sp.	KHG-1(this lab.)	Korea	--
26	<i>Diaporthe actinidia</i>	PUJ-2 (this lab.)	Korea	--
27	<i>Botryosphaeria dothidea</i>	HKI-3 (this lab.)	Korea	--
28	<i>Cladosporium carpophilum</i>	BGF-1 (this lab.)	Korea	--
29	<i>Fusarium oxysporum</i>	MJK-1 (this lab.)	Korea	--
30	<i>Rhizoctonia solani</i>	KACC 40106	Incheon, Korea	--
31	<i>Rhizoctonia solani</i>	KACC 40101	Suwon, Korea	--
32	<i>Aspergillus flavus</i>	KACC 40232	Korea	--
33	<i>Colletotrichum gloeosporioides</i>	KACC 40690	Cheongsong, Korea	--
34	<i>Alternaria kikuchiana</i>	KACC 40025	Yesan, Korea	--
35	<i>Fusarium oxysporum</i>	KACC 40052	Chuncheon, Korea	--
36	<i>Pythium ultimum</i>	KACC 40705	Chuncheon, Korea	--
37	<i>Aspergillus niger</i>	KACC 40280	Korea	--
38	<i>Pyricularia grisea</i>	KACC 40439	Naju, Korea	--
39	<i>Bipolaris oryzae</i>	KACC 41025	Dangjin, Korea	--
40	<i>Aspergillus fumigatus</i>	KACC 41016	Korea	--

^aMAFF: Gene bank of the Japanese Ministry of Agriculture, Forestry and Fisheries; KCTC: Korea Collection for Type Culture; KACC: Korea Agricultural Culture Collection

^b++ and -- indicate the presence and absence of the expected 535- and 525-bp DNA fragment amplified by the primer pairs D12F/D12R and E11F/E11R, respectively.

DNA was extracted from freshly harvested fungal mycelium using a DNeasy Plant Mini Kit (Qiagen, USA). Mycelium was ground in liquid nitrogen using a mortar and pestle to make a fine powder and total DNA was extracted following the protocol of the manufacturer. The concentration of DNA sample was determined using a DyNA Quant 200 fluorometer (Amersham Bioscience, USA).

RAPD analysis. RAPD amplification was carried out using 120 arbitrary 10-mer primers purchased from Operon (Operon Biotechnologies, USA) and used as single primer. DNA samples from three closely related *Venturia* species were amplified with the PCR primers. The reaction mixture consisted of 1 × PCR buffer (10 mM Tris-HCl (pH 9.0) 40 mM KCl, 1.5 mM MgCl₂), 0.2 mM of each dNTP, 10 pmol of RAPD primer, 2.0 U of *Taq* DNA polymerase (Bioneer, Korea), and 10 ng of template DNA in a volume of 25 µl. The amplification was carried out in a DNA Thermal Cycler (Takara Shozo, Japan). The cycle parameters were an initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 37°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 5 min. PCR amplification products were separated on a 2.0% agarose gel in TAE buffer.

Cloning and sequencing of RAPD markers. After RAPD bands unique to *V. nashicola* were excised from agarose gels, the DNA was purified from gel using an AccuPrep Gel Purification Kit (Bioneer, Korea). The purified DNA fragments were ligated into pGEM-T Easy vector (Promega, USA), after which the recombinant plasmids were transformed into competent *Escherichia coli* DH5α cells. Selection of transformed clone was carried out based on blue/white selection and digestion with *Eco*RI restriction enzyme. Plasmid DNA was isolated using a Dyne Plasmid Miniprep Kit (Dyne Bio, Korea). The inserted DNA was sequenced by SolGent Co. (Korea).

Design of SCAR primers and specificity test. Based on the nucleotide sequences of specific fragments of *V. nashicola*, two pairs of oligonucleotide primers were designed using Primer 3 software (Rozen and Skaletsky, 2000). The designed forward primer D12F and reverse primer D12R were expected to amplify a 535-bp DNA fragment with genomic DNA of *V. nashicola*. Another primer pair, E11F and E11R, was expected to produce a 525-bp DNA fragment specific for *V. nashicola*. PCR conditions for amplifying the specific fragments with the newly synthesized SCAR primers were as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing for 30 s, extension at 72°C for 45 s, and final extension at 72°C for 5 min.

