Characterization of Ribosomal DNA from *Venturia inaequalis* and Its Phylogenetic Relationship to rDNA from Other Tree-Fruit *Venturia* Species

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ABSTRACT

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A portion of the 18S ribosomal DNA (rDNA) gene, the internal transcribed spacers (ITS1 and ITS2), and the 5.8S rDNA gene were polymerase chain reaction-amplified from strains and field populations of *Venturia inaequalis* and assessed for genetic variation. A previously reported optional group I intron in the 18S rDNA gene of *V. inaequalis* was detected in 75.0% of 92 strains collected worldwide and in 61.1 and 71.2% of 54 and 59 strains from two Michigan orchards, respectively. Sequence and restriction analysis of rDNA revealed four intron alleles, three of which were present both in worldwide strains and in each field

population. Two ITS1 alleles were detected and found to be linked to specific intron alleles. The ITS1-5.8S-ITS2 sequences from *V. asperata*, *V. carpophila*, *V. cerasi*, *V. inaequalis*, *V. nashicola*, *V. pyrina*, and *Cladosporium caryigenum* were compared using phylogenetic analysis. Strains of the *Venturia* species were placed in three distinct monophyletic groups in a phylogenetic tree. The first group comprised *V. inaequalis*; the second, *V. pyrina* and *V. nashicola*; and the third, *V. cerasi*, *V. carpophila*, and *V. asperata*. The described intron and ITS1 alleles in *V. inaequalis* provide genetic markers for subdividing populations of *V. inaequalis*, and the ITS1-5.8S-ITS2 sequences are valuable in determining the relationship of the species from tree-fruit crops with other *Venturia* species.

Additional keywords: apple scab, *Carya*, coevolution, *Malus*, *Prunus*, *Pyrus*, *Venturiaceae*.

Venturia inaequalis (Cooke) G. Wint., the cause of apple scab, is a common and economically important pathogen of apples and many ornamental *Malus* species. Reliance on fungicides and the use of scab-resistant cultivars are the main strategies used for apple scab control (5,16). These strategies have resulted in difficulties with fungicide-resistant strains and with new resistance-breaking phenotypes. In response to the increase in scab control problems, scientists have determined the genetic basis for resistance to several fungicides (15) and have characterized new races (22), which has led to a better understanding of the resistance mechanism and to the development of antiresistance control strategies. Although pathogen variation leading to difficulties in control have been well characterized, very little is known about genetic variation in regions of the pathogen's genome that are not under direct selection pressure from fungicides or from host resistance genes.

The genus *Venturia* consists mostly of plant-pathogenic species; many attack the fruit and foliage of important tree-fruit crops. Members of the genus have single-septate ascospores produced in ascocarps (pseudothecia) with bitunicate asci. Sivansen (25) lists 52 species in the genus *Venturia*. The associated anamorphs are *Spilocaea*, *Fusicladium*, and *Cladosporium*, with *Fusicladium* being the most common. Separation of the genus is based on morphological criteria, and identification is aided by the narrow host specificity exhibited by each species. There have been no comparative molecular studies of the species within the genus.

In eukaryotes, the ribosomal DNA (rDNA) found in the nuclear genome in general consists of tandem repeats of three RNA genes that are transcribed as a single unit and code for the 18S, 5.8S, and

28S RNA (Fig. 1). However, in *Pythium pachycaule*, the 5.8S region also exists within the adjacent nontranscribed spacer of the rDNA repeat (2). The 18S and 28S rRNA genes are also known as nuclear small and large subunit rDNA sequences, respectively. Two internal transcribed spacers (ITS1 and ITS2) separate the three RNA genes (11). Analysis of DNA sequences from rRNA genes and the ITS region have been used in studies of phylogenetic relationships and evolution of a wide range of organisms (3). The coding rDNA sequences of the nuclear rRNA repeat units evolve relatively slowly and are useful for studying distantly related organisms, whereas the noncoding rDNA ITS sequences evolve faster than the coding sequences and may vary among species and populations (17). Besides sequence divergence in ITS sequences, polymerase chain reaction (PCR) amplification length polymorphism in nuclear rDNA due to intron insertion has also been used to assess the extent of genetic variability within populations (1,12). A previous analysis of genetic diversity within and among Swiss populations of *V. inaequalis* identified five genotypes based on restriction site polymorphism in a group I intron located in the 18S rRNA gene. The intron alleles were then used as a neutral marker for estimating the migration rate of a new race of *V. inaequalis* capable of infecting scab-resistant cultivars carrying the *Vf* gene (30).

In our study, we assessed the genetic variation in nuclear rDNA in order to examine the structure of a geographically diverse group of strains and of two local populations of *V. inaequalis*. Available ITS primers were used to PCR-amplify a portion of the 18S rRNA gene, the ITS1 and ITS2 regions, and the entire 5.8S rRNA gene of *V. inaequalis*. Variation within the 18S rDNA and ITS1 region was assessed by sequence analysis and by digesting the amplified rDNA with restriction enzymes selected to give allele-specific restriction fragment patterns. ITS1-5.8S-ITS2 sequence information from *V. inaequalis* and from other *Venturia* and a *Cladosporium* species was then used to examine phylogenetic relationships within the species of scab fungi that occur on tree-fruit crops.

