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Genetic characterization of *Gremmeniella abietina* var. *abietina* isolates from Spain

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The taxonomic placement of Spanish isolates of *Gremmeniella abietina* var. *abietina* (Gaa) (race and biotype) was determined, for the first time, using RAPD markers and comparison of RAPD patterns for Spanish isolates and those originating from different regions of Europe and North America. Fifteen Spanish isolates of Gaa were isolated from recently dead shoots of 10 *Pinus halepensis* trees. Additionally, 17 isolates of Gaa from Canada, Finland, Switzerland and the USA, previously characterized according to race and biotype, were used. Five primers, previously reported to yield readable, reproducible and polymorphic fragments, were used in PCR reactions. The results showed that the Spanish isolates represent the European race and not the Alpine or northern biotypes. Spanish isolates appear to be clearly separated from all other biotypes within the EU race and preliminary data suggest that Spanish isolates have low genetic variability.

Keywords: biotype, *Pinus halepensis*, race, RAPDs, scleroderris canker

Introduction

Gremmeniella abietina (anamorph *Brunchorstia pinea*) is a haploid ascomycete that causes stem canker (scleroderris canker) and shoot dieback on many conifer species (Donaubauer, 1972). The pathogen has been responsible for the destruction of many plantations in north and central Europe, North America and east Asia in recent decades (Yokota *et al*., 1974; Dorworth, 1979; Kaitera & Jalkanen, 1992, 1994). In Spain *G. abietina* was first reported on *Pinus pinaster* causing seedling mortality in 1929 (Martínez, 1933). It was not recorded again until 1999, when dieback caused by *G. abietina* var. *abietina* (Gaa) was seen on *P. halepensis* (Santamaría *et al*., 2003), which is currently undergoing a severe decline in Spain. The disease symptoms on *P. halepensis* include drying of needles and branches with some distortion of terminal twigs. This results in dieback and sometimes death of trees of all ages. Pycnidia are common on affected twigs and branches, but apothecia have not been seen in the field (Santamaría *et al*., 2003).

Three races, Asian, European (EU) and North American (NA), have been distinguished within Gaa (Petrini *et al*., 1989). These have been defined based on conidial morphology (Dorworth & Krywienczyk, 1975; Petrini *et al*., 1989), serology (Dorworth & Krywienczyk, 1975), soluble protein electrophoresis (Petrini *et al*., 1990; Lecours *et al*., 1994), FAST profiles (Müller & Uotila, 1997) and genetic markers (Hamelin *et al*., 1993, 1996; Bernier *et al*., 1994; Hantula & Müller, 1997; Dusabenyagasani *et al*., 2002). The Asian race has been reported only in Japan, where it causes damage to *Abies sacchalienensis* (Yokota *et al*., 1974). The NA race occurs in North America, mainly in natural stands of *P. banksiana* and *P. contorta* (Laflamme, 1993), where it affects only small trees or the lower portion of the crowns of adult trees that remain under snow cover during the winter. In the field, both conidia and ascospores are produced by both the Asian and NA races (Skilling, 1969; Yokota *et al*., 1974).

The EU race of Gaa is widely distributed throughout Europe. In 1977, it was recorded in northeastern North America (Skilling, 1977) where it occurs on *Pinus*, *Picea* and *Larix* spp. (Butin, 1995). Three biotypes have been distinguished amongst isolates of the EU race based on symptomatology (Uotila, 1983, 1993; Hellgren, 1995; Hellgren & Högberg, 1995), conidial morphology and growth rates (Morelet, 1980; Uotila, 1983; Hellgren, 1995; Hellgren & Högberg, 1995; Kaitera & Jalkanen, 1996), serology (Petäistö *et al*., 1996), FAST profiles (Müller & Uotila, 1997) and molecular markers (Hamelin *et al*., 1993, 1996; Hellgren & Högberg, 1995; Hamelin & Rail, 1997; Hantula & Müller, 1997; Dusabenyagasani *et al*., 1998).