Annealing temperatures of D12F/D12R and E11F/E11R primers were 55 and 62°C, respectively. Specificity of two primer pairs for *V. nashicola* were evaluated with nine strains of three *Venturia* species, nine strains of *V. nashicola* isolated in different regions of Korea and 20 other plant pathogenic fungi listed in Table 1.

Southern blot analysis. To confirm the specificity of SCAR primers for *V. nashicola*, DNA hybridization was performed using the amplified fragments as probes. PCR products amplified with SCAR primers were excised from agarose gel and then labeled with digoxigenin. Alkaline transfer and Southern hybridizations were carried out as described by Sambrook and Russell (2001). Probe labeling, hybridization and detection were performed with a DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics, Germany) as recommended by the manufacturer.

Nested PCR primers for *V. nashicola*. To increase detection sensitivity, nested PCR primers were designed from the first round of PCR products amplified using the E11F/E11R primer pair. Under low stringent PCR conditions, conducted with annealing temperature of 52°C, the E11F/E11R primer pair produced a 535-bp fragment with DNA of *V. pirina*, which is the closest fungus to *V. nashicola*. The PCR product was cloned and the nucleotide sequence was determined as described above. The nucleotide sequence of the amplified product was aligned with that of *V. nashicola*. Sequence patterns unique to *V. nashicola* were used to design the specific internal primer pair. The internal primer pair, Na11F and Na11R, for the second PCR was expected to amplify a 235-bp DNA fragment with *V. nashicola*. For nested PCR, fungal DNA was first subject to amplification by PCR using the external primers E11F and E11R. Subsequently, the amplified product was used as the template for the second PCR using primers Na11F and Na11R.

Sensitivity of nested PCR assay. To determine the detection limit of nested PCR, genomic DNA of *V. nashicola* was serially diluted with distilled water to yield a final concentration ranging from 50 pg/µl to 500 ag/µl. Template DNA (1 µl) was first subjected to amplification by a DNA Thermal Cycler (Takara Shozo, Japan) using the external primer pair E11F/E11R. Then, one µl of product from the first PCR was used as template for the second PCR using the internal primer pair Na11F/Na11R. PCR amplification was performed using the parameters described above, except that the annealing temperature was 65°C. Ten µl of PCR product was separated on a 1.5% agarose gel in TAE buffer. Gel was stained with ethidium bromide and photographed under ultraviolet light.

Results

Identification of RAPD markers. Five *V. nashicola* strains, one strain of *V. pirina*, and three strains of *V. inaequalis* were used for comparison and identification of specific bands representing *V. nashicola* using RAPD analysis. Among 120 primers tested in this study, two primers, OPD-12 (5'-CACCGTATCC-3') and OPE-11 (5'-GAGTCTCAGG-3'), produced polymorphic profiles that distinguished *V. nashicola* from *V. pirina* and *V. inaequalis*. Species-specific bands of approximately 580 bp and 820 bp in length, respectively, were amplified only from DNA of *V. nashicola* (Fig. 1).

Design of the SCAR primers. Two RAPD markers for *V. nashicola* amplified using OPD-12 and OPE-11 primers were excised from agarose gel and DNA fragments were cloned and sequenced. Comparison of the nucleotide sequences of two RAPD fragments did not match any known sequences available in the GenBank sequence database. Based on the nucleotide sequences of the RAPD markers, two SCAR primer pairs, D12F/D12R (5'-ACAAACAGCCTCCCTCATCT-3'/5'-TAACAAACCCACATAAAGAT-3') and E11F/E11R (5'-AACATACCTGCCACCCAAAA-3'/5'-AAGGCAAGCGAATCCTCATT-

