Specific Polymerase Chain Reaction Identification of *Venturia nashicola* Using Internally Transcribed Spacer Region in the Ribosomal DNA

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ABSTRACT

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A technique based on the polymerase chain reaction (PCR) was developed for the identification of *Venturia nashicola* using nucleotide sequence information of the ribosomal DNA region. The complete internal transcribed spacer (ITS) region of *V. nashicola* strains and phylogenetically related species was amplified with the two universal ITS1 and ITS4 primers, sequenced, and digested with five restriction enzymes. The alignment of nucleotide sequences and analyses of digestion patterns

indicated constant polymorphisms between *V. nashicola* and related species at nucleotides 126 and 127, which overlapped a *TaqI* restriction site. An oligonucleotide primer named A126 was designed for identifying this variable region. A primer set (A126 and ITS4) that allowed the amplification of a 391-bp DNA fragment within the ITS region by PCR was specific to *V. nashicola* when it was checked against fungal genomic DNAs of related fungi. This primer set was a good candidate for a species-specific reagent in a procedure for identification of *V. nashicola* by PCR.

Additional keywords: Japanese pear, pear scab, *Pyrus*, *V. pirina*.

The fungus *Venturia nashicola* Tanaka and Yamamoto is the causal agent of scab on Japanese pear (*Pyrus pyrifolia* var. *culta*) and Chinese pear (*Pyrus ussuriensis*) and one of the most important diseases of Asian pears. Reliance is placed on fungicide use to control this serious disease because of the lack of scab-resistant commercial cultivars. Development of fungicide resistant strains has prompted plant pathologists and breeders to evaluate scab resistance in pear species and cultivars. Evidence that a source of resistance carried by cv. Kinchaku that could be used in breeding programs has been reported (8,9). Within strains isolated from Japanese pear, existence of three physiological races of *V. nashicola* on Japanese pear was demonstrated (9), and all these races were avirulent on cv. Kinchaku.

Pyrus pyrifolia var. *culta* is poorly cultivated in Europe where host *Pyrus communis* (European pear) is predominant. A distinction between oriental and occidental pears was clearly established by molecular data based on restriction fragment length polymorphism (RFLP) banding pattern obtained from chloroplast DNA (6). Scab that occurred on European pear is caused by *V. pirina*, which causes severe losses in some places.

Based on nucleotide sequence of rDNA, *V. nashicola* is very close to *V. pirina* and both are genetically distinct from the *Venturia* spp. found on other fruit trees (14). Morphological differences and physiological specialization were reported between these two species by different authors (7,15,18,19). However, their distinction based on morphological characteristics remains difficult (17). In 1999, Schnabel et al. (14) considered that the question of whether *V. nashicola* could be a race of *V. pirina* rather than a distinct species was still open. More recently, Ishii and Yanase (10) reexamined the relationship between these two putative fungal species and concluded that *V. nashicola* was a species

distinct from *V. pirina*. Their results based on pathogenicity tests and mating experiments, clearly demonstrated the existence of host specificity and sexual barrier.

V. nashicola is a quarantine organism in Europe. To date, no sensitive detection technique is available. Morphological differentiation between these two species based on conidia and ascospores size is possible (10) but not sufficient to make a clear identification in practice. Moreover, it needs, in any case, to cultivate and store reference strains that pose a biohazard risk in countries in which *V. nashicola* is a quarantine pathogen. To date, there is a lack of workable molecular data within this genus (14), but because it is now demonstrated that these two scab agents belong to two distinct species, we may hypothesize that DNA workable differences exist. In this context, a molecular approach seemed capable to distinguish them. Analysis of DNA sequences has brought significant advances in identification of species in general. Several approaches may be used. Most of them are based on nondefined target DNA sequences, which include analysis of RFLP and analysis of random amplified polymorphic DNAs that have been in use for a decade (22,24), and more recent techniques such as amplified fragment length polymorphism (20) and direct amplification of length polymorphism (1). Because of their ancestral trait, rDNA sequences have been commonly used in phylogenetic studies and, therefore, as targets to differentiate species by polymerase chain reaction (PCR)-based methods. Analysis of internal transcribed spacer (ITS) nucleotide sequences has solved classification problems at the intra-generic level for many phytopathogenic fungi such as *Pythium* spp. (21) or *Colletotrichum* spp. (3,16) and enables fungal detection based on species-specific and even race-specific PCR methods (2,5).

