Evidence of Two *Formae Speciales* in *Venturia inaequalis*, Responsible for Apple and *Pyracantha* Scab

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Accepted for publication 5 November 2001.

ABSTRACT

Le Cam, B., Parisi, L., and Arene, L. 2002. Evidence of two *formae speciales* in *Venturia inaequalis*, responsible for apple and *Pyracantha* scab. *Phytopathology* 92:314-320.

Genetic relationships, mating crosses, and host specificity of *Venturia inaequalis* isolates from *Malus* spp. and of *Spilocaea pyracanthae* isolates from *Pyracantha* spp. were evaluated. Sequence analysis of the complete internal transcribed spacer (ITS) region (ITS1-5.8S to ITS2) revealed a total similarity between these two putative species. ITS restriction fragment length polymorphism carried out with five restriction enzymes on a collection of 28 isolates confirmed a lack of diversity in this region between and within these two populations. Additional isolates from three related species (*V. pirina*, *V. nashicola*, and *S. eriobotryae*)

were divided into two distinct monophyletic groups in a phylogenetic tree using ITS sequence comparison. These groups were related to their anamorph (i.e., *Spilocaea* or *Fusicladium*). When inoculated on their host of origin, fields isolates caused typical symptoms of scab disease, and a host specificity was demonstrated by cross pathogenicity of isolates from *Malus* × *domestica* and *Pyracantha* spp. Mating on dried leaves in vitro between one isolate of each putative species led to production of numerous perithecia. Ninety-six sporulating monoascosporic progenies were isolated from this cross. Based on these genetic and pathogenic data, we proposed that pathogens responsible for scab on *Malus* spp. and *Pyracantha* spp. are considered as two *formae speciales* belonging to *V. inaequalis*.

Additional keywords: cross inoculations, host specificity.

The ascomycete *Venturia inaequalis* (Cooke) G. Wint. is responsible for the most important disease of apple in temperate countries. Pathogenic behavior of this fungus is now well described. Seven races were identified and genetic studies have demonstrated that the relationships between *V. inaequalis* and *Malus* spp. fit the gene-for-gene theory (2). *V. inaequalis* is a heterothallic fungus with a sexual cycle occurring each year in winter, followed by many asexual reproduction cycles during the epidemic phase. This mixed reproduction system is conducive to a high genetic diversity within populations (28) and is supposed to be a factor facilitating adaptation of pathogens to specific resistance genes of the host both by increasing risks of avirulence losses and spreading of new virulences. The *Vm* resistance gene, introgressed from *Malus micromalus* in cultivated apples, has been rapidly overcome by race 5 while it was introduced in only six cultivars (31). Improved cultivars released by plant breeding programs carry the *Vf* gene from *M. floribunda* 821, first considered as conferring durable resistance (8). Although this gene has, to date, scarcely been used in commercial plantations, resistance breakdown has already occurred in many countries (19,20,22), demonstrating that the durability of resistance introduced in such a perennial crop remains questionable because of the capability of *V. inaequalis* to overcome resistance under monogenic determinism.

Within the genus *Venturia* (anamorphs *Fusicladium*, *Spilocaea*, and *Cladosporium*), 52 species have been described (26). Most are pathogens of fruit trees or ornamental plants. The host plant *pyracantha*, *Pyracantha* sp. Roem., is particularly susceptible to scab. A breeding program on this plant was developed in the United States by Egolf in 1970 (10); it was aimed at scab resistance as well as at evergreen or fructification criteria. The associ-

ated anamorph of pathogens responsible for scab on *Malus* × *domestica* and on *Pyracantha* spp. belong to the genus *Spilocaea*. The sexual form has never been reported for the pathogen of this shrub. Therefore, it is called *Spilocaea pyracanthae* (G. Otth) Arx. Distinction between *V. inaequalis* and *S. pyracanthae* based on morphological criteria and on cross pathogenicity remains controversial (26). Moreover, no comparative genetic studies have been reported, and current data fails to discriminate these two putative species. Defining the limits of these species is of great interest in order to evaluate diversity of the pathogen and predict the potential spread of genes involved in pathogenicity. *Pyracanthas* are commonly planted in gardens and hedges, and wind distribution may contribute to migration of the fungus in orchards (28). The host range of these two pathogens is not yet clearly defined; therefore, the role of *pyracantha* as an alternative host of *V. inaequalis* acting as source of diversity remains to be evaluated.