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MATERIALS AND METHODS

Fungal strains and ascospore progeny. Most of the strains of *V. inaequalis* were taken from a collection of strains acquired previously from colleagues worldwide in the 1980s (15,26) and stored continuously at 4°C under mineral oil at Michigan State University (Table 1). Additional strains of *V. inaequalis* were isolated from two Michigan orchards. Strains of other *Venturia* and *Cladosporium* species from deciduous tree-fruit and nut crops were supplied by colleagues in Japan, Israel, and the United States as listed in Table 1, but some *V. pyrina* and all *V. carpophila* strains we collected in 1997. Two species, *V. asperata* and *V. cerasi*, were acquired from the American Type Culture Collection. Ascospore progeny of *V. inaequalis* from selected sexual crosses made and characterized previously for benomyl phenotype by Stanis and Jones (26) were also taken from stocks stored under mineral oil. All strains and progeny were maintained on potato dextrose agar (Difco Laboratories, Detroit).

DNA isolation. A 6-mm-diameter agar plug taken from the center of 4- to 8-week-old cultures was placed in a 1.5-ml microcentrifuge tube with 180 µl of tissue lysis buffer (Qiagen, Santa Clarita, CA) and 20 µg of proteinase K (Qiagen). The mycelium agar plug was ground into small fragments with a plastic pestle, and the fragments were allowed to incubate in the buffer for 6 to 12 h at 55°C. DNA isolation was performed according to the Qiagen Tissue Kit protocol (Qiagen). Basically, the DNA was bound onto a silica membrane, washed with a buffer, and then eluted from the membrane. The eluted DNA was stored at -20°C in elution buffer (Qiagen).

PCR amplification and digestion of rDNA. Primers ITS1-F (5'-CTTGGTCATTAGAGGAAGTAA-3') or ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') or ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used to amplify DNA from the 3' end of the 18S rRNA gene to the 5' end of the 5.8S gene or to the 5' end of the 28S rRNA gene (Fig. 1) (8,31). The primers were synthesized with an automatic DNA synthesizer (model 380B; Applied Biosystems, Inc., Foster City, CA) at the Macromolecular Structure Facility, Department of Biochemistry, Michigan State University, East Lansing. PCR reactions were performed in a total volume of 50 µl containing 1 µl (20 to 60 ng) of template DNA; 1 µM each primer; 200 µM each dNTP; 1.25 U of *Taq* DNA polymerase (Gibco BRL, Gaithersburg, MD); 20 mM Tris-HCl, pH 8.4; 50 mM KCl; and 1.5 mM MgCl₂. Dimethyl sulfoxide at a final concentration of 5% was routinely added to PCR reactions performed in the later part of the study. Mineral oil was added to prevent evaporation. Cycling parameters were 94°C for 2 min followed by 30 cycles of 94°C for 30 s, 54°C for 1 min, and 72°C for 1 min. Amplifications were performed in a model PTC-150-16 minicycler (MJ Research, Inc., Watertown, MA). Amplification products were analyzed by electrophoresis through

1.5% agarose in 0.5× Tris-borate-EDTA buffer (19). Restriction enzyme digestions were performed in a total volume of 20 µl with 12 µl of PCR product (224 to 336 ng/µl) and 5 U of restriction enzyme in buffer supplied by the manufacturers. Reactions with *Cfo*I (Promega Corp., Madison, WI), *Sal*I (Gibco BRL), and *Bsp*1286I (New England Biolabs, Inc., Beverly, MA) were performed at 37°C for 1 to 3 h, and reactions with *Bst*UI (New England Biolabs, Inc.) were performed at 60°C for 1 h. Digested DNA was electrophoresed in 0.9% polyacrylamide gels run in 1× Tris-borate-EDTA buffer. Ethidium bromide-stained gels were visualized with UV light.

DNA sequencing. PCR products were purified using the Wizard PCR Preps DNA Purification System (Promega Corp.) and then partially sequenced using primers ITS1-F, ITS1, ITS2, ITS4, AJ197 (5'-GGGATGGTAACAACGGGCTCGAC-3'), and AJ196 (5'-GT-CGAGCCCCGTTGTTACCATCCC-3') at the Plant Research Laboratory Sequencing Facility, Michigan State University, East Lansing.

DNA hybridization. A 111-bp digoxigenin (DIG)-labeled DNA fragment was generated by PCR with a pair of intron-specific primers, AJ167 (5'-CGCGACTGGAAATAATGGTTG-3') and AJ166 (5'-GTCCGGCCTGGTTTGGTA-3'), and used as a probe to detect intron DNA. The PCR reaction (100 µl) consisted of 2 µl (40 to 120 ng) of template DNA from *V. inaequalis* strain 685BF; 1 µM each primer; 200 µM dATP; 200 µM dCTP; 200 µM dGTP; 190 µM dTTP; 10 µM DIG-11-dUTP (Boehringer GmbH, Mannheim, Germany); 1 U of *Taq* DNA polymerase (Gibco BRL); 20 mM Tris-HCl, pH 8.4; 50 mM KCl; and 1.5 mM MgCl₂. Mineral oil was added to prevent evaporation. Cycling parameters were 94°C for 2 min followed by 30 cycles of 94°C for 30 s, 58°C for 1 min, and 72°C for 1 min. PCR products (1 µl; 20 to 30 ng of DNA) from each strain of *V. inaequalis* amplified with primers ITS1-F and ITS2 were spotted on positively charged nylon membranes (Boehringer GmbH), soaked for 2 min twice in 1.5 M NaCl-0.5 M NaOH solution and twice in 1.5 M NaCl-0.5 M Tris-HCl solution. After air-drying, the membranes were cross-linked in a Stratalinker (Stratagene Inc., La Jolla, CA) and incubated in prehybridization solution (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 1% blocking reagent [Boehringer GmbH], 0.1% *N*-lauroylsarcosine, and 0.02% sodium dodecyl sulfate [SDS]) for 1 h at 68°C. Heat-denatured DIG-labeled probe (20 µl; 400 to 600 ng/µl) was added to the prehybridization solution and incubation continued overnight at 68°C. The membrane was then washed twice in 2× SSC-0.1% SDS for 5 min at room temperature followed by two washes in 0.1× SSC-0.1% SDS for 15 min at 68°C. The membranes were rinsed in 0.1 M maleic acid (pH 7.5), 0.15 M NaCl, and 0.3% Tween-20; soaked in 0.1 M maleic acid (pH 7.5), 0.15 M NaCl, and 1% blocking reagent for 30 min at room temperature; and after adding anti-DIG-alkaline phosphatase fragments (0.05 µl/ml; Boehringer GmbH), incubated for another 30 min at room temperature. The membranes were washed twice in 0.1 M maleic acid

