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M. Dusabenyagasani · N. Lecours · R. C. Hamelin

Sequence-tagged sites (STS) for studies of molecular epidemiology of scleroderris canker of conifers

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Abstract Scleroderris canker is a very damaging disease of conifers caused by a fungal pathogen, *Gremmeniella abietina* var 'abietina'. This fungal pathogen is now known to comprise a number of distinct races and biotypes. In North America, two races, an indigenous North American race and an introduced European race, are present. In Europe, three distinct biotypes have been reported within the European race: one in the Alps, another in Fennoscandia, and a third that overlaps with the first two. We used random amplified microsatellites (RAMS) and DNA sequencing with arbitrary primer pairs (SWAPP) to design five PCR primer pairs flanking polymorphic regions of the genome of the European race of *G. abietina*. Length polymorphisms produced by repeats of basic units in microsatellites were distinguished by electrophoresis of PCR products in agarose gels, and point mutations were identified by low-ionic-strength single-strand conformation polymorphisms (LIS-SSCP). Some primers generated private alleles in the European biotype and the psychrophilic Alpine and Fennoscandian biotypes, i.e., alleles that were fixed within the two groups but polymorphic between them. Conversely, one pair of primers amplified at least 3, 4, and 7 alleles in the Fennoscandian, Alpine, and European biotypes, respectively. The Alpine and Fennoscandian biotypes, although geographically separated, were genetically more closely related to one another than to European biotype, which has an overlapping distribution. However, both Alpine and Fennoscandian biotypes have similar ecotypic adaptation. The evolution of these biotypes could be explained by their geographic separation following the end of the last glaciation.

Key words *Gremmeniella abietina* · LIS-SSCP · Microsatellite · PCR · Psychrophilic biotype · STS markers

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M. Dusabenbyagasani · N. Lecours · R. C. Hamelin (✉)
Natural Resources Canada, Canadian Forest Service,
Laurentian Forestry Centre, 1055 du PEPS,
PO Box 3800, Sainte-Foy, Quebec, Canada, G1V 4C7
Fax: +418 648-6956
E-mail: rhamelin@cfl.forestry.ca

Introduction

Scleroderris canker is a very damaging disease of conifers caused by a fungal pathogen, *Gremmeniella abietina* (Lagerb.) Morelet that occurs throughout the northern hemisphere. This fungus is subdivided into several taxonomic entities that have been characterized by various biochemical assays such as serology (Dorworth and Krywienczyk 1975; Dorworth et al. 1977), protein electrophoresis (Benhamou et al. 1984; Lecours et al. 1994), and DNA analysis (Hamelin et al. 1993; Bernier et al. 1994; Hellgren and Högberg 1995; Hamelin et al. 1996; Hamelin and Rail 1997). *Gremmeniella abietina* var 'abietina' (*G. a. abietina*) primarily attacks pines and is the most economically important pathogen within the *G. abietina* complex. It comprises three races: an Asian race in Japan, a North American race which is believed to be indigenous to North America, and a European race which was introduced into North America earlier this century (Dorworth et al. 1977). Since its introduction, the European race of *G. a. abietina* has been spreading in North America, most likely on nursery material and by rain-splashed conidia (Skilling 1969). It occurs now throughout northeastern North America and covers four American states and four Canadian provinces (Skilling et al. 1984, 1986; Laflamme and Lachance 1987). As a result of the severe damage caused by this fungus and its spreading potential, *G. a. abietina* was quarantined in several countries, including Canada and the northeastern United States,

to prevent further spread and new introductions (Barrett 1984).