In most eukaryotic organisms, ribosomal DNA (rDNA) comprises a multigene family with copies arranged in tandem repeats. Many analyses of DNA sequences dealing with fungi have been based on the rDNA repeat because of their conserved molecular function (25), and they are commonly used for taxonomic studies. Recently, characterization of rDNA has been employed to explain phylogenetic relationships within species of the genus *Venturia* that cause scab on tree fruit crops (24). Their findings, based on internal transcribed spacer (ITS) region ITS1-5.8S to ITS2 sequences, were that *V. inaequalis* differed from other species isolated from *Pyrus* spp. or *Prunus* spp.

To elucidate the relationships between *V. inaequalis* and *S. pyracanthae*, we first carried out an analysis of variation within ITS sequences of isolates representative of these two groups and from related species. Digestions of amplified ITS of a larger number of isolates (28) with five restriction enzymes were further carried out to confirm the results. In a second step, we prepared crosses in vitro between *S. pyracanthae* (isolate 186) and two isolates of *V. inaequalis* with opposite mating type (isolates 104 and 302) in

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Publication no. P-2001-1228-01R

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order to test the hypothesis of a single species. Host range of these two putative species was then tested by cross-inoculations of *Pyracantha* spp. cuttings and *Malus* × *domestica* seedlings with *V. inaequalis* and *S. pyracanthae*.

MATERIALS AND METHODS

Polymorphism in rDNA region. *Fungal isolates.* Characteristics and origin of *Venturia* sp. and *Spilocaea* sp. strains used in this study are listed in Table 1. Strains used were isolated from apple (*Malus* × *domestica*), firethorn (*Pyracantha* sp.), European pear (*Pyrus communis*), Japanese pear (*P. pyrifolia* var. *culta*), and loquat (*Eriobotrya japonica*). They were derived from monoclonal isolates. Fungal cultures were stored on malt agar (Merck, Darmstadt, Germany) at 4°C.

Mycelial growth and DNA extraction. Each isolate initially was grown on malt agar (Merck) plates for at least 20 days at 17°C. Plugs of mycelium were deposited on the surface of a malt agar plate covered up by a sheet of cellophane. After 7 days of incubation, 10 mg of agarless mycelium was gently scrapped from the surface and placed in a 1.5-ml microcentrifuge tube containing 100 µl of lysis buffer (50 mM Tris-HCl, pH 7.2, 50 mM EDTA, 3% sodium dodecyl sulfate, 1% 2-mercaptoethanol). DNA extraction was then performed as previously described (12,16). Concen-

tration of DNA was determined using UV spectrophotometry; the final concentration was adjusted with distilled water to 10 ng/µl and stored at -20°C.

Polymerase chain reaction amplification and digestion. Amplification of the ITS was performed in a programmable thermocycler (GeneAmp PCR System 9700; Applied Biosystems, Inc., Foster City, CA). The primer sequences used were 5'-TCCGTA-GGTGAACCTGCGG-3' (ITS1) from the 18S, and 5'-TCCTCC-GCTTATTGATATGC-3' (ITS4) from the 28S rDNA (30). A 50-µl reaction mixture contained 0.8 µM each primer, 200 µM dNTP, 1 unit of red Gold Star polymerase (Eurogentec SA, Herstal, Belgium), 1× amplification buffer (75 mM Tris-HCl, pH 9), 20 mM (NH₄)₂SO₄, 0.01% (wt/vol) Tween 20, 5 mM MgCl₂, and 30 ng of template DNA. The mixture was subjected to an initial denaturation for 5 min at 94°C, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, a 45-s extension at 72°C, and 7 min of final extension at 72°C. Polymerase chain reaction (PCR) amplification was 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). Digestions with restriction enzymes were performed in a final volume of 20 µl with 12 or 15 µl of PCR products and eight units of the following enzymes: *TaqI*, *EcoRI*, *MspI*, *Tsp509I* (Eurogentec), and *RsaI* (New England Biolabs Inc., Beverly, MA). Reactions were

