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Spanish population of *Gremmeniella abietina* is genetically unique but related to type A in Europe

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ABSTRACT

Genetic structure of the European *Gremmeniella abietina* var. *abietina* was analyzed in this study. Ninety-two Spanish isolates, six Swiss isolates of Alpine biotype, 76 Finnish isolates of biotype A and 54 Finnish and seven Russian isolates of biotype B were collected. Genetic variation of different populations was analyzed using sequence analysis of specifically amplified markers GAAA1000, GAAA800 and ACA900. Variation in the GAAA1000 marker was significant, and composed of 33 alleles divided into the following four studied populations: five alleles in the Alpine type, 12 in biotype B, 16 in biotype A and two in the Spanish population. Based on variation in GAAA1000 marker, a subset of isolates were further analyzed using GAAA800 and ACA900 sequences, which showed lower overall genetic variability, and no variation among the Spanish population. Genetic differentiation analysis revealed a high genetic differentiation among populations. Finally, clustering analysis of GAAA1000 sequences showed that the Spanish isolates clearly separated from the rest of the biotypes, whereas the Alpine type was closely related to the B type. However, one of the A-type isolates had an identical GAAA1000 allele with the prevailing allele among Spanish isolates. Altogether, our data suggest that the Spanish population is genetically highly differentiated from any other *G. abietina* population in Europe with a probable A-type origin.

Introduction

Gremmeniella abietina (Lagerb.) Morelet is the causal agent of shoot dieback and Scleroderris canker on many species of conifers including spruce, fir, larch, pine and juniper around the world (Donaubauer 1972; Dorworth 1974; Yokota *et al.* 1974; Setliff *et al.* 1975; Barklund & Rowe 1981; Kaitera & Jalkanen 1996; Kaitera *et al.* 1998; Laflamme *et al.* 1998). In Spain, this fungus was first observed in its anamorphic phase (*Brunchorstia pinea* (Karst.) Höhn) on *Pinus pinaster* Ait. in 1929 (Martínez 1933), however, it was isolated again from declined stands of *Pinus halepensis* Mill. in 1999 (Santamaría *et al.* 2003).

Taxonomic classification of genus *Gremmeniella* is established according to host species, species geography,

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epidemiology, physiology and morphology, as well as serological and biochemical studies. *Gremmeniella* has been further divided into three species, each associated with its host tree; *Gremmeniella laricina* (Ett. Petrini, L.E. Petrini, Lafl. & Ouell) with genus *Larix*, *Gremmeniella juniperina* L. Holm and Holm with genus *Juniperus* (Petrini *et al.* 1989) and *G. abietina* (Lagerberg) Morelet comprised two varieties: var. *abietina*, found mainly on pines, and var. *balsamea*, which is found on spruces and firs (Petrini *et al.* 1989). Similarly, within *G. abietina* var. *abietina* three races are distinguished: Asian, North American (NA) and European (EU). The Asian race has been only isolated from *Abies sachalinensis* in Japan (Yokota *et al.* 1974). The NA race is found in natural stands of *Pinus contorta* Loud., *Pinus resinosa* Ait. and *Pinus banksiana* Lamb. in North America (Laflamme *et al.* 1998). This race infects seedlings and lower branches, which are covered in snow during winter months, and where sexual and asexual phases are produced. *G. abietina var. abietina* from Europe was introduced to North America and designated there as an EU race. It was first detected in New York State in 1977, where it caused serious damages on *P. contorta* stands (Skilling 1977).