Within the European race, three biotypes have been described and correlated to ecotypic adaptations as well as geographic origin in Europe (Morelet 1980; Uotila 1993; Hellgren and Högberg 1995; Hamelin et al. 1996). The Fennoscandian biotype (≡ Type B ≡ Small Tree Type) is only present at high altitudes or high latitudes in northern Europe, whereas the Alpine biotype (≡ var 'cembrae') is confined to the Alps at altitudes above 2000 m on *Pinus cembra*, *P. mugo*, *P. sylvestris*, and *Larix lyalli*. Both biotypes appear to be psychrophilic fungi since, like the North American race of *G. a. abietina*, they occur on shoots of seedlings or on low branches of trees covered with snow during the winter (Donaubauer 1972; Marosy et al. 1989; Hellgren and Högberg 1995). The European biotype (≡ Type A ≡ Large Tree Type) covers a much broader geographic range and has been reported throughout Europe from Fennoscandia, where it overlaps with the Fennoscandian biotype, to Italy and Switzerland, where it overlaps with the Alpine biotype. Although the three biotypes appear to be genetically differentiated based on DNA fingerprintings, the potential for hybridization between them has been reported (Hellgren and Högberg 1995; Hamelin et al. 1996; Hantula and Müller 1997; Uotila 1997). In North America, where the European biotype has been introduced, surveys have indicated the absence of the Fennoscandian and Alpine biotypes (Hamelin et al. 1996). However, the exact origin of the North American epidemic of scleroderris canker has not been determined. While DNA profiles showed that two distinct introductions took place, one in Newfoundland and the other one on continental North America, the most common DNA haplotype on continental North America was completely absent from Europe and Newfoundland, suggesting the possibility of different origins (Hamelin et al. 1997).

Studies on the genetic differentiation and molecular epidemiology of the different genetic variants of *G. a. abietina* have been mostly based on DNA fingerprinting approaches such as random amplified polymorphic DNA (RAPD; Hamelin et al. 1993, 1996) and random amplified microsatellites (RAMS; Hantula and Müller 1997). One of the weaknesses of these approaches is that pure genomic DNA is required to generate a DNA profile, and monospore fungal cultures must be obtained. This step reduces considerably the sample size that can be processed. In addition, culturing can induce a selection process that may not reflect the real makeup of the population. Another weakness of the DNA fingerprinting approaches is the inherent problem of comparing divergent DNA profiles between distant groups. Finally, even though DNA fingerprinting is appropriate to identify genetic variants, it may be not suitable to determine the evolutionary relationships between genetic variants, in part because of the potential for comigration of non-homologous RAPD markers (Quiros et al. 1995).

Population genetics studies using anonymous DNA markers combined with mutation detection techniques and direct sequencing are providing a powerful tool for testing hypotheses on population genetics and evolutionary relationships between populations and species and for studying epidemiological processes in plant pathogens (Lessa and Applebaum 1993; Bodenes et al. 1996; Liu et al. 1996; Bagley et al. 1997; Orti et al. 1997). These markers can provide not only allele frequencies, but also allelic sequence data at a population level and allelic phylogeny, and reveal more precisely processes such as migration and recolonization, population bottlenecks, and mutations (Huttley et al. 1997; Petit et al. 1997). The aim of the study described here was to develop sequence-tagged sites (STS) (Olson et al. 1989) combined with mutation detection techniques and identify sets of polymerase chain reaction (PCR) primers flanking polymorphic regions at various hierarchical levels within the European race of *G. a. abietina* in Europe and North America.

Material and methods

Fungal strains

For the development of STS markers, representative strains of the three biotypes of the European race of *G. a. abietina* previously identified (Hamelin et al. 1996) were used (Table 1). Fungal culture and DNA extraction were as previously described (Hamelin et al. 1996).

Marker development

We used 5′-end-anchored simple-sequence repeat (SSR) primers 5′-BDB9(ACA)5, 5′-DHB(CGA) 5, and 5′-YHY(GT) 7G (Hantula et al. 1996) and 11 primer pairs including ITS3 and ML6 (White et al. 1990) to generate DNA fingerprints according to amplification conditions previously described for random amplified microsatellites (RAMS) and sequencing with arbitrary primer pairs (SWAPP) (Burt et al. 1994; Hantula et al. 1996). PCR products were separated on agarose gels, and putative polymorphic DNA fragments from a same genomic region were sampled using a sterile pipette tip. DNA fragments from RAMS were reamplified in the same reaction conditions as the first PCR amplification. Restriction enzymes with a single cleavage site in the PCR product were then identified and used to digest amplicons. This generated DNA fragments with a single priming site for direct sequencing. The two fragments were separated in agarose gels, purified from the gels with the QIA quick gel extraction kit (Quiagen GmbH, Hilden, Germany), and sequenced with an ABI automatic sequencer. PCR products from SWAPP reactions were either directly purified with the QIA quick PCR purification kit (Quiagen GmbH, Hilden, Germany) or purified from an agarose gel and sequenced as above. When sequence polymorphisms were identified between strains, internal PCR primers flanking the polymorphic region were designed. Potential primers were analyzed for likelihood of primer-dimers, primer-loops, and hair-pin formation using the Generunner software.