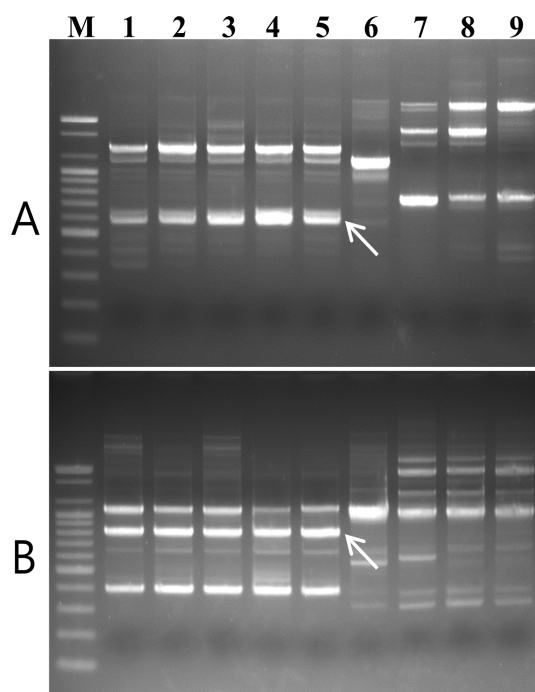


Fig. 1. RAPD fingerprints resulting from PCR amplification of genomic DNA of *V. nashicola* (lanes 1–5), *V. pirina* (lane 6), and *V. inaequalis* (lanes 7–9) using the primers (A) OPD-12 and (B) OPE-11. Fragments indicated by arrows were subsequently cloned and sequenced. M, 100 bp DNA size marker (Bioneer).

3'), were designed. These primer pairs specifically amplified DNA bands of 535 bp (Fig. 2A) and 525 bp (Fig. 2B) in length, respectively, with *V. nashicola* DNA. Southern blot hybridization with probe derived from the PCR product using SCAR primers and *V. nashicola* DNA confirmed the specificity of the primer pairs (Supplementary Fig. S1).

Specificity of primer pairs. To test the specificity of the designed primer pairs, purified DNA samples from 20 plant pathogenic fungal species and nine *V. nashicola* isolates collected in Korea were amplified using both the D12F/D12R and E11F/E11R primer pairs. No fragments were amplified from any other fungus associated with plant disease (Supplementary Fig. S2). However, these primer pairs were specific to *V. nashicola* as amplification of the expected fragments was successfully performed with DNA of all Korean isolates tested (Fig. 3).

Evaluation of internal primer pair for the nested PCR. The internal primer pair Na11F (5'-CACACCAGGTCA-GATTTCG-3')/Na11R (5'-GGTATTATCTACTCGATCAG-3') amplified an expected 235-bp fragment only with *V.*

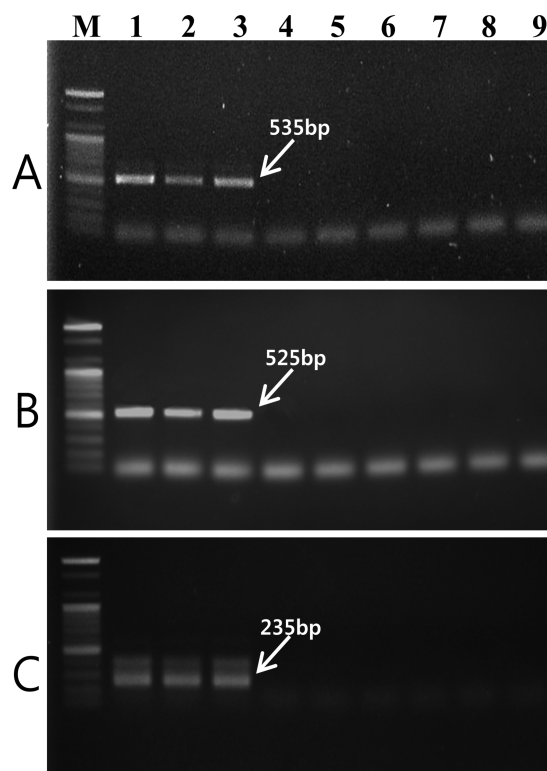


Fig. 2. Agarose gel of PCR product amplified with genomic DNA of *V. nashicola* (lanes 1–3), *V. pirina* (lane 4–6), and *V. inaequalis* (lanes 7–9) using (A) D12F/D12R, (B) E11F/E11R, and (C) Na11F/Na11R primer pairs. Lanes 1–3, 4–6, and 7–9 correspond to the strain numbers 1–3, 6–8, and 9–11, respectively, in Table 1. M, 100 bp DNA size marker (Bioneer).