The Alpine biotype (previously known as *Brunchorstia pinea* var. *cembrae*) has been recorded only in the Alps at altitudes above 2000 m on *P. cembra*, *P. mugo* and *L. lyallii*.

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The Northern biotype (Fennoscandian biotype, type B, 'STT' small tree type) occurs most frequently at high latitudes in northern Europe and mainly on *Picea abies*, *Pinus sylvestris* and *P. contorta*. Symptoms caused by this biotype resemble those caused by the NA race (Karlman *et al*., 1994). The European biotype (type A, 'LTT' large tree type) causes more severe damage in mature pines than the other biotypes (Uotila, 1990) and is more widely distributed geographically and ecologically. The European biotype produces conidia frequently, while ascospores are less common in the field (Uotila, 1992).

Analysis of randomly amplified polymorphic DNA (RAPD) markers has been used previously for genetic analysis of Gaa (Hansson *et al*., 1996; Wang, 1997; Hamelin *et al*., 1998). Some RAPD markers are correlated with races and biotypes of the pathogen (Hamelin *et al*., 1993, 1996).

The main objective of the present study was to determine the taxonomic placement of Spanish isolates of Gaa (race and biotype) using RAPD markers and by comparing RAPD patterns between the Spanish isolates and those originating in Europe and North America.

Materials and methods

Isolates

Affected twigs were collected from four planted *P. halepensis* stands located in Valle de Cerrato, Palencia (UTM, 4640475, 386450), Astudillo 1, Palencia (UTM, 4671775, 393550), Astudillo 2, Palencia (UTM, 4667465, 390225) and Matallana, Valladolid (UTM, 4620800, 391565) in 2001, 2002 and 2003 (Santamaría *et al*., 2003). The stands were located in northwest Spain at altitudes ranging from 800 to 900 m and in transitional areas where both evergreen sclerophyll broadleaf and coniferous forest occur within the temperate zone, and where the soil is less than 50 cm thick and contains limestone components with a basic pH. Hot, dry summers, and frequent frosts in winter are common in these areas, although snow cover is rare.

One- or 2-year-old recently killed shoots, located 3–4 m above the ground and bearing pycnidia of Gaa, were collected from the outside of the canopy of 10 trees (40 years old) with symptoms of scleroderris canker. The disease symptoms on *P. halepensis* included drying of needles and branches, with some distortion of terminal twigs, resulting in dieback and sometimes death of trees of all ages. Pycnidia were common on affected twigs and branches, but apothecia were not seen. *Gremmeniella abietina* var. *abientina* was isolated either from pycnidia or from small pieces of wood adjacent to pycnidia.

The wood samples and pycnidia were surface-disinfested with 2% sodium hypochlorite and rinsed twice in sterilized water prior to inoculations. Wood samples were then transferred to 1·5% malt agar with pine needle extract in Petri dishes (Kurkela, 1979) and incubated at 15°C. A droplet of conidia from each pycnidium, opened with a needle, was transferred uniformly onto the same medium and incubated in a similar way. Three single conidial isolates derived from a single pycnidium (091P1P, 091P2P and 091P3P) and four mycelial isolates derived from a single twig (0911P, 0912P, 0921P and 0922P) were selected in order to test genetic stability through mitosis and the absence of contamination with unrelated spores.

Another eight Spanish isolates were selected for the assays (Table 1). Seventeen isolates of Gaa from Canada, Finland, Switzerland and the USA, previously isolated from single pycnidia or apothecia and characterized according to race and biotype (Table 1), were included for comparison with the Spanish isolates. All 32 isolates were grown for 6 weeks on sterile potato dextrose broth (PDB) at 15°C in the dark, after which time the mycelium was harvested, lyophilized and used immediately or stored at –20°C until needed.