Amplification and DNA sequencing of markers

The mutations previously observed in the amplified fragments were confirmed by using the new set of primers to amplify and sequence amplicons from the selected strains. All strains listed in Table 1 were then assayed with the new primers to verify if there was variation between or within biotypes. The cycling protocol for the first four loci (Table 2) consisted of a first hot start step at 99°C for 10 min, followed by a 35-cycle second step, 94°C for 30 s, 58°C (54°C: ACA9 locus) for 1 min, and 68°C for 2 min, and a final extension step at 68°C for 10 min. For the last locus, the cyclin g protocol consisted of a first hot start step at 99°C for 10 min, followed by a 35-cycle second step, 92°C for 30 s, 63°C for 30 s, and 72°C for 2 min, and a final extension step at 72°C for 10 min. Some PCR products containing length polymorphisms were separated by electrophoresis in an agarose-synergel (Diversified Biotech, Newton Centre, Mass.) gel (1.5% + 1.5%) in TRIS-phosphate EDTA (TPE 0.5×) buffer. To identify point mutations or small indels, low-ionic-strength single-strand confirmation polymorphism (LIS-SSCP) (Maruya et al. 1996) was performed with 1/10 dilution of the PCR product at 4°C, 22°C, or 32°C with 8% acrylamide gels in TRIS-borate EDTA (TBE 5×) buffer. One DNA marker was digested with *Nla*III, and DNA fragments were separated by electrophoresis in a (1.5% + 1.5%) agarose-synergel gel.

Table 1 Characteristics of the *Gremmeniella abietina* strains used

Strains	Origin	Biotype[a]
P80	Germany, Rendsburg	European
P86	Germany, Rendsburg	European
P85	Germany, Nordhorn	European
P82	Germany, Schmalenbeck	European
P89	Germany, Schmalenbeck	European
P83	Germany, Haard	European
NF87-0492	Canada, Newfoundland	European
NF87-0494	Canada, Newfoundland	European
NF87-0500	Canada, Newfoundland	European
US15	United States, Vermont	European
CF87-0032	Canada, Quebec, L'Ascension	European
CF87-0036	Canada, Quebec, Lac Saguay	European
CF88-0007	Canada, Quebec, Chute St-Philippe	European
J5	Italy, Adriatic coast	European
J7	Italy, Ortesi, Alps	Alpine
M1019	Switzerland, Chilchenberg, Alps	Alpine
M1023	Switzerland, Chilchenberg, Alps	Alpine
M1024	Switzerland, Ahorni, Alps	Alpine
M1042	Switzerland, Ahorni, Alps	Alpine
Fi0003	Finland, Ylikiminki	Fennoscandian
A139p7	Sweden, Adak, Västerbotten	Fennoscandian
A319p1	Sweden, Adak, Västerbotten	Fennoscandian
SL229p3	Sweden, Adak, Västerbotten	Fennoscandian
SL149s	Sweden, Springliden, Västerbotten	Fennoscandian
SL548p1	Sweden, Springliden, Västerbotten	Fennoscandian

[a] Biotypes were determined based on RAPD profiles as previously described (Hamelin et al. 1996)

Results

Five STS markers showing various levels of polymorphisms were generated. Three of these markers had only 2 alleles, one common to the Alpine and Fennoscandian biotypes and a second one present only in the European biotype (Table 2). One of these markers, CGA3, contained a 6-bp insertion and two point mutations in the Alpine and Fennoscandian biotypes. This insertion was detectable on an agarose gel and was the result of a microsatellite, AATGAG unit, which occurred three times in the European biotype and four times in the Alpine and Fennoscandian biotypes (Table 2, Fig. 1). When PCR products from all 25 strains were analyzed by LIS-SSCP, however, no other mutations or microsatellite polymorphisms were detected within the three biotypes.