Currently, three biotypes occur in Europe: small tree type (STT), Alpine type and A type. STT, also referred to as Northern or B type, is restricted to moderately high altitudes in northern Europe. It affects *Picea abies* (L.) Karst., *Pinus sylvestris* L. and *P. contorta* (Uotila 1983; Hellgren & Högberg 1995; Hamelin *et al.* 1996). The Alpine type is found in high altitudes (~2000 m) of the central European Alps infecting *Pinus cembra* L., *P. mugo* Turra, *Larix lyallii* Parl. and *P. sylvestris* (Hamelin *et al.* 1996). Both pathogens grow in harsh conditions and produce pycnidia and apothecia in buds of seedlings or lower branches of adult trees covered by snow during winter months (Hellgren & Högberg 1995).The most pathogenic of the three biotypes is type A (Uotila 1990), a widely distributed form of *G. abietina* var. *abietina*, ranging from Italian Apennines to northern Sweden. It infects *Pinus resinosa*, *P. sylvestris* and *P. contorta*, *Pinus pinea* L. and *P. abies*. It rarely produces apothecia in forests (Kaitera & Jalkanen 1996), and it has also spread to North America. In Europe no local genetic differentiation has been observed among the A type populations (Hamelin *et al.* 1996).

In Spain, *G. abietina* has been collected from *P. halepensis* planted forest stands in north-western Spain at altitudes between 800 and 900 m in transitional areas, where both evergreen sclerophyll broad-leaf and coniferous forest occur within the temperate zone. Hot and dry summers, and frost days (60 d per year on average) with minimal snowfall in winter are common. In Spanish population, apothecia are not produced in the field (Santamaría *et al.* 2003) contrasting the remaining European populations. Detailed phylogenic and genetic studies of *G. abietina* populations contribute to our understanding of its epidemiology and potential impact on *P. halepensis* stands (Dusabenyagasani *et al.* 2002; McDonald & Linde 2002). In this sense, numerous genetic analyses have provided fundamental information about this pathogen. Various studies have been carried out to elucidate not only the molecular variability of this fungus but also the relationship to different environments where *G. abietina* is established (Uotila 1983; Hellgren & Högberg 1995; Hamelin *et al.* 1996; Hamelin & Rail 1997; Hantula & Müller 1997; Hantula *et al.* 1998; Dusabenyagasani *et al.* 1998, 2002; Santamaría *et al.* 2005; Kraj & Kowalski 2008).

The main objectives of this study were: to clarify the genetic differentiation status of Spanish *G. abietina* population within the European diversity using sequence-based data; and to determine phylogenic relationships among all European biotypes.

Materials and methods

Gremmeniella *sampling*

Ninety-two Spanish isolates were compiled for this study, most of them collected during the autumn of 2007 (Table 1). The rest of Spanish isolates was obtained from the collection of *Gremmeniella abietina* at the Department of Plant Production of the E.T.S.I.I.A.A. (Palencia). Isolates were obtained from twigs of symptomatic *Pinus halepensis* located in provinces of Valladolid and Palencia: Villalba de los Alcores (UTM, 4620 800, 391 565) in Valladolid; Valle de Cerrato (UTM, 4640 475, 386 450), Hontoria (UTM, 4638 684, 383 877) and Astudillo (UTM, 4667 465, 390 225) in Palencia. The southern Finnish isolates were collected between 2003 and 2005 from four locations: Somero (UTM, 611 549.6, 6653 253), Nummi-Pusula (UTM, 611 760.5, 665 3016.4), Hyytiälä (UTM, 337 995.2, 6766 411) and Karhula (337 773.6, 6766 663.5), the northern Finnish and Russian (Kola peninsula) isolates were acquired between 1994 and 1995 from 11 locations (Kaitera *et al.* 2000), and Swiss isolates from the surrounding areas of Davos (UTM, 500 000, 5094 143.8) (Table 1).

DNA analyses

Isolates were grown for 2 weeks at 20 °C on modified orange serum (MOS) agar plates (Müller *et al.* 1994) supplemented with cellophane membranes. DNA from these cultures was isolated following the protocol described by Vainio *et al.* (1998).

Polymerase chain reaction (PCR) was performed following the recommended conditions described by the manufacturer of Dynazyme II DNA-polymerase (Finnzymes Ltd, Espoo, Finland). For species identification random amplified microsatellite (RAMS) markers were amplified using primers CGA (5′DHB $(CGA)_5$) and CCA (5′DDB$(CCA)_5$), where B = C, G or T; H = C, A or T; D = T, A or G (Hantula *et al.* 1996) in addition to sample DNA concentration of 2 μM.