DNA extraction

DNA was extracted following the protocol of Möller *et al*. (1992). Approximately 10 mg of lyophilized mycelium were ground in a 1·5 mL microtube using disposable plastic pestles. Five hundred microlitres of extraction buffer [100 mM Tris-HCl (pH 8), 10 mM EDTA, 2% SDS, 1 mg proteinase K mL^{-1}] were added to the mycelium and incubated at 60°C for 60 min. After CTAB 10% (cetyltrimethyl-ammonium bromide) precipitation and chloroform-isoamyl alcohol (24:1) extraction, the mixture was centrifuged for 5 min at 12 000 ***g***. The upper phase was mixed with 225 μL ammonium acetate (5 M) and centrifuged again. DNA was precipitated with isopropanol. The resulting pellet was washed twice with 70% ice-cold ethanol, dried and resuspended in 50 μL of sterile distilled water. All DNA was diluted 1:50 in sterile distilled water prior to amplification.

DNA amplification

Amplifications were performed in volumes of 25 μL containing 1× PCR buffer II [100 mM Tris HCl pH 8·3; 500 mM KCl, autoclaved; GeneAmp PCR Core Reagents (Applied Biosystems), 200 μM of each dNTP (Applied Biosystems), 0·2 μM of the specific oligonucleotides, 2 mM $MgCl_2$, 1 U AmpliTaq® DNA Polymerase (Applied Biosystems) and approximately 10 ng of genomic DNA]. PCR amplifications were carried out in a thermal cycler PTC 100 (MJ Research Inc.) programmed as described by Hamelin *et al*. (1996): a denaturation step at 94°C for 3 min, followed by one cycle at 35°C for 4 min and 72°C for 2 min, then 45 cycles of 94°C for 1 min, 35°C for 1 min and 72°C for 2 min. The reactions ended with a 10-min extension step at 72°C.

PCR products were analysed by electrophoresis on 1·2% agarose gels in 1× TAE buffer at 3 V cm^{-1}. The gels were stained with ethidium bromide and RAPD profiles were visualized under UV light. Five oligonucleotides (10-mers from Qiagen Operon) were used for the amplifications: OPA03 (5′-AGTCAGCCAC-3′), OPA08 (5′-GTGACGTAGG-3′), OPA12 (5′-TCGGCGATAG-3′),