TABLE 1. Fungal strains used in this study^a

Fungal strains	Isolation (country and date)	Host plant
<i>Venturia inaequalis</i>		
93 race 1	URSS - 1974	<i>Malus</i> × <i>domestica</i> 'McIntosh'
104 race 1 ^b	St. Lézin, France - 1978	<i>Malus</i> × <i>domestica</i> 'Golden Delicious'
119 race 1	Romania - 1980	<i>Malus</i> × <i>domestica</i> 'Golden Delicious'
1105 race 1	The Netherlands - 1994	<i>Malus</i> × <i>domestica</i> 'Golden Delicious'
330 race 2	Germany - 1988	<i>Malus</i> × <i>domestica</i>
855 race 3	Germany - 1991	<i>Malus</i> × <i>domestica</i>
856 race 3	Germany - 1991	<i>Malus</i> × <i>domestica</i>
405 race 4	Bordeaux, France - 1988	<i>Malus</i> × <i>domestica</i>
740 race 4	Beaucouzé, France - 1991	<i>Malus</i> × <i>domestica</i>
147 race 5	Beaucouzé, France - 1985	<i>Malus</i> × <i>domestica</i>
105 race 5	New York, USA - 1979	<i>Malus</i> × <i>domestica</i>
844 race 5	Germany - 1991	<i>Malus</i> × <i>domestica</i>
302 race 6 ^{b,c}	Ahrensburg, Germany - 1988	<i>Malus</i> × <i>domestica</i> 81/11-22 ^d
1060 race 7 ^e	Beaucouzé, France - 1993	<i>Malus floribunda</i> 821
1144 race 7	The Netherlands - 1994	<i>Malus floribunda</i>
1066 race 7 ^{c,e}	Beaucouzé, France - 1993	<i>Malus floribunda</i> 821
<i>Spilocaea pyracanthae</i>		
D 38.2	Beaucouzé, France - 1993	<i>Pyracantha koïdzumii</i> × <i>P. coccinea</i> 'Mohave'
D 38.5	Beaucouzé, France - 1993	<i>P. koïdzumii</i> × <i>P. coccinea</i> 'Mohave'
D 38.6	Beaucouzé, France - 1993	<i>P. koïdzumii</i> × <i>P. coccinea</i> 'Mohave'
D 38.7 ^c	Beaucouzé, France - 1993	<i>P. koïdzumii</i> × <i>P. coccinea</i> 'Mohave'
D 38.8	Beaucouzé, France - 1993	<i>P. koïdzumii</i> × <i>P. coccinea</i> 'Mohave'
D 38.9	Beaucouzé, France - 1993	<i>P. koïdzumii</i> × <i>P. coccinea</i> 'Mohave'
D 19.3	Beaucouzé, France - 1993	<i>P. coccinea</i> 'Lalandei'
186 ^{b,c}	Ireland - 1987	<i>Pyracantha</i> sp.
1381	La Meignanne, France - 1998	<i>Pyracantha</i> sp.
1383	France - 1998	<i>Pyracantha</i> sp.
1388	France - 1998	<i>Pyracantha</i> sp.
V55 ^f	Dossenheim, Germany - 1995	<i>Pyracantha</i> sp.
<i>Venturia pirina</i>		
P11 ^c	Beaucouzé, France - 1991	<i>Pyrus communis</i> 'Président Drouart'
P64 ^c	The Netherlands - 1995	<i>Pyrus communis</i> 'Doyenne'
<i>Venturia nashicola</i>		
P17 ^c	Lanxade, France - 1991	<i>Pyrus pyrifolia</i> 'Culta'
P63 ^c	Bergerac France - 1996	<i>Pyrus pyrifolia</i> 'Culta'
<i>Spilocaea eriobotryae</i>		
1389 ^{c,g}	Montpellier, France - 1998	<i>Eriobotrya japonica</i>

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

^b Strains tested for sexual crosses.

^c Strains in which the complete internal transcribed spacer (ITS) region was sequenced and aligned.

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

^e Strains originating from the Fl 1 isolate described by Roberts and Crute (22).

^f Strain V55 was supplied by A. Kollar, Institute for Plant Protection in Fruit Crops, Dossenheim, Germany.