Fig. 1. Map of a portion of the ribosomal DNA (rDNA) repeat from *Venturia inaequalis* showing the location of primers used to amplify and sequence rDNA fragments. The expanded map in 5' to 3' orientation shows the region amplified with primers ITS1-F and ITS2 and the nucleotide location for variable and nonvariable restriction sites C, S, and B of enzymes *Cfo*I, *Sal*I, and *Bst*UI, respectively. The intron sequence extends from nucleotide 38 to nucleotide 425 and is not designated specifically in the expanded map.

(pH 7.5) and 0.15 M NaCl₂ for 15 min at room temperature, soaked in CDP-Star buffer (0.1 M Tris-HCl and 0.1 M NaCl, pH 9.5; Boehringer GmbH) in 0.25 mM CDP Star reagent (Boehringer GmbH), and exposed to X-ray film.

Computer analysis. Nucleotide sequences from PCR-amplified fragments were assembled in SeqMan version 3.57 (DNASTAR Inc., Nevada City, CA) and the consensus sequence reconfirmed by comparing it with the original sequencing data output. Consensus sequences were aligned and sequence similarities determined with MegAlign (DNASTAR Inc.). Nucleotide substitutions within the ITS1-5.8S-ITS2 region (amplified fragments of the 18S and 28S coding regions were edited out) were analyzed using unweighted parsimony analysis as implemented in PAUP 3.1.1 (Illinois Natural History Survey, Champaign, IL). A branch-and-bound search was used. Bootstrap analysis was conducted with 1,000 replications (6). Branch lengths were determined using accelerated transformation (ACCTRAN). Insertions, deletions, and unidentified nucleotides (N) were treated as missing information. rDNA sequences obtained from *Monilinia fructicola* were used initially as the outgroup for the phylogenetic analysis, but it introduced much homoplasy due

to the high level of ITS sequence divergence between *M. fructicola* and the ingroup species. Since this analysis revealed that *C. caryigenum* was a sister group to the remaining ingroup *Venturia* species, *C. caryigenum* was then used as a functional group for phylogenetic reconstruction (18).

Nucleotide sequence accession numbers. Sequences of the group I intron of *V. inaequalis* strains 5BF, 75-26, 885F, 75-150, 685BF, and 890F were assigned GenBank accession numbers AF065827 to AF065832, respectively. The GenBank accession number for the sequence of the 660-bp product amplified with primers ITS1-F and ITS2 from *V. inaequalis* strain 685BF is AF065831. The accession number for the introns of *C. caryigenum* strains CJ, FRD, and LCF are AF065833 to AF065835, respectively. The ITS1-5.8S-ITS2 sequences for *V. inaequalis* strains 890F, 685BF, 5BF, and 826BF; for *V. pyrina* strains MPS8, Akita FB-1, IL-4, IL-4, IL11, and IL18; for *V. nashicola* strains Mamenashi 12A1-1 and Mamenashi 12A1-3; for *V. cerasi* strain ATCC 12119; for *V. asperata* strain ATCC 34052; for *V. carpophila* strain BS5; and for *C. caryigenum* strains JD and LCF were assigned accession numbers AF065836 to AF065851, respectively.

TABLE 1. Strains of *Venturia* species studied and their relevant characteristics