Markers GT6 and MLITS also contained only 2 alleles, one common to the Alpine and Fennoscandian biotypes and the second one only present in the European biotype (Table 2). These 2 alleles differed by a single basepair substitution. For marker MLITS, an extra *Nla*III restriction site was present in the Alpine and Fennoscandian biotypes and allowed differentiation by digestion of the PCR product followed by gel electrophoresis (Fig. 2). The 2 different alleles in both markers were clearly separated by LIS-SSCP (Fig. 3).

Two of the five loci, CGA6 and ACA9, were particularly informative since they yielded multiple alleles in all biotypes. A total of 14 putative alleles were detected for CGA6 among the 25 strains studied (Table 2). Both indels and point mutations were observed in DNA sequences of that region of the genome. These indels often resulted in length polymorphisms when the PCR

Table 2 Characteristics of primers designed in this study

Putative locus	Number of alleles	Primer	Sequence	Tm[a] (°C)	Size of PCR product (bp)
CGA3	2	GAcga3-350D	5′ gttacctctgcctctctct 3′	58	
		GAcga3-int	5′ atgagatgccgcttgccaa 3′	58	214-220
CGA6	14	GAcga6-61f	5′ cttcgaagatcggaggacgg 3′	64	
		GAcga6-511rc	5′ cagagccagcggatgatatac 3′	64	380-420
GT6	2	GAgt6-134f	5′ ctctcgtccatgcgtctcc 3′	58	
		GAgt6-568rc	5′ aaaaggtgagactatgatgcag 3′	56	456
ACA9	5	GAaca9-452f	5′ acatgggatgcatacgatg 3′	56	
		GAaca9-800rc	5′ agacgagacagacacagtac 3′	58	386-400
MLITS	2	GAml6its3-11f	5′ tgcatagggtcgtatacgca 3′	60	
		GAml6its3-280rc	5′ aaaaagaagcatcgcagaggc 3′	62	290

[a] Tm was calculated using the CG/AT method from the Generunner software

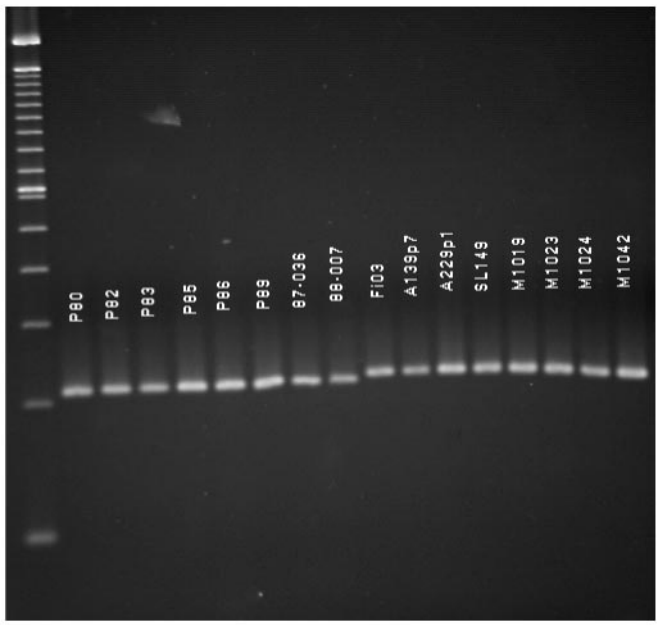

Fig. 1 Agarose-Synergel electrophoresis of *Gremmeniella abietina* PCR products amplified by GAcga-350D and GAcga-int primers. *Lane 1* 100-bp molecular-weight ladder, *lanes 2–9* strains of the European biotype P80, P82, P83, P85, P86, P89, CF87-0036, and CF87-0036, and CF88-0007, respectively, *lanes 10–13* strains of the Fennoscandian biotype, Fi0003, A139p7, A229p3, and SL149s, respectively, *lanes 14–17* strains of the Alpine biotype, M1019, M1023, M1024, and M1042, respectively