^g Strain 1389 was supplied by J. L. Nottetghem, ENSA, Montpellier, France.

carried out under conditions as recommended by the manufacturers. The digested DNA fragments were separated on 4.5% Nusieve GTG agarose gels (FMC BioProducts, Rockland) and visualized as described above. A 100-bp molecular weight ladder (Eurogentec) was used as size standard.

DNA sequencing. PCR products were purified using a Qiagen kit (Qiagen, Hilden, Germany) and then sequenced by using a Taq Dye Deoxy terminator sequencing kit and a model ABI 377 automatic sequencer (Applied Biosystems). Primers used were ITS1 and ITS4 as described above. Two isolates of each species were sequenced (*V. inaequalis*: strains 302 and 1066; *S. pyracanthae*: strains 186 and D38.7; *V. pirina*: strains P64 and P11; and *V. nashicola*: strains P63 and P17), except for *S. eriobotryae* (only one strain: 1389).

Sexual crossing. A strain of *S. pyracanthae* (186) was crossed with two strains of *V. inaequalis* of known mating type (13): strain 302 (+) and strain 104 (−). Crosses were carried out according to protocol as described (13). They were performed under sterile conditions on 56 air-dried foliar disks from *M. domestica* (cv. Gala) and from *Pyracantha* × (cv. Mohave). Disks were inoculated with mixed mycelia of the two strains and incubated for

21 days at 17°C in the darkness and then at 8°C for 140 days. Perithecia obtained were crushed and spread on water agar plates (Merck), and germinated ascospores were isolated individually under optical microscope after 48 h at 20°C.

Cross pathogenicity tests. *Fungal material and inoculum preparation.* *V. inaequalis* and *S. pyracanthae* inoculum consisted of local populations collected in diverse apple orchards or nurseries of *P. coccinea* in the area of Angers, France. Initial conidial suspensions derived from scabbed leaves of apple and pyracantha or were obtained in Roux Flasks as previously described (15). These conidial suspensions were thereafter sprayed either on batches of apple or firethorn seedlings according to Parisi et al. (20). This first inoculation allowed numerous scabbed leaves to collect that were stored at −20°C. Leaves then were rinsed with distilled water: this new conidial suspension was adjusted to 250,000 conidia per ml and was used next for plant inoculation. Strains 186 (*S. pyracanthae*) and 302 (*V. inaequalis*) used in mating experiment also were tested independently for their pathogenicity on apple seedlings.

Plant material. Seedlings originating from two crosses of *Malus* × *domestica* ('Golden Delicious' × 'Idared' and 'Florina' ×

	ITS1→	100
Vi 302	ATGGGTTAGCCTTCGGGCGCATCCCCACCTCTGTAAACCGCGCCCGGATTTCGGCGCCTGGCGG-GACCACCCCCCGTTTGGCGAGGGGGCCCCGCTGCCG	
Vi 1066		
Sp 186		
Sp 38.7		
Se 1389		
Vn P63	C.....C.....A.T.....T.....A.GG.....C.C...G...C.T.....	
Vn P17	C.....C.....A.T.....T.....A.GG.....C.C...G...C.T.....	
Vp P64	C.....C.....A.T.....T.....C.A.....A.GGT.....C...G...C.T.....	
Vp P11	C.....C.....A.T.....T.....A.....A.GGT.....C...G...C.T.....	
	101	5.8S→ 200
Vi 302	GAATTAGCCAACCCTGCCTGGAAAATTGAAGTCTGAGAACAAAGTTAAATAAAACAAAACCTTTCAACAACGGATCTCTTGGTTCTGGCAACGATGAAGAAC	
Vi 1066		
Sp 186		
Sp 38.7		
Se 1389		
Vn P63	...C.....C.....G.....CG.....	
Vn P17	...C.....C.....G.....CG.....	
Vp P64	...C.....C.....G...C...CG.....	
Vp P11	...C.....C.....G...C...CG.....	
	201	300
Vi 302	GCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCACACCTGT	
Vi 1066		
Sp 186		
Sp 38.7		
Se 1389		
Vn P63		
Vn P17		
Vp P64		
Vp P11		
	301	ITS2→ 400
Vi 302	TCGAGCGCCATTCTACCTGGAGCCCTGCTCTGTGATGGGCCCCGCTCCTCGCGGACGGGCCCCGAAACCCGTGGGCGCCGTCGTCGGCCCTGAGCGGTAG	
Vi 1066		
Sp 186		
Sp 38.7		
Se 1389		
Vn P63	AC.....A.....	
Vn P17	AC.....A.....	
Vp P64	C.....	
Vp P11	C.....	
	401	462
Vi 302	CAAGAGAAATCCCTCGCTCGGAGTGTCTGTGCGCAGGCCGCCCGAAACCCATATTTACAAG	
Vi 1066		
Sp 186		
Sp 38.7		
Se 1389		
Vn P63	C...G...C..T.....C...-..T.C.....	
Vn P17	C...G...C..T.....C...-..T.C.....	
Vp P64	C...G...C..T.....C...T.....	
Vp P11	C...G...C..T.....C...T.....	