Table 1 *Gremmeniella abietina* var. *abietina* isolates used in this study

Isolate number	Previous number	Origin	Year of isolation	Host	Race[a]/ biotype
001P	–	Spain: Valle de Cerrato (Palencia)	2001	*Pinus halepensis*	ND
002P	–	Spain: Valle de Cerrato (Palencia)	2001	*Pinus halepensis*	ND
003P	–	Spain: Valle de Cerrato (Palencia)	2002	*Pinus halepensis*	ND
004P	–	Spain: Valle de Cerrato (Palencia)	2002	*Pinus halepensis*	ND
005P	–	Spain: Valle de Cerrato (Palencia)	2002	*Pinus halepensis*	ND
006P	–	Spain: Valle de Cerrato (Palencia)	2003	*Pinus halepensis*	ND
06P	–	Spain: Astudillo 1 (Palencia)	2003	*Pinus halepensis*	ND
0911P	–	Spain: Astudillo 2 (Palencia)	2003	*Pinus halepensis*	ND
0912P	–	Spain: Astudillo 2 (Palencia)	2003	*Pinus halepensis*	ND
0921P	–	Spain: Astudillo 2 (Palencia)	2003	*Pinus halepensis*	ND
0922P	–	Spain: Astudillo 2 (Palencia)	2003	*Pinus halepensis*	ND
091P1P	–	Spain: Astudillo 2 (Palencia)	2003	*Pinus halepensis*	ND
091P2P	–	Spain: Astudillo 2 (Palencia)	2003	*Pinus halepensis*	ND
091P3P	–	Spain: Astudillo 2 (Palencia)	2003	*Pinus halepensis*	ND
VA1	–	Spain: Villalba de los Alcores (Valladolid)	2003	*Pinus halepensis*	ND
FI1	Siika 1·7	Finland: Siikakangas (Ruovesi)	1997	*Pinus sylvestris*	Eu/Northern[b]
FI2	Siika 1·2	Finland: Siikakangas (Ruovesi)	1997	*Pinus sylvestris*	Eu/Northern[b]
FI3	Valkea 1·1	Finland: Valkeajärvi (Ruovesi)	1997	*Pinus sylvestris*	Eu/European[b]
FI4	Valkea 1·8	Finland: Valkeajärvi (Ruovesi)	1997	*Pinus sylvestris*	Eu/European[b]
SZ1	M1047	Switzerland: Brienzer Rothorn	1982	*Pinus mugo*	Eu/Alpine[c,d]
SZ2	M1027	Switzerland: Chilchenberg	1992	*Pinus cembra*	Eu/Alpine[c]
SZ3	M1061	Switzerland: Val die Lei	1982	*Pinus cembra*	Eu/Alpine[c,d]
SZ4	M1019	Switzerland: Chilchenberg	1982	*Pinus mugo*	Eu/Alpine[c,d]
USA1	US-79-0045	USA: Franklin (New York)	1979	*Pinus resinosa*	Eu[e]
USA2	US-79-0048	USA: St-Lawrence Co. (New York)	1979	*Pinus resinosa*	Eu[e]
USA3	US-81-0111	USA: Watson, Lewis Co. (New York)	1981	*Pinus resinosa*	Eu[e]
USA4	US-81-0109	USA: Diana, Lewis Co. (New York)	1981	*Pinus resinosa*	Eu[e]
USA5	US-81-0105	USA: Russia, Herkimer Co. (New York)	1981	*Pinus resinosa*	Eu[e]
USA6	US-78-0052	USA: Bayfield Co. (Wisconsin)	1978	*Pinus resinosa*	NA[e]
USA7	US-78-0061	USA: Bayfield Co. (Wisconsin)	1978	*Pinus resinosa*	NA[e]
USA8	US-78-0072	USA: Birchgrove, Bayfield Co. (Wisconsin)	1978	*Pinus resinosa*	NA[e]
CA1	CF-91-0032	Canada: Ste-Anne du Lac (Québec)	1991	*Pinus banksiana*	NA[e]

ND, not determined.
[a]Race: Eu, European; NA, North American.
[b]Biotype designation determined by random amplified microsatellites (A. Uotila, Hyytiälän Metsäasema, Hyytiäläntie 124, 355 00 Korkeakoski, Finland, personal communication).
[c]Biotype designation determined by soluble protein electrophoresis (Petrini *et al.*, 1990).
[d]Biotype designation determined by RAPDs and internal transcribed spacers of the ribosomal DNA repeat subunit amplified by PCR and digested with the restriction enzymes *Hae*III and *Msp*I (Hamelin *et al.*, 1996).
[e]Race designation determined by RAPDs (Hamelin *et al.*, 1993).

OPC08 (5′-TGGACCGGTG-3′) and OPD05 (5′-TGAGCGGACA-3′). These primers were selected because they had yielded reproducible intra/interracial polymorphisms in previous work (Hamelin *et al.*, 1993, 1996, 1998). Fragment lengths were estimated by comparing them to a 100-bp DNA ladder (Pharmacia) using AlphaEaseFC image analysis software (V.3·1, Alpha Innotech Corp.).

Data analysis

Genetic distance matrices were obtained by using a Dice index (Nei & Li, 1979) with the RAPDistance software, version 1·04, for the analysis of patterns of RAPD fragments (J. Armstrong, A. Gibbs, R. Peakall, G Weiller, Australian National University, Canberra). Neighbour joining and upgma analysis were performed with phylip software (version 3·5c) and resulting trees were visualized with Treeview32 software (J. Felsenstein, Department of Genetics, University of Washington, Seattle).

Results

The five oligonucleotides produced polymorphic and distinguishable fragment patterns, which are shown in Table 2 and Fig. 1. The 16 PCR fragments selected as RAPD markers, ranging from 250 to 1800 bp, appeared as bright bands after gel electrophoresis and were consistently amplified in at least three PCR reactions.