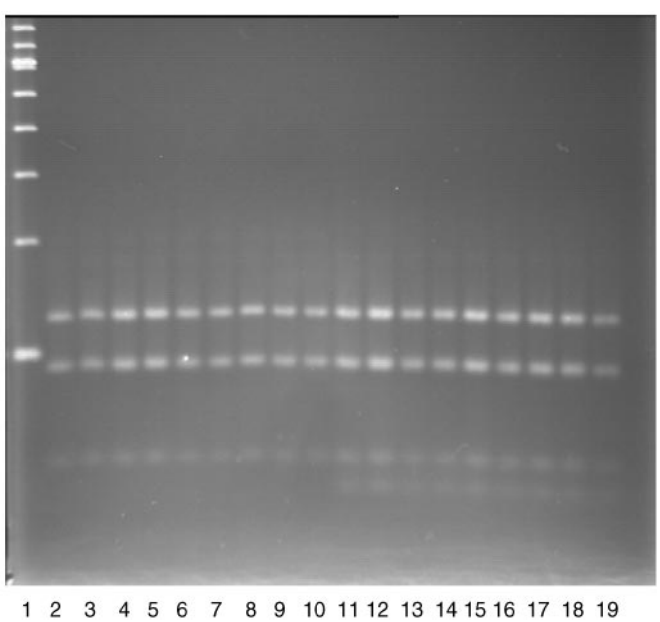

Fig. 2 Agarose-Synergel electrophoresis of *Gremmeniella abietina* *Nla*III digests from PCR products amplified by GAm16its3-11f and GAm16its3-280rc primers. *Lane 1* 100-bp molecular-weight ladder, *lanes 2–10* strains of the European biotype, P80, P85, P86, P89, NF87-0492, NF87-0494, NF87-0500, US15, and CF87-0032, respectively, *lanes 11–15* strains of the Alpine biotype, J7, M1019, M1023, M1024, and M1042, respectively, *lanes 16–19* strains of the Fennoscandian biotype, Fi0003, A139p7, A319p1, and SL548p1, respectively

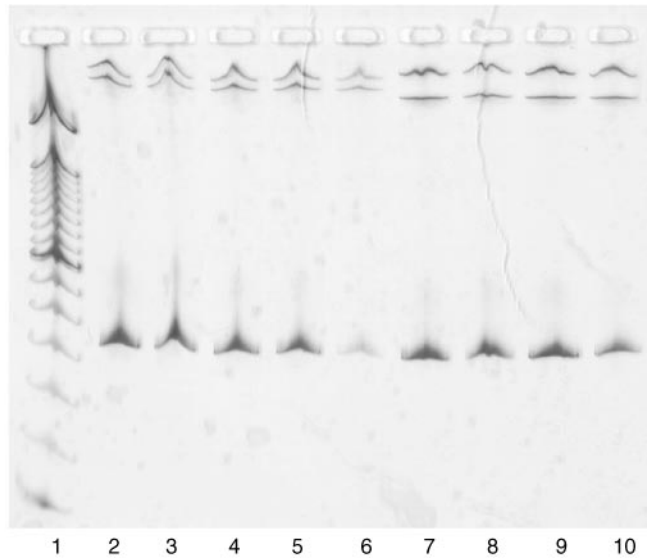

Fig. 3 LIS-SSCP analysis of *Gremmeniella abietina* PCR products amplified by GAgt6-134F and gt6-568rc primers. *Lane 1* 100-bp molecular-weight ladder, *lanes 2–6* strains of the European biotype, P85, P86, P89, NF87-0500, and US15, respectively, *lanes 7*, *8* strains of the Alpine biotype, J7 and M1024, *lanes 9*, *10* strains of the Fennoscandian biotype, Fi0003 and A139p7

products were electrophoresed in agarose gels. Seven different alleles were observed in the European biotype, 4 alleles in the Alpine biotype, and 3 alleles in the Fennoscandian biotype (Fig. 4). The 7 alleles present in the European biotype were represented by single strains such as P80, P85, P86, NF87-0500, and J5. Other alleles were present in more than 1 strain, sometimes from different continents. For example, P89 is from Europe and NF87-0492 and NF87-0494 are from North America, but all shared the same allele at locus CGA6. Similarly, US15 from the United States and CF87-0032 and CF88-0007 from Canada also had a common allele. All Alpine and Fennoscandian strains possessed unique alleles for marker CGA6 (Fig. 4). At locus ACA9, sequences were obtained for 6 strains. Five alleles were detected, and 1 of them was common to the Alpine and European biotypes and 2 alleles were detected in the Fennoscandian biotype (Tables 2 and 4, Fig. 5).