The aim of this study was to develop a PCR-specific assay for the identification of *V. nashicola*. A combination of PCR and RFLP analyses was carried out to characterize ITS regions of 25 isolates of *V. nashicola* and *V. pirina*. Nucleotide sequences of two strains representing each species were aligned in order to design specific primers, and experimental PCR conditions were defined to allow identification of *V. nashicola*.

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MATERIALS AND METHODS

Fungal strains. Strains of *V. nashicola* and *V. pirina* used in this study are listed in Table 1 and their characteristics and provenance are detailed. Strains of related *Venturia* spp. (anamorph *Spilocaea*) used to validate specificity of PCR primers were collected from fruit trees and ornamental hosts as listed in Table 1. Strains were stored continuously at 4°C on malt agar medium in the laboratory.

DNA isolation. A quick method was developed to obtain DNA from *Venturia* cultures suitable for use in PCR. Plugs of mycelium from a 3-week-old culture were placed on an autoclaved cellophane sheet first deposited on the surface of a malt agar (Merck, Darmstadt, Germany) petri dish. Ten milligrams of mycelium was collected with a scraper after 7 days of incubation at 17°C. DNA extraction was derived from the technique described by Goodwin and Lee (4). Each sample was placed in a 1.5-ml microcentrifuge

tube containing 100 µl of lysis buffer (50 mM Tris-HCl at pH 7.2, 50 mM EDTA, 3% sodium dodecyl sulfate, and 1% 2-mercaptoethanol) heated at 80°C. Tubes were then placed in a microwave oven. Samples were treated with three successive sequences (15 s, 10 s, 5 s) at 750 W. Three hundred microliters of lysis buffer was added to each tube. Tubes were incubated at 80°C for 10 min, extracted once with 400 µl of phenol/chloroform/isoamyl alcohol (25:24:1), and centrifuged at 14,000 × g for 15 min. The supernatant was transferred to a new microtube, and DNAs were precipitated in 1 volume of cold isopropanol with 10 µl of 5 M potassium acetate and centrifuged at 14,000 × g for 4 min. Pellets were washed with 200 µl of 70% ethanol, air dried, resuspended in 50 µl of distilled water, and stored at -20°C.

ITS rDNA amplification. ITS1-5.8S-ITS2 rDNA was amplified by PCR. The primer sequences were 5'-TCCGTAGGTGAAC-CTGCGG-3' (ITS1) from the 18S and 5'-TCCTCCGCTTATTG-ATATGC-3' (ITS4) from the 28S rDNA (23). PCR was performed