Primer OPA03 allowed differentiation between the EU and NA races, producing highly reproducible patterns

Table 2 Randomly amplified polymorphic DNA (RAPD) profiles for *Gremmeniella abietina* var. *abietina* isolates

	RAPD markers[a]															
Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
001P	1[b]	0	0	1	0	1	0	0	1	0	0	1	1	0	0	0
002P	1	0	0	1	0	1	0	0	1	0	0	1	1	0	0	0
003P	0	0	0	1	0	0	0	0	1	0	0	1	1	0	0	0
004P	0	0	0	1	0	1	0	0	1	0	0	1	1	0	0	0
005P	0	0	0	1	0	1	0	0	1	0	0	1	1	0	0	0
006P	0	0	0	1	0	1	0	0	1	0	0	1	1	0	0	0
06P	1	0	0	1	0	0	0	0	1	0	0	1	1	0	0	0
0911P	1	0	0	1	0	1	0	0	1	0	0	1	1	0	0	0
0912P	1	0	0	1	0	1	0	0	1	0	0	1	1	0	0	0
0921P	1	0	0	1	0	1	0	0	1	0	0	1	1	0	0	0
0922P	1	0	0	1	0	1	0	0	1	0	0	1	1	0	0	0
091P1P	1	0	0	1	0	1	0	0	1	0	0	1	1	0	0	0
091P2P	1	0	0	1	0	1	0	0	1	0	0	1	1	0	0	0
091P3P	1	0	0	1	0	1	0	0	1	0	0	1	1	0	0	0
VA1	1	0	0	1	1	1	0	0	1	0	0	1	1	0	0	0
FI1	0	0	1	0	0	1	0	0	1	0	0	1	0	1	0	0
FI2	0	0	1	0	0	1	0	1	1	0	0	1	0	1	0	0
FI3	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0
FI4	0	0	0	1	0	0	0	0	1	0	0	1	0	1	0	0
SZ1	0	0	0	1	0	1	1	0	0	0	0	1	0	1	0	0
SZ2	0	0	0	1	0	1	1	0	0	0	1	0	0	1	0	0
SZ3	0	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0
SZ4	0	0	0	1	0	1	1	0	0	0	0	1	0	0	0	0
USA1	0	0	0	1	0	1	0	0	1	0	1	0	1	0	0	0
USA2	0	0	1	1	0	1	0	0	1	0	0	1	0	1	0	0
USA3	0	0	0	1	0	1	0	0	1	0	1	0	1	0	0	0
USA4	0	0	0	1	0	1	0	0	1	0	0	0	1	0	0	0
USA5	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0
USA6	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	1
USA7	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	1
USA8	0	1	0	0	0	0	0	0	1	1	0	0	0	0	1	1
CA1	0	1	0	0	0	0	0	0	1	1	0	0	0	0	1	1

[a]RAPD markers were named according to the primer used and their molecular weight in base pairs (bp), and they are, respectively: (1) OPA03-750, (2) OPA03-1600, (3) OPA08-600, (4) OPA08-1050, (5) OPA08-1200, (6) OPA08-1300, (7) OPA08-1800, (8) OPA12-800, (9) OPA12-1200, (10) OPC08-250, (11) OPC08-1200, (12) OPC08-1250, (13) OPD05-350, (14) OPD05-550, (15) OPD05-600 and (16) OPD05-800.

[b]Binary coding used: 1, presence of RAPD marker; 0, absence of RAPD marker.

(Hamelin *et al*., 1993). Marker OPA03-1600, previously reported as present only in the North American race, was absent from all samples (including the Spanish isolates) except isolates USA6, USA7, USA8 and CA1, previously reported to belong to the NA race (Table 1). RAPD marker OPA03-750 was present only in Spanish samples, although four Spanish isolates (003P, 004P, 005P and 006) lacked it. RAPD profiles generated with primer OPA03 were homogeneous among all analysed samples (Fig. 1a).