Species Strain grouping Strain	Intron ^a	Strain (no.)	Origin; source or reference ^b
<i>Venturia inaequalis</i>			
Worldwide collection			
WB, GAVIN 43, CR2C	—	3	Michigan; (15,26)
MANITOU, FS6C-12, RH-4, RH-1-11B, SH1-9B, SPINKS 79, NU-26 DPX-5, PAJ-13C-P3, SH1-2B, SH2-10B, SH2-9B, VAN DRESSEN 1, VAN DRESSEN 2, VAN DUSER 3, WM, WL, WRR, FP-TC 139-4, FS6C-12, FS-17C P3, AB-2C-B1, BIGLER, KV3C, MSU-3, MSU-12, MSU-20, MSU-33, MSU-40, PAJ-13C-P3	+	29	Michigan; (15,26)
OLD HICKORY 6-3, MINN 5-3B, MINN, 6-1B, MINN 5-4B	—	4	Minnesota; (26)
LAUTZ 10-5, MINN 3, MINN 5C, MINN 8-2B, MIN FRUIT ACRES 7, MINN LAUTZ 4	+	6	Minnesota; (26)
#42 MINNS 118, #6 MITCHELL 94-1, 1771-2 ^c	—	3	New York; HA, (15,26)
#32 MINNS 121, #60 MINNS 86-8, VERDINE 2, #74 PRESTON 7, 1805-2, 0356-7, 1797-9, 1810-1 ^c	+	8	New York; HA, (15,26)
V-565	—	1	Maine; (26)
MAINE 6, MAINE 8, SIS-16, V-558, V-560	+	5	Maine; (26)
N.C. STATE 1 298	+	1	North Carolina; (26)
PURDUE 1820-5	+	1	Indiana; (26)
IL-23, IL-24, IL-25, IL-26	+	4	Israel; (15)
R I, R II	+	2	Australia; (15,26)
75-71	—	1	New Zealand; (26)
75-26, 75-78, 75-150	+	3	New Zealand; (15)
CHILE 24B	—	1	Chile; (26)
B2, B3, B4, B5, B6, B7, B8, B9, W10, W11	—	10	Germany; (26,27)
GERMANY G3-2, W12	+	2	Germany; (26,27)
I1-1, I4-1, I5-1, I6-1, I7-1	+	5	Italy; (26)
TUM2, TUFR1, UZ4	+	3	France; (15)
1996 collection from two orchards ^d			
East Lansing strains	—	17	Michigan; this study
East Lansing strains	+	42	Michigan; this study
Fennville strains	—	21	Michigan; this study
Fennville strains	+	33	Michigan; this study
<i>Venturia asperata</i> ATCC 34052	—	1	New Zealand; apple (23)
<i>Venturia cerasi</i> ATCC 12119	—	1	Germany; sour cherry
<i>Venturia nashicola</i>			
JS-115, Yasato 2-1-1, Mamenashi 12A1-1, Mamenashi 12A1-3, Mamenashi 12B1-1, Mamenashi 12B1-2	—	6	Japan; pear, HI
<i>Venturia pyrina</i>			
MPS1, MPS2, MPS3, MPS4, MPS5, MPS6, MPS7, MPS8, MPS9, MPS10, MPS11, MPS12, MPS13	—	13	Michigan; pear, this study
IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-11, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18	—	15	Israel; pear, ES
Akita FB-1, Akita SK-1	—	2	Japan; pear, HI
<i>Venturia carpophila</i>			
BS1, BS2, BS3, BS4, BS5, BS6	—	6	Michigan; peach, this study
<i>Cladosporium caryigenum</i>			
JD, SFV, WD	—	3	Georgia; pecan, KS
TC, LEE-P, PWD, FRD	+	4	Georgia; pecan, KS
GCS	+	1	Alabama; pecan, KS
LCF	+	1	Louisiana; pecan, KS
CJ	+	1	Georgia; pecan, KS

^a + = Intron-containing strains; — = intron-lacking strains.

^b ES = E. Shabi, Agricultural Research Organization, the Volcani Center, Bet Dagan, Israel; HA = H. Aldwinckle, Cornell University, Geneva, NY; HI = H. Ishii, National Institute of Agro-Environmental Sciences, Ibaraki, Japan; and KS = K. L. Stevenson, University of Georgia, Athens.

^c Strains 1805-2, 0356-7, 1771-2, 1797-9, and 1810-1 were supplied by H. Aldwinckle, Cornell University, Geneva, NY, as race 1 to 5 strains, respectively.

^d Each strain is a monoconidial isolate, collected from a different apple scab lesion.

RESULTS

Length polymorphism within the 18S rRNA gene of *V. inaequalis* and its relatives. The PCR product amplified with primers ITS1-F and ITS2 showed length variation among the strains of *V. inaequalis* and *C. caryigenum*, but not among the strains of *V. asperata*, *V. cerasi*, *V. nashicola*, *V. pyrina*, and *V. carpophila* (Fig. 2, Table 1). Of the 92 strains in the worldwide collection, 25% yielded an estimated 270-bp product and 75% yielded an estimated 660-bp product (Table 1). The 660-bp product was also detected in 61.1 and 71.2% of the strains isolated in 1996 from apple orchards near Fennville and East Lansing, MI, respectively. Sequence anal-

Fig. 2. Variation in the size of ribosomal DNA fragments polymerase chain reaction-amplified from *Venturia* and *Cladosporium* species using primers ITS1-F and ITS2. Lane M, 100-bp ladder; lanes 1 and 2, *V. inaequalis* strains 965F and 247BF; lanes 3 and 4, *C. caryigenum* strains JD and FRD; and lanes 5 to 9, *V. asperata* strain ATCC 34052, *V. cerasi* strain ATCC 12119, *V. nashicola* strain Mamenashi 12B1-2, *V. pyrina* strain IL-7, and *V. carpophila* strain BS6, respectively.