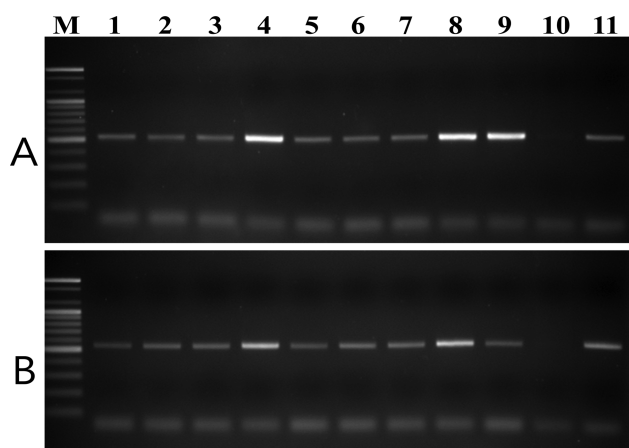


Fig. 3. PCR amplification of genomic DNA of *V. nashicola* strains isolated in Korea with primer pairs (A) D12F/D12R and (B) E11F/E11R. Lanes 1–9 correspond to *V. nashicola* strain numbers 12–20 listed in Table 1; lane 10, *V. pirina* MAFF 615009; lane 11, *V. nashicola* MAFF 615023; lane M, 100 bp DNA size marker (Bioneer).

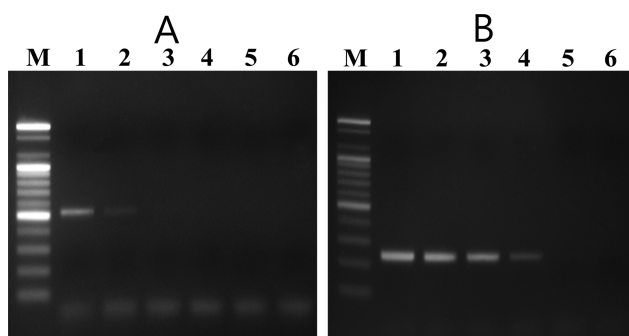


Fig. 4. Sensitivity of *V. nashicola* primer pairs. (A) Single step PCR amplification with external primer pair E11F/E11R and (B) subsequent nested PCR with internal primer pair Na11F/Na11R for detection of *V. nashicola* KCTC 6484 DNA. Lane M, 100 bp DNA size marker (Bioneer); lanes 1–6: 50 pg, 5 pg, 500 fg, 50 fg, 5 fg, and 500 ag of genomic DNA of *V. nashicola*.

nashicola DNA (Fig. 2C). No amplified product was generated from other *Venturia* species using this primer pair. Southern hybridization result also confirmed the specificity of this primer set to *V. nashicola* (Supplementary Fig. S1). When used as an internal primer pair for nested PCR, in combination with external primer pair E11F/E11R, Na11F/Na11R gave an amplification pattern identical to that of a single step PCR assay.

Sensitivity of nested PCR assay. In the nested PCR system, the external and internal primers amplified 525-bp and 235-bp DNA fragments, respectively, from all *V. nashicola* strains tested (data not shown). Sensitivity of the nested PCR system was determined by using different

amounts of *V. nashicola* KCTC 6484 DNA as a template. The first PCR with primer pair E11F/E11R detected the specific fragment in 5 pg of *V. nashicola* DNA. Nested PCR assay using 1 µl of the first PCR product as a template was 100 times more sensitive than the single round PCR and could detect the expected DNA fragment in as little as 50 fg of *V. nashicola* DNA (Fig. 4).

Discussion

V. nashicola, the causal agent of scab on Asian pear cultivars, is one of the most serious pear pathogens and is listed as a quarantine organism by many countries where Asian pear is poorly cultivated. It has been difficult to differentiate *V. nashicola* from *V. pirina*, the pathogen of European pear scab, as the two species are closely related. Recently, the phylogenetic relationship between *V. nashicola* and *V. pirina* was clarified on the basis of multiple nucleotide sequence analyses using ITS region of rDNA, β -tubuline and elongation factor 1 α genes (Beck et al., 2005; Zhao et al., 2012).

V. nashicola and *V. pirina* can be distinguished by morphological characterization of the size of their conidia and ascospores. Conidia of *V. nashicola* are shorter than those of *V. pirina*, which have longer and wider ascospores (Ishii and Yanase, 2000). However, morphological differentiation of these two species is tedious, time consuming, and not suitable in practice. Morphological and biochemical characterizations of either fungus require its isolation in culture. However, isolation of *Venturia* species on selective media are subject to limitations since these pathogens grow slowly and could be masked by overgrowth of other faster growing fungi. For identification of *V. nashicola*, therefore, rapid and specific detection methods that can be applied to environmental samples are required.