Primers OPA08 and OPA12 were selected because they were previously shown to correlate with ecotypic origin (Hamelin *et al*., 1996) and were able to distinguish each of the three biotypes belonging to the EU race. Marker OPA08-600 (characteristic of the Northern biotype) was present in FI1, FI2, SZ3 and USA2, but absent from all other isolates, including Spanish ones. Marker OPA08-1800 (characteristic of the Alpine biotype) was present only in SZ1, SZ2 and SZ4 (samples from the Alpine region; Fig. 1b). Markers OPA08-800, OPA08-1900, OPA12-1350, OPA12-1400 and OPA12-1500, previously reported to be biotype-correlated (Hamelin *et al*., 1996), were not reproducible and therefore were not included in the analysis (Fig. 1b and c).

OPC08 and OPD05 oligonucleotides were polymorphic within biotypes. Markers OPC08-250 and OPD05-800 were present only in isolates of the NA race (Fig. 1d and e). Spanish isolates possessed the OPD05-350 RAPD marker, while all other isolates, except for three EU-race isolates from North America (USA1, USA3 and USA4), lacked it (Fig. 1e).

RAPD data analysis (Fig. 2) showed that samples separated clearly into two groups. The four isolates previously reported to belong to the NA race (Table 1) were included in the first group and all other isolates belonging to the EU race in the second one. Within this second group, isolates belonging to each one of the three known biotypes clustered together and two novel groups appeared to be clearly

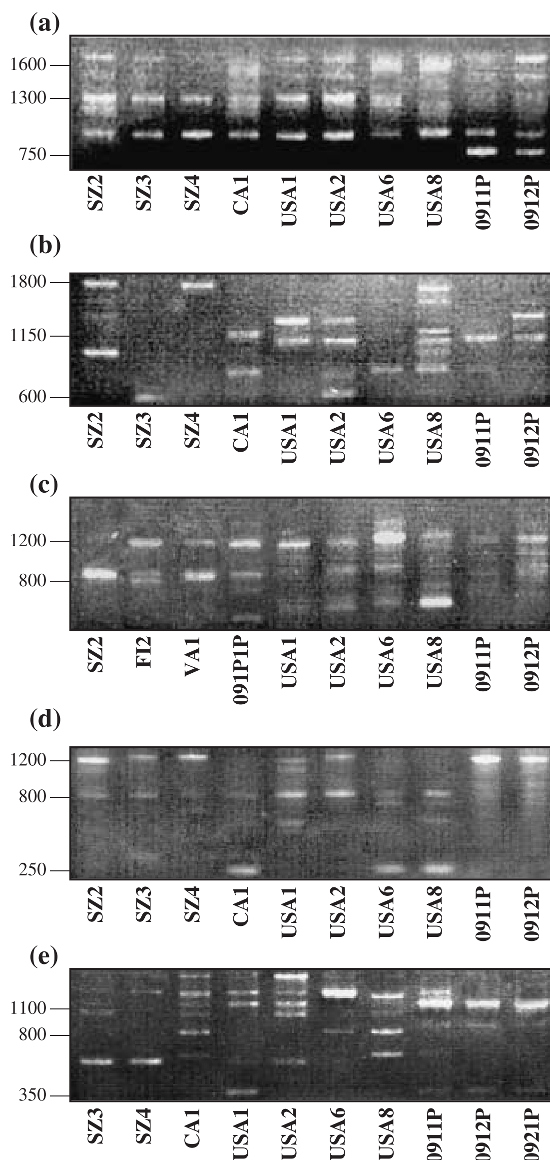

Figure 1 Examples of randomly amplified polymorphic DNA (RAPD) products from *Gremmeniella abietina* var. *abietina* using primers OPA03 (a), OPA08 (b), OPA12 (c), OPC08 (d) and OPD05 (e).

separated from all other biotypes at a similar genetic distance. The Spanish isolates formed one of these new groups, and the isolates belonging to the EU race from North America formed a second group, with the exception of USA2, which fell into the Northern biotype group.