Fig. 3. *SalI* and *CfoI* restriction digests of ribosomal DNA amplified from *Venturia inaequalis* with primers ITS1-F and ITS2. Lane 1, *SalI* digest of DNA from German strain G3-2; and lanes 2 to 7, *CfoI* digest of DNA from German strains W12 and G3-2, Michigan strains 1001F and 981F, Israeli strain IL-23, and Italian strain I1-1, respectively. Lanes M contain 100-bp ladder.

ysis of the estimated 660-bp amplification product from six *V. inaequalis* strains revealed the presence of a 388-bp intron in the 18S rRNA gene of each strain located at a site corresponding to position 1,506 of the rDNA sequence of *Escherichia coli* (GenBank accession numbers AF065827 to AF065832, respectively) (Fig. 1) (9). That length variation in PCR-amplified fragments was due to the presence of the intron was confirmed with an intron-specific probe. The probe hybridized to DNA amplified from all strains of *V. inaequalis* with the 660-bp product, but not to DNA amplified from any strain with the 270-bp product (data not shown). Strains containing DNA that hybridized with the probe were designated as intron⁺ strains, the remaining strains were designated as intron⁻ strains. Furthermore, no nucleotide sequences associated with the intron were detected when the 270-bp amplification product from strain 826BF was sequenced (data not shown).

Among 10 strains of *C. caryigenum* collected from three neighboring states in the southern United States, seven exhibited an amplification product of about 630 bp and three a product of about 270 bp (Table 1). Sequence analysis of the 630-bp product from *C. caryigenum* strains CJ, FRD, and LCF revealed the presence of a 360-bp intron (GenBank accession numbers AF065833 to AF065835, respectively) inserted at the same position in the 18S rRNA gene as the intron for *V. inaequalis*. The intron from *C. caryigenum* showed 86% similarity with the intron from *V. inaequalis*.

Intron and ITS1 alleles in *V. inaequalis*. DNA amplified from *V. inaequalis* with primers ITS1-F and ITS2 was digested with the restriction enzymes *SalI*, *CfoI*, and *BstUI*. Digestion with *SalI* cut the PCR product amplified from intron⁺ strains into two fragments of 191 and 469 bp or it failed to cut the DNA (Fig. 3). Digestion with *CfoI* cut the PCR product into three fragments: a variable 319- or 254-bp fragment and two additional fragments of 150 and 144 bp (Fig. 3). *BstUI* digested the PCR product into either three or four clearly visible fragments, not counting fragments smaller than 80 bp (Fig. 4). The three fragments were 284, 125, and either

Fig. 4. *BstUI* restriction digests of ribosomal DNA amplified from *Venturia inaequalis* with primers ITS1-F and ITS2. Lane M, 100-bp ladder; and lanes 1 to 3, strains 1164F, IL-24, and 1370F, respectively. The joint 125-bp fragment found for strains IL-24 and 1370F indicates the presence of a variable *BstUI* restriction site within the ITS1 region.

102 or 86 bp. The four fragments were 284, 165, 109, and 102 bp. The nucleotide location for each restriction site including variable sites was determined by sequence analysis (Fig. 1). A variable *Bst*UI site was located in the ITS1 region. The resulting α and β ITS1 alleles were identified based on the absence (allele α) or presence (allele β) of a 125-bp *Bst*UI fragment (Fig. 4). Intron⁺ strains lacking the *Bst*UI variable site always revealed a 165-bp fragment. None of the intron⁻ strains contained the variable *Bst*UI restriction site; *Bst*UI cut the 270-bp product amplified from 17 intron⁻ strains into two fragments of 165 and 105 bp (data not shown). An interesting pattern of intron and ITS alleles emerged when the restriction digest data for two Michigan field populations and for several foreign strains were analyzed (Table 2). Four intron alleles were identified based on variations in *Cfo*I, *Sal*I, and *Bst*UI restriction patterns. Allele I consisted of intron⁻ strains, while alleles II, III, and IV consisted of intron⁺ strains distinguished based on their respective restriction patterns (Table 2). Alleles I, II, and IV were common in all three population groups, while allele III was only detected in the group of foreign strains. Strains with the α allele exhibited intron alleles I and II; those with the β allele exhibited intron alleles III and IV (Table 2, Fig. 4). When one strain of each of five races were examined, race 2, 4, and 5 strains belonged to intron allele II; race 1 strain, to intron allele IV; and the race 3 strain, to intron allele I (data not shown).

Inheritance of the intron. Progeny from crosses between intron⁺ and intron⁻ strains showed a 1:1 segregation of the intron, and segregation of the intron did not depend on segregation of the allele for benomyl resistance (Table 3). The intron was detected in all progeny from a cross between two intron⁺ strains and was absent in progeny from a cross between two intron⁻ strains.

Nucleotide variation in the ITS1-5.8S-ITS2 region of *V. inaequalis*. In addition to sequence variation in the intron, there was sequence variation in the ITS1-5.8S-ITS2 region of *V. inaequalis*. Variation took the form of single base substitutions or single base insertions/deletions. Strains 5BF and 826F exhibited a nucleotide

base insertion (G) between positions 65 and 66 (counted from the 5' end of ITS1), increasing the length of the ITS1 region from 154 to 155 bp (Fig. 5). In addition to an insertion, there were base substitutions at positions 79 and 83, resulting in the variable ITS1 *Bst*UI site described above (Figs. 1 and 6). Both strains also contained two base substitutions at positions 434 and 450 in the ITS2 region (Fig. 5). A single substitution was found at position 300 in the 5.8S rRNA gene of strain 826BF, but this substitution was not verified by sequencing a second amplification product. In all strains, the 5.8S rRNA gene and the ITS2 region were 159 and 149 bp, respectively.