TABLE 1. Fungal strains used and analysed for primer specificity^a

Fungal strain	Isolation or reference (country and date)	Host plant	Polymerase chain reaction amplification with primers A126/ITS4
<i>Venturia pirina</i>			
P1	Haute Savoie, France - 1988	<i>Pyrus communis</i> cv. Conférence	—
P11 ^b	Beaucouze, France - 1991	<i>Pyrus communis</i> cv. Président Drouart	—
P13	Sarthe, France - 1986	<i>Pyrus communis</i> cv. Pierre Corneille	—
P20	Beaucouze, France - 1987	<i>Pyrus communis</i> cv. Duchesse de Mouchy	—
P30	Haute Savoie, France - 1987	<i>Pyrus communis</i> cv. Conférence	—
P52	Beaucouze, France - 1992	<i>Pyrus communis</i> cv. Williams	—
P55	Beaucouze, France - 1992	<i>Pyrus communis</i> cv. Williams	—
P22	Beaucouze, France - 1987	<i>Pyrus communis</i> cv. Louise Bonne	—
P64 ^b	The Netherlands - 1995	<i>Pyrus communis</i> cv. Doyenne	—
Vp1 ^c	Dossenheim, Germany - 1995	<i>Pyrus communis</i> cv. Vereinsdechant	—
Vp2 ^c	Dossenheim, Germany - 1995	<i>Pyrus communis</i> cv. Gute Luise	—
Vp3 ^c	Braunschweig, Germany - 1999	<i>Pyrus communis</i>	—
Vp4 ^c	Dossenheim, Germany - 1999	<i>Pyrus communis</i> cv. Williams Christ	—
<i>Venturia nashicola</i>			
P6	Balma, France - 1988	<i>Pyrus pyrifolia</i> var. <i>culta</i>	+
P8	Balma, France - 1988	<i>Pyrus pyrifolia</i> var. <i>culta</i>	+
P17 ^b	Lanxade, France - 1991	<i>Pyrus pyrifolia</i> var. <i>culta</i>	+
P49	Lanxade, France - 1991	<i>Pyrus pyrifolia</i> var. <i>culta</i>	+
P57	Clermont Ferrand, France - 1993	<i>Pyrus pyrifolia</i> var. <i>culta</i>	+
P60	Clermont Ferrand, France - 1993	<i>Pyrus pyrifolia</i> var. <i>culta</i>	+
P63 ^b	Bergerac, France - 1996	<i>Pyrus pyrifolia</i> var. <i>culta</i>	+
Mamenashi 12A1-3 ^{d,e} race 2	Tottori, Japan - 1991	Wild pear strain Mamenashi 12	+
Mamenashi 12B 1-1 ^e race 3	Tottori, Japan - 1991	Wild pear strain Mamenashi 12	+
Mamenashi 12B 1-2 ^e race 3	Tottori, Japan - 1991	Wild pear strain Mamenashi 12	+
JS 115 ^e race 1	Oita, Japan - 1980	<i>Pyrus pyrifolia</i> var. <i>culta</i> cv. Hakko	+
Yasato 2-1-1 ^e race 1	Ibaraki, Japan - 1992	<i>Pyrus pyrifolia</i> var. <i>culta</i> cv. Kosui	+
<i>Spilocaea eriobotryae</i>			
1389 ^f	Montpellier, France - 1998	<i>Eriobotrya japonica</i> (loquat)	—
<i>Venturia inaequalis</i>			
301 race 1	Ahrensburg, Germany - 1988	<i>Malus × domestica</i> 81/19-53 ^g	—
302 ^b race 6	Ahrensburg, Germany - 1988	<i>Malus × domestica</i> 81/11-22 ^h	—
V82 ^c	Colombia - 1997	<i>Malus × domestica</i> cv. Golden dorsett	—
V71 ^c	Turkey - 1996	<i>Malus × domestica</i>	—
1066 ⁱ race 7	Beaucouze, France - 1993	<i>Malus floribunda</i> 821	—
<i>Spilocaea pyracanthae</i>			
186	Ireland - 1987	<i>Pyracantha</i> sp.	—
38.2	Beaucouze, France - 1993	<i>Pyracantha</i> sp.	—
38.7	Beaucouze, France - 1993	<i>Pyracantha</i> sp.	—
V56 ^c	Dossenheim, Germany - 1995	<i>Pyracantha</i> sp.	—
V55 ^c	Dossenheim, Germany - 1995	<i>Pyracantha</i> sp.	—

^a Unless otherwise specified, the strains were from our laboratory.

^b Strains in which internal transcribed spacer (ITS) region was sequenced.

^c Strains were supplied by A. Kollar, Institute for Plant Protection in Fruit Crops, Dossenheim, Germany.

^d ITS sequence of strain was published by Schnabel et al. (14) (GenBank Accession No. AF065846) and was aligned in Figure 2.