Sequence data showed that the genomic region flanked by primers GAcga6-61f and GAcga6-511rc (marker CGA6) was rich in polymorphic microsatellites such as (G)n/G:A, (GGCA)n, (GACA)n, (CAGA)n/G:C, and (ATCT)n (Table 3). Strains belonging to all three biotypes of the European race were polymorphic with regard to several of these repeats. For example, the GGCA motif was repeated 8-16 times in the European biotype but only six times in the Fennoscandian and Alpine biotypes (Table 3). ATCT was repeated 6–16 times in the European biotype, 13 or 14 times in the Alpine biotype, and 10 or 14 times in the Fennoscandian

biotype. Several repeat units were also found at the ACA9 locus (Table 4). For example, TCCAC was repeated four times in the Fennoscandian biotype, three or five times in the Alpine biotype, and three times in the European biotype. Sequence data from the ACA9 locus also indicated that Fi0003, a strain from Finland belonging to the Fennoscandian biotype, and US15, a strain from the United States belonging to the European biotype, had identical microsatellites except for a single additional TCCAC repeat in Fi0003.

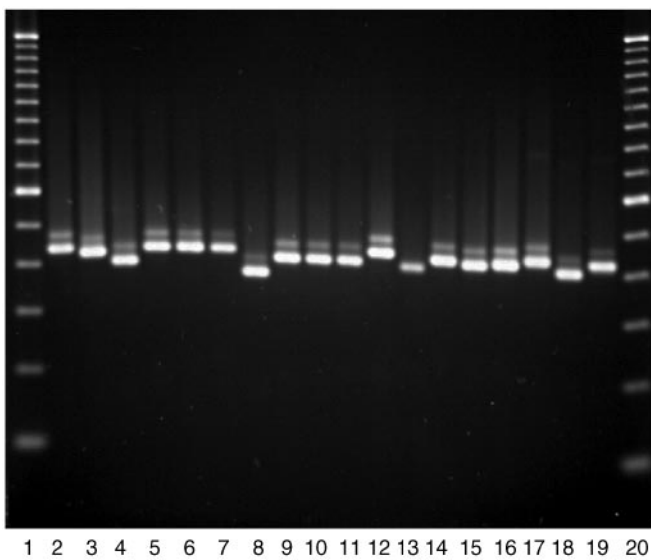

Fig. 4 Agarose-Synergel electrophoresis of *Gremmeniella abietina* PCR products amplified by GAcga6-61f and GAcga6-511rc primers. *Lane 1* 100-bp molecular-weight ladder, *lanes 2–12* strains of the European biotype P80, P85, P86, P89, NF87-0492, NF87-0494, NF87-0500, US15, CF87-0032, CF88-0007, and J5, respectively, *lanes 13–16* strains of the Alpine biotype, J7, M1019, M1024, and M1042, respectively, *lanes 17–19* strains of the Fennoscandian biotype, Fi0003, A139p7, and A319p1, respectively

Discussion

Three differentiated biotypes of *G. a. abietina* have been reported in Europe (Morelet 1980; Hellgren and