The dendrogram (Fig. 2) generated from RAPD data also revealed that Spanish isolates were closely related and that genetic distance among them was low. Indeed, most Spanish isolates collected from a single stand (isolates sharing the two first digits in their identification number) had identical haplotypes. The Spanish isolates derived from a single pycnidium (091P1P, 091P2P and 091P3P), as well as the Spanish mycelial isolates from a single twig (0911P, 0912P, 0921P and 0922P), had identical RAPD patterns for all five primers tested (Table 2; Fig. 2).

Discussion

This study has shown that the RAPD marker OPA03-1600 was consistently absent in the Spanish isolates of Gaa. Hamelin *et al.* (1993) showed that this marker is present in the NA race. The Spanish isolates therefore belong to the EU race. This result was confirmed by the dendrogram analysis, which showed the Spanish isolates to be much more closely related to those of the EU race than to those of the NA race. In the present work, two new markers (OPC08-250 and OPD05-800) were shown to differentiate the EU and NA races. It would, however, be advisable to test the OPC08 and OPD05 primers with a greater number of isolates having a wider range of genetic variability to acquire greater confidence in this finding.

Hamelin *et al.* (1996) described other RAPD markers that distinguished three biotypes within the EU race. The Alpine biotype was characterized by the presence of markers OPA08-1800 and OPA12-1400 and the absence of OPA08-600, OPA12-1350 and OPA12-1500. The Northern biotype was characterized by the presence of OPA08-600, OPA12-1400 and OPA12-1500. The European biotype was characterized by the presence of OPA08-800, OPA08-1900, OPA12-1350 and OPA12-1400 RAPD markers and by the absence of OPA08-600 and OPA12-1500. Based on these markers, the results indicate that Spanish isolates do not belong to either the Alpine or the Northern biotype. It was not possible to determine whether the Spanish isolates belong to the European biotype because the markers characteristic of this biotype were not observed.

The present study included two isolates (SZ3 and USA2), previously characterized by other authors as being of the Alpine biotype and EU race (Petrini *et al.*, 1990; Hamelin *et al.*, 1993, 1996), that did not show the characteristic markers of the Alpine biotype and EU race suggested by Hamelin *et al.* (1996). Taking into account that some RAPD markers previously described were not completely reproducible in the present study, other fungal features were considered. Based on conidial morphology (Santamaría *et al.*, 2004), Spanish isolates, which produce mainly short and tri-septate conidia, probably do not belong to the Alpine biotype, whereas Ettlinger (1945) stated that the Alpine biotype produces significantly longer and seven-septate conidia. Geographically, the result is in agreement with the fact that the Alpine biotype has been recorded only at altitudes above 2000 m, while in Spain, Gaa was found only at 800 m above sea level. The symptoms of the disease found in the field also confirm that Spanish isolates do not belong to the Northern biotype, since this form of the fungus infects only small seedlings, saplings or the lower canopy of adult pine trees near the ground and produces abundant apothecia (Uotila, 1983; Kaitera *et al.*, 1998). The Spanish isolates attack trees of all sizes, causing dieback and some distortion of terminal twigs, and do not produce apothecia in field.

Analysis of RAPD data showed that two new groups appeared to be clearly separated from all other biotypes at