Fig. 1. Alignment of the complete internal transcribed spacer (ITS) region ITS1-5.8S to ITS2 sequences of *Venturia inaequalis* (Vi) strains 302 and 1066; *Spilocaea pyracanthae* (Sp) strains 186 and 38.7; *S. eriobotryae* (Se) strain 1389; *V. nashicola* (Vn) strains P63 and P17; and *V. pirina* (Vp) strains P64 and P11; - indicates a nucleotide deletion.

'Melrose') were used for the pathogenicity tests. The protocol for seed production, stratification, and seedling growth in the greenhouse was described previously (2). Concerning tests on *Pyracantha* spp., six clones were analyzed: 'Golden Charmer' (*P. coccinea* × *P. rogersiana*), 'Mohave' (*Pyracantha* ×), 'Mozart' (*P. atalantioides*), 'Saphyr Rouge' (*Pyracantha* ×), 'Sunshine' (*P. coccinea*), and 'Watereri' (*Pyracantha* ×). The pyracantha clones were cultivated in a greenhouse in the same conditions as apple seedlings.

Inoculation and grading. The inoculation tests were carried out threefold. The batches of apple seedlings were inoculated and incubated in a growth chamber according to the technique previously described (2). For each experiment, 146 to 219 apple seedlings of each cross were inoculated with each putative species. The plantlets of pyracantha were inoculated in the greenhouse for two experiments and in a growth chamber for the third. For each clone and experiment, 12 plantlets (2-month-old) produced from cuttings were tested with *V. inaequalis* and *S. pyracanthae*. Scab occurrence on seedlings was assessed after 11 days of incubation using the scale of Chevalier et al. (7). Symptoms were classified into six classes: seedlings in classes 0 to 3a were considered as resistant and seedlings in classes 3b and 4 as susceptible. The incidence of scab on pyracantha (percentage of scabbed plantlets) was scored after 22 to 26 days of incubation. For apple seedlings, the results are expressed as the percentage mean of scab-infected seedlings in each class. Concerning pyracantha, the percentage is calculated taking into account the three replications of the test. Strains 302 and 186 used in the mating experiment were tested further on a single experiment on 146 seedlings from the cross 'Golden Delicious' × 'Idared' following the same protocol.

Sequence analysis. ITS sequences were aligned using ClustalW (29). Virtual enzymatic restriction and phylogenetic tree were performed with Seqweb Version 1.1 (Wisconsin Package Version 10) from GCG software.