For sequence analysis, a hypervariable marker GAAA1000 (Uotila *et al.* 2006) was used to characterize our isolates using primers GAAA1000 forward (5′-GAT GGA GAT CAG GAA TCG G-3′) and GAAA1000 reverse (5′-CGA TTT AGA GAA TTT TCA AAG GT-3′). Similarly, markers GAAA800 and ACA900 were amplified using GAAA800 forward (5′-CTC AAC CCA CTC CCG C-3′) and reverse (5′-CGA GAG AGT AAG GAA TAA ATG A-3′), and ACA900 forward (5′-CCC CTC AGT CCG TAC GTA C-3′) and reverse (5′-CCC TCA ATT TAG TCA ACC CT-3′), respectively.

Samples were initially denatured for 10 min at 95 °C, followed by 37 amplification cycles consisting of 30 s of denaturation at 95 °C for CCA and CGA and 1 min for GAAA1000, GAAA800 and ACA900, 45 s annealing at 50 °C (GAAA1000), 55 °C (GAAA800), 51 °C (ACA900) and 61 °C (CCA and CGA), and 2 min (CCA and CGA) or 1 min (GAAA1000, GAAA800,

Table 1 – Isolates studied and their GenBank sequence accession numbers.

Isolate code	Host tree	Origin[a]	Year[b]	Race/biotype[c]	Accession numbers for EMBL		
					GAAA1000	GAAA800	ACA900
Samples from Spain							
O6P	*P. halepensis*	Astudillo	2003	n.d.	FN663377	n.d.	n.d.
O9P-1	*P. halepensis*	Astudillo	2003	n.d.	FN663378	n.d.	n.d.
O9P-1B	*P. halepensis*	Astudillo	2003	n.d.	FN663379	n.d.	n.d.
O9P-7	*P. halepensis*	Astudillo	2003	n.d.	FN663380	n.d.	n.d.
O9P-8	*P. halepensis*	Astudillo	2003	n.d.	FN663381	FN663177	FN663278
O9P-9	*P. halepensis*	Astudillo	2003	n.d.	FN663382	n.d.	n.d.
O9P-10	*P. halepensis*	Astudillo	2003	n.d.	FN663383	n.d.	n.d.
O9P-10B	*P. halepensis*	Astudillo	2003	n.d.	FN663384	n.d.	n.d.
O9P-11	*P. halepensis*	Astudillo	2003	n.d.	FN663385	n.d.	n.d.
O9P-12	*P. halepensis*	Astudillo	2003	n.d.	FN663386	n.d.	n.d.
O9P-13	*P. halepensis*	Astudillo	2003	n.d.	FN663387	n.d.	n.d.
HON2-1	*P. halepensis*	Hontoria	2007	n.d.	FN663405	n.d.	n.d.
HON2-3	*P. halepensis*	Hontoria	2007	n.d.	FN663406	n.d.	n.d.
HON2-33	*P. halepensis*	Hontoria	2007	n.d.	FN663408	n.d.	n.d.
HON2-4	*P. halepensis*	Hontoria	2007	n.d.	FN663407	n.d.	n.d.
HON3-1	*P. halepensis*	Hontoria	2007	n.d.	FN663409	n.d.	n.d.
HON3-11	*P. halepensis*	Hontoria	2008	n.d.	FN663417	n.d.	n.d.
HON3-2	*P. halepensis*	Hontoria	2007	n.d.	FN663410	n.d.	n.d.
HON3-22	*P. halepensis*	Hontoria	2007	n.d.	FN663418	n.d.	n.d.
HON3-3	*P. halepensis*	Hontoria	2007	n.d.	FN663411	n.d.	n.d.
HON3-33	*P. halepensis*	Hontoria	2008	n.d.	FN663419	n.d.	n.d.
HON3-4	*P. halepensis*	Hontoria	2007	n.d.	FN663412	FN663181	FN663282
HON3-44	*P. halepensis*	Hontoria	2007	n.d.	FN663420	n.d.	n.d.