Polymerase chain reaction has provided a sensitive, rapid, and reliable method for the identification and detection of plant pathogenic fungi (Martin et al., 2000). The sequences of the ITS1 and ITS2 spacer regions of fungal rDNA are variable and have potential to distinguish fungi at the species level. A PCR-based method using ITS sequence for the detection of *V. nashicola* was described previously by Le Cam et al. (2001). They aligned the complete ITS1 nucleotide sequences of *V. nashicola*, *V. pirina* and *V. inaequalis* and designed PCR primers specific to *V. nashicola*. The forward primer A126 was designed from a polymorphic region between *V. nashicola* and *V. pirina*, whereas the reverse primer was the “universal” ITS4 oligonucleotide from 28S rDNA (White et al., 1990). However, *V. nashicola* and *V. pirina* show only two base substitutions in the primer A126 region. The primer pair A126/ITS4, therefore, has the potential to amplify false positive band

with DNA of *V. pirina* depending on the PCR conditions. We found that this PCR primer set produced a 391-bp band under recommended conditions with *V. pirina* when DNA hybridization was performed using the amplified fragment as a probe (data not shown).

In the present study, we designed two pairs of specific primers for *V. nashicola* using SCAR markers derived from RAPD analyses. As shown in Fig. 1, *V. nashicola* produced polymorphic and distinguishable RAPD band patterns comparable with its close relatives, *V. pirina* and *V. inaequalis*. RAPD markers were subsequently converted to SCAR markers by developing longer, and hence, more specific primers from RAPD sequences. After confirmation, the developed SCAR primer pairs, D12F/D12R and E11F/E11R, were validated with 76 additional Korean isolates. All Korean isolates tested produced expected fragments using both primer pairs.

Nested PCR was employed to increase the detection limit of the pathogen. In this study, we used the Na11F/Na11R pair as an internal primer of the second PCR. The specificity of this primer pair for *V. nashicola* was also confirmed by DNA hybridization as shown in Fig. 2C and supplementary Fig. S1. Therefore, three sets of PCR primers designed in this work can each be used as a primer for the first PCR to detect *V. nashicola*. Nested PCR employing *V. nashicola*-specific primers in both PCR rounds would satisfy the specificity and sensitive requirements for the monitoring applications. This approach shows high specificity and sensitivity for the detection of *V. nashicola* in a high background of other fungi and plant tissues.

In conclusion, the PCR primers designed in this work are sensitive and specific for the detection of *V. nashicola*. The detection method reported here could be used for the quarantine inspection of *V. nashicola* and the diagnosis of scab of Asian pears.