RESULTS

Interspecific variation in the ITS1-5.8S to ITS2 region. All DNA samples were amplified with the ITS1/ITS4 primers, and a single band product was visualized by electrophoresis in an agarose gel. Sequencing of the entire length of the ITS region showed no length variation between isolates of *V. inaequalis*, *S. pyracanthae*, *V. nashicola*, and the strain isolated from loquat (461 bp; Fig. 1) while ITS size of *V. pirina* was 462. Alignment of the ITS1-5.8S to ITS2 sequences of the two strains of *V. inaequalis* (302 and 1066) revealed a total homology with the two sequenced strains of *S. pyracanthae* (186 and D38.7) and with the strain isolated from loquat (1389) (Fig. 1). Their nucleotide sequences showed more than 92.4% of similarity with *V. nashicola* and *V. pirina*. Tree fruit pathogens were divided into two groups: a first group composed of strains related to the anamorph *Spilocaea* and the second group of the anamorph *Fusicladium* (Fig. 2). The amplified products from 17 strains of *V. inaequalis* and 12 strains of *S. pyracanthae* were digested with *Eco*RI, *Tsp*509I, *Rsa*I, *Msp*I, and *Taq*I. Whatever the enzymes used, digestion obtained from DNAs of *V. inaequalis* and *S. pyracanthae* presented the same banding patterns as those obtained from DNA extracted from *S. eriobotryae* (data not shown). No polymorphism was revealed within *V. inaequalis* and *S. pyracanthae* (Fig. 3). Number and size of visualized DNA fragments were in accordance with virtual restriction obtained from sequence data of each species using application Seqweb, version 1.1, from GCG software.

Mating of *V. inaequalis* and *S. pyracanthae*. Only the cross between strain 302 (*V. inaequalis*) and strain 186 (*S. pyracanthae*) was fertile. A total of 974 mature and large perithecia were produced on the 112 foliar disks inoculated with mycelia of these two strains. No fruiting body was observed on leaves of the other tested cross. Disks from apple leaves produced 6.6 times more

perithecia than those from pyracantha leaves. From a few crushed perithecia, we isolated 96 sporulating monoasporic strains originating from the two types of leaves, which confirms the success of the cross.

Pathogenicity of *V. inaequalis* and *S. pyracanthae* isolates on apple seedlings and firethorn clones. *Apple seedlings.* Progenies obtained from the cross 'Golden Delicious' × 'Idared' were all susceptible to *V. inaequalis* inoculum (Fig. 4A); the distribution into six classes of symptoms in the progeny 'Florina' × 'Melrose' showed a resistance/susceptible ratio close to 1:1 (282 resistant individuals and 223 susceptible individuals), corresponding to the segregation of the *Vf* resistance gene carried by cv. Florina. Progenies from the two crosses inoculated with *S. pyracanthae* showed typical resistance reaction, lack of symptoms (class 0) to symptoms of chlorosis or necrosis without sporulation (class 2) (Fig. 4B). Strain 302 (*V. inaequalis*) exhibited a compatible reaction when inoculated on *Malus* × *domestica* seedlings, whereas inoculation with strain 186 (*S. pyracanthae*) did not lead to any symptoms (class 0).

Pyracantha clones. The six clones inoculated with *S. pyracanthae* were susceptible (Table 2). Strong variation of susceptibility was observed between clones in our controlled experimental conditions. Data obtained with *V. inaequalis* inoculation showed that all clones were resistant. However, a plant of cv. Saphir Rouge that scored in the third experiment presented slight sporulation.

DISCUSSION

Based on alignment of ITS1-5.8S to ITS2 region sequences, a perfect homology has been observed between *V. inaequalis* (strains 302 and 1066), and *S. pyracanthae* (strains 186 and D38.7). These sequence data have been confirmed on a larger set of isolates by screening nucleotide variation with five restriction enzymes that had previously permitted clear differentiation among other *Venturia* spp. (16). Although the set of *V. inaequalis* included isolates of race 1 to 7 from diverse geographic origins, no vari-

Fig. 2. Phylogram from analysis of the sequences of the internal transcribed spacer (ITS) ITS1-5.8S to ITS2 rDNA region of *Venturia* and *Spilocaea* spp. related to their anamorph and major host. The tree was constructed with the unweighted pair group method with arithmetic means method. Se: *S. eriobotryae*, Sp: *S. pyracanthae*, Vi: *V. inaequalis*, Vp: *V. pirina*, Vn: *V. nashicola*. Branches indicated numbers of substitutions per 100 bases.

ation was detected. Within *V. inaequalis*, such a homology of nucleotide sequences of rDNA was expected regarding results obtained by Schnabel et al. (24), who reported slight variation that does not exceed 1.3%. Within *S. pyracanthae*, no intraspecific variation was observed. Considering that many reports dealing with fungal species showed that intraspecific nucleotide variation of the total region of the ITS may exceed 10% (1), our results could be considered as self sufficient to propose that these two fungi belong to the same species.