HON3-5	*P. halepensis*	Hontoria	2007	n.d.	FN663413	n.d.	n.d.
HON3-55	*P. halepensis*	Hontoria	2007	n.d.	FN663421	n.d.	n.d.
HON3-6	*P. halepensis*	Hontoria	2007	n.d.	FN663414	n.d.	n.d.
HON3-66	*P. halepensis*	Hontoria	2007	n.d.	FN663422	n.d.	n.d.
HON3-7	*P. halepensis*	Hontoria	2007	n.d.	FN663415	FN663182	FN663283
HON3-77	*P. halepensis*	Hontoria	2006	n.d.	FN663423	n.d.	n.d.
HON3-8	*P. halepensis*	Hontoria	2007	n.d.	FN663416	n.d.	n.d.
HON3-88	*P. halepensis*	Hontoria	2008	n.d.	FN663424	n.d.	n.d.
HON4-1	*P. halepensis*	Hontoria	2007	n.d.	FN663426	n.d.	n.d.
HON4-11	*P. halepensis*	Hontoria	2007	n.d.	FN663430	n.d.	n.d.
HON4-2	*P. halepensis*	Hontoria	2007	n.d.	FN663427	n.d.	n.d.
HON4-4	*P. halepensis*	Hontoria	2007	n.d.	FN663428	n.d.	n.d.
HON4-5	*P. halepensis*	Hontoria	2007	n.d.	FN663429	n.d.	n.d.
HON5-3	*P. halepensis*	Hontoria	2008	n.d.	FN663431	n.d.	n.d.
HON6-3	*P. halepensis*	Hontoria	2009	n.d.	FN663425	n.d.	n.d.
HON8-1	*P. halepensis*	Hontoria	2007	n.d.	FN663432	n.d.	n.d.
HON8-2	*P. halepensis*	Hontoria	2007	n.d.	FN663433	n.d.	n.d.
HON8-3	*P. halepensis*	Hontoria	2007	n.d.	FN663434	n.d.	n.d.
HON8-33	*P. halepensis*	Hontoria	2007	n.d.	FN663435	n.d.	n.d.
HON9-2	*P. halepensis*	Hontoria	2007	n.d.	FN663436	FN663183	FN663284
H1-4	*P. halepensis*	Hontoria	2007	n.d.	FN663388	n.d.	n.d.
H1-13	*P. halepensis*	Hontoria	2007	n.d.	FN663389	FN663178	FN663279
H4-1	*P. halepensis*	Valle de Cerrato	2005	n.d.	FN663390	n.d.	n.d.
H4-2	*P. halepensis*	Valle de Cerrato	2005	n.d.	FN663391	FN663179	FN663280
H4-3	*P. halepensis*	Valle de Cerrato	2005	n.d.	FN663392	n.d.	n.d.
H4-4	*P. halepensis*	Valle de Cerrato	2005	n.d.	FN663393	n.d.	n.d.
H4-6	*P. halepensis*	Valle de Cerrato	2005	n.d.	FN663394	n.d.	n.d.
H4-9	*P. halepensis*	Valle de Cerrato	2005	n.d.	FN663395	n.d.	n.d.
H4-9B	*P. halepensis*	Valle de Cerrato	2005	n.d.	FN663396	n.d.	n.d.
H4-15	*P. halepensis*	Valle de Cerrato	2005	n.d.	FN663397	n.d.	n.d.
H4-19	*P. halepensis*	Valle de Cerrato	2005	n.d.	FN663398	FN663180	FN663281
H4-19B	*P. halepensis*	Valle de Cerrato	2005	n.d.	FN663399	n.d.	n.d.
H8-2	*P. halepensis*	Valle de Cerrato	2005	n.d.	FN663400	n.d.	n.d.
H8-3	*P. halepensis*	Valle de Cerrato	2005	n.d.	FN663401	n.d.	n.d.
H8-4	*P. halepensis*	Valle de Cerrato	2005	n.d.	FN663402	n.d.	n.d.
HA	*P. halepensis*	Valle de Cerrato	2006	n.d.	FN663403	n.d.	n.d.
HA-2	*P. halepensis*	Valle de Cerrato	2005	n.d.	FN663404	n.d.	n.d.
P1-1	*P. halepensis*	Valle de Cerrato	2005	n.d.	FN663437	n.d.	n.d.