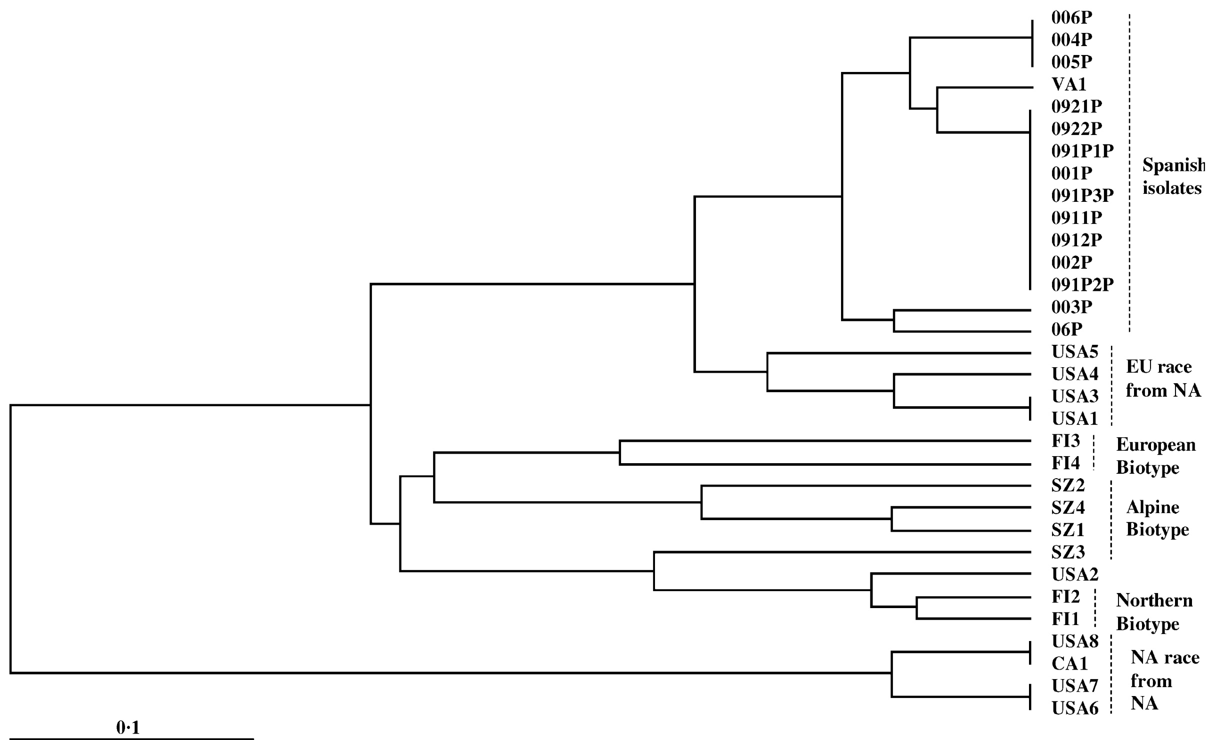

Figure 2 Dendrogram showing phylogenetic relationship among 32 *Gremmeniella abietina* var. *abietina* isolates.

a similar genetic distance, within the EU race. This result suggests that two novel biotypes may occur within the EU race. One of these represents Spanish isolates and the other represents the isolates belonging to the EU race from North America. This is consistent with the findings of Hamelin *et al*. (1998) who stated that European populations of the Gaa European biotype were genetically very different from continental North American populations of the EU race. However, based on symptomatology, growth rates in culture and conidial morphology, no differences were found among Gaa isolates from Spain, those of the EU race from North America and the European biotype from North Europe (Santamaría *et al*., 2004). Phylogenetic studies, including a larger sampling of isolates from Europe and North America, would be required to clarify this point.

Since the main aim of the present work was not to conduct a population study, but was taxonomic, the number of samples collected was insufficient for a genetic diversity study and other molecular techniques would be more suitable than RAPDs for this purpose. However, based on this preliminary study, it seems that diversity among the Spanish isolates might be low when compared with foreign isolates. This result could be explained by the relative geographic proximity (max. 50 km between Astudillo 1 and Matallana sites) among the only four *P. halepensis* stands where the pathogen has been recovered in Spain. Nevertheless, a detailed population will have to be collected to confirm this. No variation in RAPD profiles was observed among monosporic isolates derived from a single pycnidium or among mycelial isolates derived from a single twig, indicating genetic stability through mitosis and within a twig. This result agrees with those obtained previously by Wang (1997). In the present work, samples with both multispore and mycelial origins (isolates 001P, 002P, 003P, 004P and 005P) could be thus considered as a single genotype.

Results of this study show that Spanish isolates of Gaa belong to the European race and not the Alpine or Northern biotypes. Genetically, the Spanish isolates appear to be clearly separated from all other biotypes within the EU race.

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