One isolate of *S. eriobotryae* (Cav.) Hughes (1389) from loquat (*Eriobotrya japonica*) (Thunb.) Lindl. was included in the comparison. This isolate showed a sequence identical to strains of *V. inaequalis* and *S. pyracanthae*. According to Raabe and Gardner (21), loquat scab as well as firethorn scab are caused by *S. pyracanthae* which would have been recently introduced in the United States. Sequence data obtained in this study would tend to confirm their previous conclusion based on cross pathogenicity and morphology criteria. A perfect stage has never been reported on pyracantha; however, D'Oliviera and D'Oliviera reported immature perithecia in loquat leaves and, as a result, they named the

fungus *V. eriobotrya* (Cav.) D'Oliviera (9). The two related species *V. nashicola* and *V. pirina* also were included in the study. Our results support previous reports (24) by demonstrating that these two fungi are closely related, but genetically distinct, from *V. inaequalis* and also from *S. pyracanthae*.

Mating tests were carried out to determine if fertility barriers existed between isolates from apple and firethorn. Using two parental strains of *V. inaequalis* with opposite mating types, we demonstrated that mating was possible with a strain isolated from pyracantha. High capacity for sporulation of ascospores produced both from apple and pyracantha leaves attested to the fertility of this cross. Keitt and Palmiter (15) demonstrated that *V. inaequalis* is a heterothallic fungus with two compatibility groups whose intragroup crosses are sterile. Our results clearly showed the capability of these two putative species to mate in vitro. These data confirmed our results in nucleotide sequencing, indicating that both entities represent the same species.

Cross-pathogenicity tests of *S. pyracanthae* and *V. inaequalis* strains on *Pyracantha* spp. and *M. domestica* demonstrated a host range specificity of each putative species. Typical sporulation le-

Fig. 3. Enzyme restriction digests of ribosomal DNA amplified from *Venturia inaequalis* (strain 302) and *Spilocaea pyracanthae* (strain D 38.9) with universal primers internal transcribed spacer 1 (ITS1) and ITS4 using *EcoRI*, *RsaI*, *TaqI*, *MspI*, and *Tsp509I*. Lane M, 100-bp ladder.

Fig. 4. Distribution of two apple progenies originating from 'Golden delicious' × 'Idared' (■) and 'Florina' × 'Melrose' (□) into different classes of symptoms after inoculation with **A**, *Venturia inaequalis* and **B**, *Spilocaea pyracanthae*. Symptoms were classified using the scale of Chevalier et al. (7): class 0 = no symptom; class 1 = hypersensitivity (pinpoint pits); class 2 = resistance, chlorosis, either together or separate, without sporulation; class 3a = weak resistance, chlorosis, and necrosis, either together or separate, with slight sporulation; class 3b = weak susceptibility, chlorosis, and necrosis, either together or separate, with sporulation; and class 4 = susceptibility, abundant sporulation without chlorosis and necrosis, either together or separate. Results are the mean of three experiments; standard errors of the mean are indicated by bars.

sions observed on pyracantha clones sprayed with inoculum of *S. pyracanthae* provided evidence of virulence of this inoculum. Analyzing distribution into the different classes of symptoms in the progenies 'Golden Delicious' × 'Idared' and 'Florina' × 'Melrose' clearly indicated a resistance reaction. Concerning *V. inaequalis* inoculations, all progeny of the cross 'Golden Delicious' × 'Idared' showed severe symptoms characteristic of a compatible reaction; 44.2% of the progeny of the cross 'Florina' × 'Melrose' did not: Florina contains the *Vf* gene (17) and fungal inoculum used carried the avirulence *avrVf*; incompatible reaction occurred in the progeny with a segregation resistance/susceptible close to 1:1. In our experimental conditions, all tested firethorn clones were classified as resistant to *V. inaequalis*. Slight scab lesions were observed on the clone 'Saphir Rouge'; however, it was seen on a single plant tested in a growth chamber where conditions for disease development were optimal. Moreover, symptoms observed on this plant presented only slight sporulation scored as a 3a class on the scale of Chevalier (7). This class was created to score resistant plants carrying the *Vf* gene that expressed a slight sporulation in an incompatible reaction (7,11), as observed with 'Prima' (14). Consequently, the phenotype observed on this plant was then considered resistant.