^e Strains were supplied by H. Ishii, National Institute of Agro-Environmental Sciences, Ibaraki, Japan.

^f Strain 1389 was supplied by J. L. Notteghem, ENSA Montpellier, France.

^g Progeny (vf/vf) of a Prima (Vf/vf) × KI 40 (vf/vf) cross.

^h Progeny (Vf/vf) of a Prima (Vf/vf) × A143/24 (vf/vf) cross.

ⁱ Strain originating from the Fl 1 isolate described by Roberts and Crute (13).

in a 0.2-ml tube with a total volume of 50-μl reaction mixture containing 0.8 μM forward and reverse primer, 200 μM each dNTP, 1 unit of red Gold Star polymerase (Eurogentec SA, Herstal, Belgium), and 30 ng of genomic DNA in a 1× amplification buffer (75 mM Tris-HCl buffer at pH 9 supplemented with 20 mM (NH₄)₂SO₄, 0.01% (wt/vol) Tween 20, and 5 mM MgCl₂). PCR was carried out in a thermocycler (GeneAmp PCR System 9700; Applied Biosystems Inc., Foster City, CA) programmed for 30 cycles consisting of a denaturing step at 94°C for 30 s, an annealing step at 60°C for 30 s, and an extension step at 72°C for 45 s. These cycles were preceded by an initial denaturation step at 94°C for 5 min and ended with a final extension at 72°C for 7 min. The PCR products were analyzed by electrophoresis through 1.4% agarose gel in a 0.5× Tris-borate-EDTA buffer, stained with ethidium bromide, and visualized under UV light (300 nm).

DNA sequencing. ITS regions were sequenced for two strains of each species. PCR products were purified by a Qiagen Kit (Qiagen, Hilden, Germany) and sequenced automatically by an ABI 377 DNA sequencer with a *Taq* Dye Deoxy terminator

sequencing kit (Applied Biosystems). Primers ITS1 and ITS4 were used for sequencing reactions.

Restriction of amplified products. To assess sequence variability within and between species, amplified ITS products of *V. nashicola* and *V. pirina* isolates were digested with restriction endonucleases *TaqI*, *EcoRI*, *MspI*, *Tsp509I* (Eurogentec), and *RsaI* (New England Biolabs Inc., Beverly, MA). Reactions were carried out following the manufacturer's recommendations. Digestions were performed in a final volume of 20 μl with 12 or 15 μl of PCR products and 8 units of enzymes. Restriction fragments were separated on 3.2% Nusieve GTG agarose gels (FMC BioProducts, Rockland, ME) and visualized as previously described. A 100-bp molecular weight ladder (Eurogentec) was used as size standard.

Primer design and PCR conditions for specific identification of *V. nashicola*. In addition to DNA sequences obtained in this study, data on ITS1-5.8S-ITS2 sequences from other strains belonging to *Venturia* spp. were obtained from GenBank. It included data from two Japanese strains of *V. nashicola* (Mamenashi 12A1-1 and Mamenashi 12A1-3) and from four strains of *V. pirina* (MPS8

Fig. 1. Enzyme restriction digests of ribosomal DNA amplified with universal primers (internal transcribed spacer 1 [ITS1] and ITS4) from *Venturia pirina* (strains P11 and P64) and *V. nashicola* (strains P17 and P68) using A, *RsaI* and *TaqI*, and B, *MspI*. Lane M, 100-bp ladder.