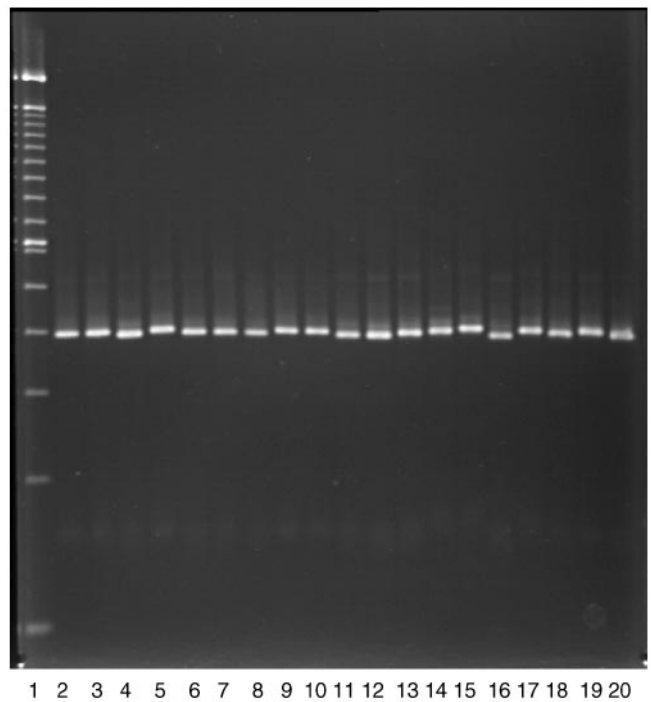

Fig. 5 Agarose-synergel electrophoresis of *Gremmeniella abietina* PCR products amplified by GAaca9-452f and GAaca9-800rc primers. *Lane 1* 100-bp molecular-weight ladder *lanes 2–11* strains of the European biotype, P80, P85, P86, P89, NF87-0492, NF87-0494, NF87-0500, US15, CF87-0032, and J5, respectively, *lanes 12–16* strains of the Alpine biotype, J7, M1019, M10124, and M1042, respectively, *lanes 17–20* strains of the Fennoscandian biotype Fi0003, A139p7, A319p1, and SL548p1, respectively

Table 3 Polymorphic microsatellites amplified by CGA6 primers

Samples	Microsatellites				
	(G)n/G:A[a]	(GGCA)n	(GACA)n	(CAGA)n/G:C[a]	(ATCT)n
Alpine biotype					
M1019	14	6	10	5	14
M1024	9	6	10	5	14
M1042	10	6	10	5	14
J7	14	6	6	5	13
Fennoscandian biotype					
A139p7	14	6	9	5	10
Fi0003	14	6	10	5	14
European biotype					
P80	6	16	8	5	11
P85	6	8	8	5	16
P86	6	10	10	5	6
J5	6	16	8	5	11
NF87-0492	6	11	8	5	15
NF87-0500	6	12	7	0	6
US15	6	10	9	5	11

[a] Imperfect microsatellite; the nucleotide to the left of the colon is substituted at least once by the nucleotide to the right of the colon

Table 4 Polymorphic microsatellites amplified by ACA9 primers

Strains	Microsatellites				
	(G) n	(CCAA) n/C : T [a]	(A) n/A : G [a]	(TAGA)n	(TCCAC)n
Alpine biotype					
M1024	10	6	16	4	5
M1042	11	6	16	3	3
Fennoscandian biotype					
A139	11	6	16	4	4
Fi0003	11	5	19	4	4
European biotype					
P86	11	6	16	3	3
US15	11	5	19	4	3

[a] Imperfect microsatellite; the nucleotide to the left of the colon is substituted at least once by the nucleotide to the right of the colon

Högberg 1995; Hamelin et al. 1996). The Alpine and Fennoscandian biotypes are geographically isolated but share very similar ecological adaptations. They appear to be adapted to snowy conditions and are restricted to ecological niches in the Alps and Fennoscandia characterized by cold winters with long-lasting snow cover (Hellgren and Högberg 1995). These biotypes were grouped together by the first axis in a principal component analysis based on RAPD (Hamelin et al. 1996). In contrast, the European biotype occurs throughout Europe from Fennoscandia to the Adriatic coast and overlaps with the other two biotypes. Our survey with anonymous DNA markers supports the hypothesis that the Alpine and Fennoscandian biotypes are evolutionarily closely related but that the European biotype is a more distant taxon (Petrini et al. 1990).