(*continued on next page*)

Table 1 – (*continued*)

Isolate code	Host tree	Origin[a]	Year[b]	Race/biotype[c]	Accession numbers for EMBL		
					GAAA1000	GAAA800	ACA900
P1-2	*P. halepensis*	Valle de Cerrato	2005	n.d.	FN663438	n.d.	n.d.
P1-3	*P. halepensis*	Valle de Cerrato	2007	n.d.	FN663439	FN663184	FN663285
P1-5	*P. halepensis*	Valle de Cerrato	2007	n.d.	FN663440	FN663185	FN663286
P1-6	*P. halepensis*	Valle de Cerrato	2007	n.d.	FN663441	n.d.	n.d.
P1-8	*P. halepensis*	Valle de Cerrato	2007	n.d.	FN663442	n.d.	n.d.
P1-10	*P. halepensis*	Valle de Cerrato	2007	n.d.	FN663443	n.d.	n.d.
P1-11	*P. halepensis*	Valle de Cerrato	2007	n.d.	FN663444	n.d.	n.d.
P1-12	*P. halepensis*	Valle de Cerrato	2007	n.d.	FN663445	n.d.	n.d.
P1-13	*P. halepensis*	Valle de Cerrato	2007	n.d.	FN663446	n.d.	n.d.
P1-16	*P. halepensis*	Valle de Cerrato	2007	n.d.	FN663447	n.d.	n.d.
P1-18	*P. halepensis*	Valle de Cerrato	2007	n.d.	FN663448	n.d.	n.d.
P1-63	*P. halepensis*	Valle de Cerrato	2007	n.d.	FN663449	n.d.	n.d.
P3-3	*P. halepensis*	Valle de Cerrato	2007	n.d.	FN663450	n.d.	n.d.
P3-4	*P. halepensis*	Valle de Cerrato	2008	n.d.	FN663451	n.d.	n.d.
P3-7	*P. halepensis*	Valle de Cerrato	2007	n.d.	FN663452	n.d.	n.d.
P3-9	*P. halepensis*	Valle de Cerrato	2007	n.d.	FN663453	n.d.	n.d.
P3-12	*P. halepensis*	Valle de Cerrato	2007	n.d.	FN663454	n.d.	n.d.
P3-18	*P. halepensis*	Valle de Cerrato	2007	n.d.	FN663455	n.d.	n.d.
P3-20	*P. halepensis*	Valle de Cerrato	2007	n.d.	FN663456	n.d.	n.d.
P3-21	*P. halepensis*	Valle de Cerrato	2007	n.d.	FN663457	n.d.	n.d.
P4-1	*P. halepensis*	Valle de Cerrato	2007	n.d.	FN663458	n.d.	n.d.
OOP-1	*P. halepensis*	Valle de Cerrato	2007	n.d.	FN663373	n.d.	n.d.
OOP-2	*P. halepensis*	Valle de Cerrato	2007	n.d.	FN663372	FN663176	FN663277
OOP-22	*P. halepensis*	Valle de Cerrato	2001	n.d.	FN663376	n.d.	n.d.
OOP-3	*P. halepensis*	Valle de Cerrato	2001	n.d.	FN663374	n.d.	n.d.
OOP-7	*P. halepensis*	Valle de Cerrato	2001	n.d.	FN663375	n.d.	n.d.
VAI-1	*P. halepensis*	Valle de Cerrato	2001	n.d.	FN663459	n.d.	n.d.
VAI-12	*P. halepensis*	Valle de Cerrato	2001	n.d.	FN663461	n.d.	n.d.
VAI-13	*P. halepensis*	Villalba de los Alcores	2003	n.d.	FN663462	n.d.	n.d.
VAI-3	*P. halepensis*	Villalba de los Alcores	2003	n.d.	FN663460	FN663186	FN663287
VAI-33	*P. halepensis*	Villalba de los Alcores	2003	n.d.	FN663463	n.d.	n.d.
Samples from Finland and Russia							
AHT9b	*P. sylvestris*	Alahyytiäläntie	2003	Eu/Biotype A	FN663536	FN663221	FN663332