	ITS1 →	MspI site (#45)	RsaI site (#69)
Vn P17	CTGGGTTAGCCTCCGGGCGCAACTCCACCCTTTGTAAACCGCGGCCCGGATTTCGGCGCCTGGCGGAGGGCACC		
Vn P63			
Vn M12A1-1			
Vn M12A1-3			
Vp P11		A.....	T.....T
Vp P64		C.....A.....	T.....T
Vp IL4		A.....	T.....T
Vp MPS8	N.....	A.....	T.....T
Vp IL11	G.....C.....		T.....T
Vp IL18	G.....C.....		T.....T
Vi 302	A.....T.....T.C.....C.....		AC.....T

	101	A126 primer	TaqI (#126)
Vn P17	TCGCGGGGGCCTCCGCCTGCCGGAATCAGCCAACCTGCGTGGAAATCGAAGTCTGAGAACGAGTTAAATCGAACAA		
Vn P63			
Vn M12A1-1			
Vn M12A1-3			
Vp P11		CT.....	C.....
Vp P64		CT.....	C.....
Vp IL4		CT.....	
Vp MPS8		C.....	
Vp IL11	A.....	CT.....	
Vp IL18	A.....	CT.....	
Vi 302	.T...A...G.C.....T.....		T.....A.....AA.....

Fig. 2. Alignment of the complete internal transcribed spacer 1 (ITS1) nucleotide sequences (5' to 3') for *Venturia nashicola* (Vn) strains P17, P63, M12A1-1, and M12A1-3; *V. pirina* (Vp) strains P11, P64, IL4, MPS8, IL11, and IL18; and *V. inaequalis* (Vi) strain 302. Underlined nucleotides identify the internal forward primer (A126) used to amplify specifically DNA from *V. nashicola* strains.

from the United States and IL4, IL11, and IL18 from Israel); their accession numbers are AF065845, AF065846, AF065840, and AF065842 to AF065844, respectively (14). All these sequences were aligned. Differences in nucleotides between these sequences and data obtained by PCR/RFLP were used to design PCR primers putatively specific to *V. nashicola*. PCR conditions used to specifically amplify *V. nashicola* were the same as those described previously.

RESULTS

ITS products PCR. A single amplification product of 501 to 502 bp was obtained for tested DNA samples originating from all of the strains listed in Table 1 that corresponded to the entire length of the ITS region (ITS1, 5.8S ribosomal gene, ITS2), plus a small portion of the small and large subunits of rRNA genes. Data obtained with nucleotide sequencing limited to ITS1, 5.8S, and ITS2 region showed polymorphism in length of amplicons, ranging from 461 bp (*V. nashicola*) to 462 bp (*V. pirina*). Analysis of additional sequences published by Schnabel et al. (14) confirmed this difference of at least one base between these two species.

PCR-RFLP on ITS region. Two enzymes of the five tested did not produce any polymorphism between species: the six-base restriction enzyme *EcoRI* cut DNA amplicons at one site and the four-base restriction enzyme *Tsp509I* had two restriction sites. *RsaI*, *TaqI*, and *MspI* enzymes provided different patterns between the two species (Fig. 1). The four-base restriction enzyme *RsaI* had one restriction site in the ITS region of *V. pirina* but none in that of *V. nashicola*. Analysis of *TaqI* restriction patterns indicated the occurrence of four sites in both species but differences in band size were observed. Five *MspI* sites were observed in the ITS region of *V. pirina* and six *MspI* sites in that of *V. nashicola*. No polymorphism was observed within each fungal species using these five restriction enzymes, except one strain of *V. pirina* (Vp3) that had three sites for the restriction with *RsaI* instead of two.

DNA sequencing. Sequencing of ITS regions of two strains of each species was carried out to confirm the lack or the presence of restriction sites revealed by RFLP. Sequence alignment of additional strains of *V. nashicola* and *V. pirina* with diverse geographical origins displayed some mutations within species. Therefore, *RsaI* restriction site 69, which distinguished *V. nashicola* from *V. pirina* strains according to RFLP data, appeared variable. At this restriction site, no difference was observed between two *V. pirina* strains originating from Israel (IL11 and IL18) and all *V. nashicola* strains. Moreover, these two *V. pirina* strains and strains

of *V. nashicola* had an *MspI* restriction site at nucleotide 45, whereas other strains of *V. pirina* did not. Alignment of all sequences confirmed a polymorphic region (nucleotides 126 and 127) between *V. nashicola* and *V. pirina* (Fig. 2). This region overlapped a *TaqI* site and corresponded to one or two nucleotides that mismatched, depending on *V. pirina* strains.