Interspecific variation in the ITS1-5.8S-ITS2 region. The ITS1-5.8S-ITS2 nucleotide sequences for six *Venturia* species were aligned with corresponding sequences for *C. caryigenum* strains JD and LCF (Fig. 5). The nucleotide length of the ITS1 region varied from 152 bp for *C. caryigenum* strain JD to 157 bp for *V. cerasi*. The 5.8S DNA in all strains and species was 159 bp long. The ITS2 region varied in length from 146 bp for *V. carpophila* strain BS5 to 150 bp for *V. pyrina* strains IL-11 and IL-18. The ITS1-5.8S-ITS2 nucleotide sequences from the strains of *V. inaequalis*, *V. pyrina*, *V. nashicola*, and *C. caryigenum* revealed more than 95% similarity within and more than 83% similarity between the species.

Further analysis of the nucleotide sequences for *V. pyrina* and *V. nashicola* indicated that it should be possible to differentiate the species based on differences in their *Bsp*1286I restriction digestion patterns. DNA amplified with primers ITS1-F and ITS4 from 10 strains of *V. pyrina* and 6 strains of *V. nashicola* was examined by *Bsp*1286I restriction analysis. Four restriction patterns, designated as PI, PII, PIII, and PIV, were identified among the 16 strains (Fig. 6, Table 4). Variable restriction sites were found at positions 69, 342, and 361; a nonvariable site was found at position 292 (Fig. 5). DNA amplified from five strains of *V. nashicola* and two of *V. pyrina* gave nearly identical restriction fragment patterns of 224, approximately 155, and 137 bp (Fig. 6, lanes 1 and 2). However, sequence anal-

TABLE 2. Intron and ITS1 restriction site polymorphism and linkage in strains of *Venturia inaequalis* from two orchards in Michigan and from geographical regions outside North America

Allele	18S ribosomal DNA				Fennville orchard			East Lansing orchard			Other orchard sites ^c		
	Variable restriction site ^a				Strain (n = 38)	ITS1 allele ^b		Strain (n = 47)	ITS1 allele		Strain (n = 24)	ITS1 allele	
						α	β		α	β		α	β
I ^d	—	—	—	—	8	8	0	9	9	0	5	5	0
II	—	+	—	—	11	11	0	16	16	0	8	8	0
III	—	+	+	—	0	...	...	0	...	...	3	0	3
IV	+	—	+	+	19	0	19	22	0	22	8	0	8

^a C = *Cfo*I site at position 408; S = *Sal*I site at position 191; B = *Bst*UI site at positions 60 and 409, respectively. Positions were counted from the 5' end of primer ITS1-F (Fig. 1).

^b α = no variable *Bst*UI site within the ITS1 region; β = variable *Bst*UI site at position 80 within the ITS1 region (Fig. 5).

^c Australia, one strain; Germany, seven strains; Israel, four strains; Italy, five strains; New Zealand, three strains; and France, four strains.

^d I = intron-lacking strains.

^e ... = Allele not existent in our study.

TABLE 3. Segregation of an intron in the 18S ribosomal RNA gene among single-ascospore isolates of *Venturia inaequalis* and among progenies with different levels of resistance to benomyl

Parental intron phenotype Cross ^a	Progeny tested (no.)			Parental benomyl phenotype ^b	Reaction to benomyl within intron group	
	Total	Intron ⁻	Intron ⁺		Intron ⁻	Intron ⁺
Intron ⁺ × Intron ⁻						
WB × RH-4	16	8	8	S × HR	5S : 3HR	5S : 3HR
Maine 6 × CR2C	7	3	4	MR × VHR	2MR : 1VHR	2MR : 2VHR
Intron ⁺ × Intron ⁺						
WL × RH-1-11B	10	0	10	S × HR	0	5S : 5HR
Intron ⁻ × Intron ⁻						
WB × CR2C	10	10	0	S × VHR	5S : 5VHR	0

^a Intron⁺ = intron-containing parental strain; intron⁻ = intron-lacking parental strain.

^b S = sensitive to benomyl, no growth at 0.5 µg/ml; MR = medium resistance, growth at 0.5 µg/ml, but not at 50 µg/ml; HR = high resistance, growth at 50 µg of benomyl per ml and 0.5 µg of isopropyl-N-(3,4-diethoxyphenyl)-carbamate (NPC) per ml; and VHR = very high resistance, growth at 50 µg of benomyl per ml, but not at 0.5 µg of NPC per ml (15).

ysis showed that the middle fragment from the two *V. pyrina* strains was actually 2 bp longer (a 157-bp fragment) due to the presence of additional nucleotides in the ITS2 region. The enzyme cut the PCR fragment amplified from the sixth strain of *V. nashicola* into three fragments of 224, 175, and 137 bp (designated as pattern PII [Fig. 6, lane 3]). DNA amplified from six strains of *V. pyrina* was cut into fragments of 361 and 157 bp, while DNA from two other strains of *V. pyrina* was cut into fragments of 361 and 175 bp (designated as patterns PIII and PIV, respectively [Fig. 6, lanes 4 and 5,

respectively]). Strains of *V. nashicola* exhibited restriction patterns PI and PII and strains of *V. pyrina* exhibited restriction patterns PI, PIII, and PIV. There was no correlation between restriction pattern and pathogenic race (Table 4). All restriction site variations were confirmed by sequence analysis of the corresponding DNA of selected strains.