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References

- Asano, T., Senda, M., Suga, H. and Kageyama, K. 2010. Development of multiplex PCR to detect five *Pythium* species related to turfgrass disease. *J. Phytopathol.* 158:609–615.
- Beck, A., Ritschel, A., Schubert, K., Braun, U. and Triebel, D. 2005. Phylogenetic relationships of the anamorphic genus *Fusicladium* s. lat. as inferred by ITS nrDNA data. *Mycol. Prog.* 4:111–116.
- Chen, L. S., Chu, C., Liu, C. D., Chen, R.S. and Tsay, J. G. 2006. PCR-based detection and differentiation of anthracnose pathogens, *Colletotrichum gloeosporioides* and *C. truncatum*, from vegetable soybean in Taiwan. *J. Phytopathol.* 154:654–662.
- Chiocchetti, A., Sciaudone, L., Durando, F., Garibaldi, A. and Migheli, Q. 2001. PCR detection of *Fusarium oxysporum* f. sp. *basilici* on basil. *Plant Dis.* 85:607–611.
- Grosch, R., Schneider, J. H. M., Peth, A., Waschke, A., Franken, P., Kofoet, A. and Jabaji-Hare, S. H. 2006. Development of a specific PCR assay for the detection of *Rhizoctonia solani* AG 1-IB using SCAR primers. *J. Appl. Microbiol.* 102:806–819.
- Grote, D., Olmos, A., Kofoet, A., Tuset, J. J., Bertolini, E. and Cambra, M. 2002. Specific and sensitive detection of *Phytophthora nicotianae* by simple and nested-PCR. *Eur. J. Plant Pathol.* 108:197–207.
- Ishii, H. and Yanase, H. 2000. *Venturia nashicola*, the scab fungus of Japanese and Chinese pears: a species distinction from *V. pirina*. *Mycol. Res.* 104:755–759.
- Le Cam, B., Devaux, M. and Parisi, L. 2001. Specific polymerase chain reaction identification of *Venturia nashicola* using internally transcribed spacer region in the ribosomal DNA. *Phytopathology* 91:900–904.
- Li, H. B., Wu, X. Q., Peng, H. Z., Fu, L. Z., Wei, H. L., Wu, Q. Q., Jin, Q. Y. and Li, N. 2008. New available SCAR markers; potentially useful in distinguishing a commercial strain of the superior type from other strains of *Lentinula edodes* in China. *Appl. Microbiol. Biotechnol.* 81:303–309.
- López-Mondéjar, R., Beaulieu, R., Ros, M. and Pascual, A. 2012. SCAR-based real-time TaqMan PCR for early detection of *Fusarium oxysporum* in melon seedlings under greenhouse nursery conditions. *Crop Prot.* 33:1–6.
- Ma, Z. and Michailides, T. J. 2002. A PCR-based technique for identification of *Fusicoccum* sp. from pistachio and various other hosts in California. *Plant Dis.* 86:515–520.
- Martin, R. R., James, D. and Lévesque, C. A. 2000. Impacts of molecular diagnostic technologies on plant disease management. *Annu. Rev. Phytopathol.* 38:207–239.
- Ni, H. F., Yang, H. R., Chen, R. S., Hung, T. H. and Liou, R. F. 2012. A nested multiplex PCR for species-specific identification and detection of *Botryosphaeriaceae* species on mango. *Eur. J. Plant Pathol.* 133:819–828.
- Rigotti, S., Gindro, K., Richter, H. and Viret, O. 2002. Characterization of molecular markers for specific and sensitive detection of *Botrytis cinerea* Pers.: Fr in strawberry (*Fragaria × ananassa* Duch.) using PCR. *FEMS Microbiol. Lett.* 209:169–174.
- Rozen, S. and Skaletsky, H. J. 2000. Primer 3 on the WWW for general users and for biologist programmers, In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology*, ed. by S. Krawetz and S. Misener, pp. 365–386. Humana Press, Totowa, USA.
- Sambrook, J. and Russell, D. 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, New York, USA. 6.33–6.43 pp.
- Schnabel, G., Schnabel, E. L. and Jones, A. L. 1999. Characterization of ribosomal DNA from *Venturia inaequalis* and its phylogenetic relationship to rDNA from other tree-fruit *Venturia*

- species. *Phytopathology* 89:100–108.
- Shabi, E. 1990. Pear scab, In: *Compendium of Apple and Pear Diseases*, ed. by A. L. Jones and H. S. Aldwinckle, pp 22–23. APS Press, St. Paul, USA.
- Shabi, E., Roten, J. and Loebenstein, G. 1973. Physiological races of *Venturia pirina* on pear. *Phytopathology* 63:41–43.
- Tanaka, S. and Yamamoto, S. 1964. Studies on pear scab. II. Taxonomy of the causal fungus of Japanese pear scab. *Ann. Phytopathol. Soc. Jpn.* 29:128–136.
- Vandemark, G. J., Kraft, J. M., Larsen, R. C., Gritsenko, M. A. and Boge, W. L. 2000. A PCR-based assay by sequence-characterized DNA markers for the identification and detection of *Aphanomyces euteiches*. *Phytopathology* 90:1137–1144.
- White, T. J., Bruns, T., Lee, S. and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, In: *PCR Protocol: A Guide to Methods and Applications*, ed. by M. A. Innis, D. H. Gelfand, J. J. Sninsky and T. J. White, pp 315–322. Academic Press, New York, USA.
- Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A. and Tingey, S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucl. Acids Res.* 18:6531–6535.
- Zhao, P., Kakishima, M., Uzuhashi, S. and Ishii, H. 2012. Multi-gene phylogenetic analysis of inter- and intraspecific relationships in *Venturia nashicola* and *V. pirina*. *Eur. J. Plant Pathol.* 132:245–258.