A primer, named A126, was designed, including the mismatches at the 3' end (5'-ACCCTGCCTGGAAAATC-3'), to amplify only the strains of *V. nashicola*, whereas the second primer was the "universal" ITS4 oligonucleotide. The expected size of the PCR product was 391 bp. Three other regions specific to *V. nashicola* were pointed out: two transition mismatches at nucleotides 78 (Fig. 2) and 327, and a deletion of one base at nucleotide 451 observed in all *V. nashicola* (data not shown). No primer was designed in these regions because the differences were too slight and there was a lack of data on variability of these mismatches in other strains (Table 1).

Conditions for specific amplification. Validity of primer specificity was tested with genomic DNA extracted from all strains listed in Table 1. Standard conditions described for PCR of ITS region was not modified for this specific amplification, except annealing temperature was increased to 62°C. All *V. nashicola* strains provided a single 391-bp amplicon. DNA extracted from *V. inaequalis*, *Spilocaea pyracanthae*, and *S. eriobotryae* do not amplify using these primers (Fig. 3); ITS rDNA amplification of these strains constituted a positive control.

DISCUSSION

The aim of this study was to characterize the ITS region of *V. nashicola* in order to provide tools for identification of strains belonging to this species. Our first approach, a restriction study carried out on ITS region, clearly identified three enzymes (*TaqI*, *RsaI*, and *MspI*) that enabled differentiation of *V. nashicola* from *V. pirina*. Very low intraspecific polymorphism was detected with these enzymes.

Sequence data on these strains confirmed the mismatches between the two species. Alignment of additional sequences available in databanks indicated that at least one mutation at nucleotide 126 or 127, which overlapped a *TaqI* restriction site, was always present in *V. nashicola* strains. On the other hand, sequence alignment showed that other mutation points previously visualized by RFLP using *RsaI* and *MspI* enzymes presented intraspecific polymorphism and could not be considered good candidate sites to distinguish *Venturia* spp. The PCR primers we designed with these data were successfully used to identify *V. nashicola*

Fig. 3. Polymerase chain reaction amplifications of *Venturia nashicola* strains (P6 to Y 2.1.1) with specific primers set (A126, 5'-ACCCTGCCTGGAAAATC-3'; internal transcribed spacer 4 [ITS4], 5'-TCCTCCGCTTATTGATATGC-3') provided an expected DNA fragment of 391 bp. This primer set does not allow any amplification with DNA from *V. pirina* or with other *Venturia* and *Spilocaea* pathogens. Lane M, 100-bp ladder.

regardless of the geographic origin (Japan and France). The specificity of these primers was checked by the absence of cross reactivity with DNA from different species of *Venturia* isolated from European pear and *Malus* $\times$ *domestica*, which is genetically close to *V. nashicola* (14), and also from species less close to *Pyrus* such as *Pyracantha* and *Eriobotrya japonica*.

We also showed in this study that of the 10 DNA sequences aligned, the size of ITS region from *V. pirina* was always longer by one or two bases than that of *V. nashicola*. This characteristic and the existence of mismatches between the two species could be visualized by single-strand conformation polymorphism studies (SSCP). Kumeda and Asao (12) differentiated species of *Aspergillus* section *Flavi* using this technique on the ITS region that differed in a range of only 20 bases. SSCP is a powerful tool, nevertheless, it appears much more difficult to develop for a strategy of routine detection than a molecular identification with species-specific primers.

The proposed PCR identification protocol is rapid, and the results are unambiguous in regard to what is particularly useful to countries where *V. nashicola* is a quarantine pathogen. However, a step of fungal culture remains necessary. In order to use these specific primers in the detection of this organism, further development is needed to detect the fungus from infected host. Protocols for direct DNA extraction from leaves are established for many fungi including *V. inaequalis* (11). They must be optimized to set up a procedure in a certification scheme for *Pyrus*.

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