Thus, we demonstrated that *V. inaequalis* and *S. pyracanthae* were able to mate successfully and that pathogenicity tests carried out on a range of cultivars indicated occurrence of a host-specific response. We conclude that they are not sibling species as defined by Brasier and Rayner (4) because they are not distant enough genetically to have lost their capacity to cross. The three analyzed criteria (ITS nucleotide sequences, mating, and cross-pathogenicity) strongly suggest that these fungi are in a process of isolation due to host specialization that fits the definition of a "special form" (*forma specialis*). Suitable conditions for successful mating have been created in the laboratory on apple and firethorn leaves, suggesting that the thermostable component that stimulated pseudothecial development is also present in pyracantha leaves. The teleomorph of *S. pyracanthae* has never been seen in the field; therefore, we do not know whether such an event may occur between these two formae speciales. However, firethorn is an evergreen plant, so the need for a perfect stage in *S. pyracanthae* is probably not essential in its life cycle (21). Moreover, according to our results, isolates from pyracantha were not able to develop lesions on apple seedlings tested. These two facts reduced considerably the probabilities for these two formae speciales to mate in natural conditions. DNA fingerprinting using microsatellites developed for *V. inaequalis* (27) is underway in the laboratory to characterize the structure of these two populations and, thus, to look for occurrence of effective crossings in natural conditions.

Schnabel et al. (24) suggested that coevolution could explain the classification of *Venturia* spp.; the common ancestor of *Venturia* might have infected the common ancestor of the family *Rosaceae*. Host-specific differentiation in the anther smut fungus *Microbotryum violaceum* revealed by microsatellites clearly demonstrated that fungi from more distantly related host species are themselves more divergent (5). In our study, strains from fire-

thorn and loquat are more closely linked to strains isolated from apple than from other tree-fruit hosts. Regarding distant taxonomic position of these two ornamental plants to *Malus* spp. and *Pyrus* spp. (6,23), coevolution cannot explain the phylogenetic proximity of *V. inaequalis*, *S. pyracanthae*, and *S. eriobotryae*. Apple trees and firethorns have a common Asian origin, except the species *P. coccinea*, which would have originated from European countries around the Mediterranean to Caucase (3). Whether *V. inaequalis* was introduced in Europe with its host 2,000 to 2,500 years ago by the Greeks and Romans (18), no data are reported concerning geographical origin and historical spreading of *S. pyracanthae*. Therefore, further genetic diversity comparison of populations from Europe and Asia would permit a conclusion to be made regarding these two aspects.

Results obtained in this study allowed us to conclude that pyracantha does not act as a source of diversity within populations of *V. inaequalis* and thus does not provide strains with new patterns of pathogenicity in apple orchards both in terms of virulence and aggressiveness. However, sexual mating is possible in the laboratory; therefore, the genetic basis of host specificity would be worth investigating within the genus *Venturia*. The 96 progenies obtained in this study will permit the elucidation of genetic determinants of pathogenicity of these two pathogens. On the basis of the results of this study, we propose that *Venturia* isolates from *Malus* × *domestica* and from *Pyracantha* spp. be considered as two distinct special forms respectively named as *V. inaequalis* f. sp. *pomi* and *V. inaequalis* f. sp. *pyracanthae*.

ACKNOWLEDGMENTS

We thank A. Kollar and J. L. Notteghem for providing fungal isolates, M. Devaux and J. Gaudin for their helpful technical assistance, and J. P. Paulin for critical reading of the manuscript.

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TABLE 2. Incidence of the scab disease after inoculation of *Spilocaea pyracanthae* and *Venturia inaequalis* on six clones of *Pyracantha* spp.^a

<i>Pyracantha</i> spp. clones	<i>S. pyracanthae</i>	<i>V. inaequalis</i>
'Golden Charmer'	8.3	0
'Watereri'	13.8	0
'Mozart'	14.3	0
'Saphyr Rouge'	18.2	3.5
'Mohave'	33.3	0
'Sunshine'	61	0

^a Results, expressed in percentage of scabbed plantlets, are obtained from the whole inoculated plants of the three replicates.

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