Parsimony analysis of the ITS regions and the 5.8S subunit of *V. inaequalis*, *V. pyrina*, *V. nashicola*, *V. cerasi*, *V. asperata*, and *V. carpophila* revealed two equally most-parsimonious trees (tree length = 123,

Fig. 5. Alignment of the complete ITS1-5.8S-ITS2 nucleotide sequences (5' to 3') for *Venturia inaequalis* (Vi) strains 890BF, 685BF, 5BF, and 826BF; *V. pyrina* (Vp) strains MPS8, Akita (A) FB-1, IL-4, IL-11, and IL-18; *V. nashicola* (Vn) strains Mamenashi (M) 12A1-1 and Mamenashi 12A1-3; *V. cerasi* (Vce) strain ATCC 12119; *V. asperata* (Va) strain ATCC 34052; *V. carpophila* (Vca) strain BS5; and *Cladosporium caryigenum* (Cc) strains JD and LCF.

consistency index = 0.805, and retention index = 0.883). The trees were distinguishable only by the location of *V. nashicola* strains Mamenashi 12A1-1 and Mamenashi 12A1-3V and *V. pyrina* strains IL-11 and IL-18. In one tree, both strains from *V. nashicola* were phylogenetically related closer to *V. pyrina* strains MPS8, Akita FB-1, and IL-4 than to IL-11 and IL-18. In the second tree, strains IL-11 and IL-18 were related closer to all other *V. pyrina* strains than to *V. nashicola* strains Mamenashi 12A1-1 and Mamenashi 12A1-3. A strict consensus tree was created that revealed a tree length of 124, consistency index of 0.798, and retention index of 0.879 (Fig. 7). All tree-fruit pathogens formed a monophyletic group that was a sister group of the nut pathogen *C. caryigenum* (Fig. 7). Within the group of tree-fruit pathogens, three well-supported monophyletic groups were obtained. Group I consisted of *V. inaequalis* strains only; group II, *V. pyrina* and *V. nashicola* strains; and group III, strains of *V. cerasi*, *V. carpophila*, and *V. asperata* (bootstrap values of equally most-parsimonious tree of

100, 99, and 99%, respectively). That group III was different from the group I-group II monophyletic group was supported by a bootstrap value of equally most-parsimonious tree of 71%.

DISCUSSION

Although nucleotide variation in the ITS1 region within our sample of *V. inaequalis* strains was notable, it was variation in an optional group I intron that was most useful for differentiating genotypes within *V. inaequalis*. Four intron alleles were identified based on restriction fragment patterns of DNA amplified with primers ITS1-F and ITS2 from a geographically diverse group of *V. inaequalis* strains. Intron⁻ and intron⁺ strains of *V. inaequalis* were widely distributed geographically, and we would expect to find both types of strains wherever pathogen populations exist. Among intron⁺ strains, a higher frequency of alleles II and IV than of allele III would be expected, because allele III was rare among the strains we examined. Tenzer and Gessler (30) also noted variation associated with the intron, and they identified five intron alleles within

Fig. 6. *Bsp*1286I restriction digests of ribosomal DNA amplified with primers ITS1-F and ITS4 from *Venturia nashicola* and *V. pyrina*. Lane M, 100-bp ladder; and lanes 1 to 5, DNA from *V. nashicola* strain Mamenashi 12A1-3, *V. pyrina* strain IL-11, *V. nashicola* strain Yasato2-1-1, and *V. pyrina* strains Akita FB-1 and IL-15, respectively.

Fig. 7. Phylogram of the unrooted strict consensus tree from parsimonious analysis of the ITS1-5.8S-ITS2 region DNA sequences from strains of *Venturia* and *Cladosporium* species related to their anamorph and major hosts. Vi = *Venturia inaequalis*, Va = *V. asperata*, Vce = *V. cerasi*, Vn = *V. nashicola*, Vp = *V. pyrina*, Vca = *V. carpophila*, and Cc = *C. caryigenum*. Branch lengths indicate the equivalent of numbers of substitutions.

TABLE 4. ITS1-5.8S-ITS2 restriction site polymorphism in races of *Venturia pyrina* and *V. nashicola* from Israel, Japan, and Michigan

ITS1-5.8S-ITS2 rDNA												
Variable <i>Bsp</i> 1286I restriction sites ^a			Races of <i>V. pyrina</i> (n = 10) ^b							Races of <i>V. nashicola</i> (n = 6) ^c		
Allele	1	2	3	R1	R2	R3	R4	R5	Race unknown	R1	R2	R3
PI	+	+	+	IL-11				IL-18		JS-115	M 12A1-1, M 12A1-3	M 12B1-1, M 12B1-2
PII	+	+	—							Yasato2-1-1		
PIII	—	+	+		IL-3	IL-13			MPS8, MPS10, Akita SK-1, Akita FB-1			
PIV	—	+	—				IL-15, IL-16					

^a 1 = *Bsp*1286I site at position 69 (counted from the 5' end of ITS1; Fig. 5); 2 = *Bsp*1286I site at position 342; and 3 = *Bsp*1286I site at position 361.

^b R1 to R5 = *V. pyrina* races 1 to 5; race designations were made by Shabi et al. (24).