The presence of identical alleles in samples from Newfoundland and Europe and the lack of shared alleles in samples from continental North America and Europe is consistent with recent DNA fingerprinting studies suggesting distinct introductions of the European biotype into Newfoundland and continental North America, possibly from different origins (Hamelin et al. 1997). Nevertheless, the presence of common alleles between the European and Alpine biotypes, and of identical microsatellite repeats between the European and Fennoscandian biotypes, suggests the possibility of hybridization and introgression between these biotypes (Uotila 1997).

Sequence analyses of the rDNA-ITS (Hamelin and Rail 1997) and of β-tubulin, glyceraldehyde 3-phosphate dehydrogenase (GPD), and mtDNA (Hamelin and Dusabenyagasani, unpublished) failed to reveal any sequence divergence in coding regions and introns among the Alpine, European, and Fennoscandian biotypes. The time of divergence between these biotypes might be very recent. However, markers developed in the current study apparently sampled regions evolving at different rates. Three of these markers, CGA3, GT6, and MLITS, were more variable than the rDNA-ITS, ß-tubulin, mtDNA, and GPD and indicated divergence between biotypes, but were more conserved than markers CGA6 and ACA9. The pattern of sequence divergence in the three more conserved markers (CGA3, GT6 and MLTIS) suggests that all three biotypes share a common ancestor but that Alpine and Fennoscandian biotypes diverged much more recently. One possible explanation is that these two biotypes may have shared a common ancestor during glaciation but diverged as the psychrophilic ancestor was isolated in the Alps and northern Europe.

The two highly polymorphic markers, CGA6 and ACA9, apparently represent genomic regions that are evolving at a much faster rate than the other three markers in the present study or the rDNA-ITS (Hamelin and Rail 1997). These two loci could be very informative with regard to the molecular epidemiology of scleroderris canker. The distribution of the different alleles throughout the geographic range of the disease could indicate the dissemination pattern of the pathogen, particularly in North America where the introduction event occurred fairly recently (Dorworth et al. 1977).

Some alleles could be clearly separated on agarose gels with or without prior digestion with a restriction enzyme. On the other hand SSCP (Orita et al. 1989), a technique widely used in medical diagnostics, proved to be a more powerful tool to detect indels as well as point mutations. For example, we have successfully differentiated samples with a single point base substitution in a DNA fragment of 456bp. Therefore, this technique could be easily applied to determine biotypes of unknown samples of *G. a. abietina*. SSCP was also useful in some strains where indels that occurred at different sites within the marker could compensate for one another and produced whole DNA fragments differing in length by a few base pairs but possessing very divergent sequences. For example, the length difference between PCR products from P86 and A139p7 samples was 4bp because the four repeats inserted at the GGCA microsatellite in P86 was compensated for by a deletion of the same length at the ATCT microsatellite and *vice versa* in the case of A139p7 (results not shown).

We did not observe an obvious correlation between microsatellite length and the degree of polymorphism as has been observed in some plant species (Smulders et al. 1997; Thomas and Scott 1993). Four of the ten microsatellite regions in the CGA6 and ACA9 DNA fragments were imperfect microsatellites because some bases of the repeated units were substituted. Since DNA regions with imperfect microsatellites displayed reduced variation compared with those with perfect repeats, our results support the hypothesis that long microsatellites are stabilized by the accumulation of point mutations. These point mutations decrease the likelihood of slippage and, thereby, the microsatellite might remain unchanged over a long period of time (Vosman and Arens 1997).

From a practical standpoint, the three conserved markers identified in this work are useful for diagnostic purposes, while the two more polymorphic markers are useful in various epidemiological studies. One of the advantages of using the markers developed here for population studies is that they can be used directly with the very small (< 1 mm) fruiting bodies. Previous techniques required single spore isolation and cultures, a tedious process that may prevent large population sampling.

The DNA markers sampled by this study showed that despite geographic separation, Alpine and Fennoscandian biotypes are more closely related than their European counterpart which, however, has an overlapping geographical distribution. Our results are in agreement with those from observations of spore septation (Morelet 1980; Uotila 1993) and soluble protein electrophoresis (Petrini et al. 1990). They also support the hypothesis of a more recent common ancestor of the Alpine and Fennoscandian biotypes and a more distant one with the European biotype. Larger sample sizes for the three biotypes should be carried out for that hypothesis to be validated.

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