^c R1 to R3 = *V. nashicola* races 1 to 3; race designation according to H. Ishii, personal communication; M = Mamenashi.

the intron locus of *V. inaequalis*. We were able to confirm three of these alleles (A, B, and E), except sequence analysis revealed that one *Cfo*I cutting site in allele B was located at a different mapping site than previously reported (30). Although other restriction patterns were occasionally observed, these patterns were not reproducible nor were restriction sites identified in strains with variable restriction patterns. Because we utilized *Bst*UI restriction sites in addition to *Cfo*I and *Sal*I sites, the intron alleles we described overlapped but were not identical to those found in Swiss populations of *V. inaequalis*. The presence of variant forms of the intron could be useful for subdividing populations of *V. inaequalis* as already shown by Tenzer and Gessler (30). Molecular characterization of races or pathogenicity group based on rDNA polymorphism was described previously (7,10,20). No correlation was found between intron alleles I to IV and the physiological races of *V. inaequalis* tested. Either there is no correlation between the physiological race and the rDNA alleles or our intron allele types do not adequately reflect the genetic diversity within the rDNA for the purpose of race discrimination. However, the ITS1 regions in strains of *V. inaequalis* revealed two distinguished alleles (α and β) that differed in three locations within the ITS1 region and in two locations within the ITS2 region. The differences between the ITS types may be conserved, because they were confirmed by sequencing a second strain of each type (Fig. 5). The two strains of the α type were from different orchards.

The probable origin of the group I intron found in *V. inaequalis* is not clear. In both our study and the Swiss study (30), the frequency of intron⁺ strains was always higher than the frequency of intron⁻ strains. No intron⁻ strain was found to contain the ITS1 β allele, but intron⁺ strains were found to contain either an α or β allele. If the intron was originally present in all strains of *V. inaequalis* and is now slowly being lost, our data would suggest that it is not as stable in strains with the ITS1 α genotype. Interestingly, the intron was inherited in *V. inaequalis* by half of the progeny when intron⁺ strains were crossed with intron⁻ strains. This is consistent with inheritance studies for *Tetrahymena pigmentosa* (21). rDNA is present in multiple copies in nuclear DNA, but it is not always distributed in a variable number of chromosomes in the genome (13,17). Whether the rRNA genes and ITS sequences we examined are present on several or all chromosomes in *V. inaequalis* is unknown. Restriction of tandem repeats of rDNA to one or two chromosomes was reported for the ascomycete *Leptosphaeria maculans* (13). The simplest explanation for a 1:1 segregation of the intron is that all rDNA repeats in *V. inaequalis* are located on a single chromosome.

C. caryigenum was used as an outgroup species to root the *Venturia* species phylogenetic tree, because its rDNA sequence exhibited a high level of nucleotide sequence divergence to each of the *Venturia* species we examined. Strains of this species were included in this study because some *Venturia* species have *Cladosporium* anamorphs. *C. caryigenum* has no known teleomorph. Although *C. caryigenum* was separate phylogenetically from the tree-fruit *Venturia* species based on an analysis of their ITS sequences, some strains of *C. caryigenum* and *V. inaequalis* exhibited length variation in their 18S rRNA gene due to the presence of a group I intron. However, the introns detected in *C. caryigenum* and in *V. inaequalis* were distinct. Their presence in these pathogenic fungi from diverse hosts cannot be readily explained by common ancestry alone.

The analysis of the sequence variation in the ITS1-5.8S-ITS2 region of *V. pyrina* and *V. nashicola* indicated that the fungi were closely related but genetically distinct from the *Venturia* species found on other tree-fruit hosts. Previously, the *Venturia* species from *Pyrus* were differentiated by their morphological differences (29) and physiological specialization (14,24,28). However, Sivansen (25) noted that the size of the conidia and ascospores of *V. nashicola* fell within those for *V. pyrina*. The question of whether *V. nashicola* should be considered a race of *V. pyrina* rather than a

species distinct from *V. pyrina* cannot be answered on the basis of the rDNA sequence similarity alone. Unfortunately, the morphology, physiology, and genetic data reported for *V. pyrina* and *V. nashicola* only represent a small number of isolates (14,24,25,28,29). Although we investigated a range of isolates, we did not evaluate their morphological and physiological characters. A comprehensive evaluation of a geographically diverse set of isolates of *V. pyrina* and *V. nashicola* is needed to determine whether these species should be considered conspecific.

The strain of *V. asperata* used in this study was the type culture deposited at the American Type Culture Collection by Samuels and Sivansen (23). It was isolated from dead apple leaves coinfecting with *V. inaequalis*. The ITS sequences of *V. asperata* and *V. inaequalis* were phylogenetically different, which supports their status as separate species based on morphological characters (23). Interestingly, the strain of *V. asperata* from *Malus* was grouped with two species of *Venturia* from *Prunus*, not with the *Malus* pathogen *V. inaequalis*. The close relationship between *V. asperata* and *V. carpophila* was previously shown based on morphological characters (23). As only one strain each of *V. asperata*, *V. cerasi*, and *V. carpophila* was included in this study, it would be premature to assign a definitive position to them before examining more strains.

The three monophyletic groups found for the *Venturia* species were, with the exception of *V. asperata*, linked to the corresponding host rather than to their anamorph. In addition, the pathogens isolated from *Prunus* were a sister group of the two groups comprising pathogens isolated from *Malus* and *Pyrus*. Considering that *Prunus* was previously identified as a sister group of *Malus* and *Pyrus* (4), we suggest coevolution. This would mean that the common ancestor of the pathogen might have infected the common ancestor of the family Rosaceae. However, this hypothesis is not valid for *C. caryigenum*, since this would require much higher sequence diversity of the pathogen. More sampling is needed to support our hypothesis. Nucleotide variation within ITS1 and ITS2 in *M. domestica*, *Pyrus calleryana*, and *Prunus cerasifera* was greater than in their corresponding parasites, suggesting that the hosts evolved faster than the parasites.

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