AHT31	*P. sylvestris*	Alahyytiäläntie	2003	Eu/Biotype A	FN663537	FN663222	FN663322
AHT36	*P. sylvestris*	Alahyytiäläntie	2003	Eu/Biotype A	FN663538	FN663223	FN663333
AHT42	*P. sylvestris*	Alahyytiäläntie	2003	Eu/Biotype A	FN663539	FN663224	FN663323
AHT44	*P. sylvestris*	Alahyytiäläntie	2003	Eu/Biotype A	FN663540	FN663225	FN663324
AHT48	*P. sylvestris*	Alahyytiäläntie	2003	Eu/Biotype A	FN663541	FN663226	FN663325
AHT49	*P. sylvestris*	Alahyytiäläntie	2003	Eu/Biotype A	FN663542	FN663227	FN663326
AHT50	*P. sylvestris*	Alahyytiäläntie	2003	Eu/Biotype A	FN663543	FN663232	FN663327
AHT51	*P. sylvestris*	Alahyytiäläntie	2003	Eu/Biotype A	FN663544	FN663228	FN663328
AHT58	*P. sylvestris*	Alahyytiäläntie	2003	Eu/Biotype A	FN663545	FN663229	FN663329
AHT60	*P. sylvestris*	Alahyytiäläntie	2003	Eu/Biotype A	FN663546	FN663230	FN663330
AHT2a	*P. sylvestris*	Alahyytiäläntie	2003	Eu/Biotype A	FN663533	FN663219	FN663331
AHT5a	*P. sylvestris*	Alahyytiäläntie	2003	Eu/Biotype A	FN663534	FN663231	FN663320
AHT7a	*P. sylvestris*	Alahyytiäläntie	2003	Eu/Biotype A	FN663535	FN663220	FN663321
Kuusi5	*Picea abies*	Alahyytiäläntie	2003	Eu/Biotype A	FN663590	FN663259	FN663360
Kuusi10	*Picea abies*	Alahyytiäläntie	2003	Eu/Biotype A	FN663591	FN663260	FN663361
Kuusi11	*Picea abies*	Alahyytiäläntie	2003	Eu/Biotype A	FN663592	FN663261	FN663362
Kuusi24	*Picea abies*	Alahyytiäläntie	2003	Eu/Biotype A	FN663593	FN663262	FN663363
SmearS4	*P. sylvestris*	Alahyytiäläntie	2003	Eu/Biotype A	FN663594	FN663263	FN663364
SmearS9	*P. sylvestris*	Alahyytiäläntie	2003	Eu/Biotype A	FN663595	FN663264	FN663365
KAR2B	*P. sylvestris*	Karhula	2003	Eu/Biotype A	FN663581	FN663249	FN663350
KAR8	*P. sylvestris*	Karhula	2003	Eu/Biotype A	FN663491	FN663250	FN663351
KAR11	*P. sylvestris*	Karhula	2003	Eu/Biotype A	FN663582	FN663251	FN663352
KAR18	*P. sylvestris*	Karhula	2003	Eu/Biotype A	FN663583	FN663252	FN663353
KAR23	*P. sylvestris*	Karhula	2003	Eu/Biotype A	FN663584	FN663253	FN663354
KAR30	*P. sylvestris*	Karhula	2003	Eu/Biotype A	FN663585	FN663254	FN663355
KAR49	*P. sylvestris*	Karhula	2003	Eu/Biotype A	FN663588	FN663257	FN663358
KAR44	*P. sylvestris*	Karhula	2003	Eu/Biotype A	FN663587	FN663256	FN663357
KAR31	*P. sylvestris*	Karhula	2003	Eu/Biotype A	FN663586	FN663255	FN663356

(*continued on next page*)

Table 1 – (continued)

Isolate code	Host tree	Origin[a]	Year[b]	Race/biotype[c]	Accession numbers for EMBL		
					GAAA1000	GAAA800	ACA900
KAR53	*P. sylvestris*	Karhula	2003	Eu/Biotype A	FN663589	FN663258	FN663359
Ka04-4	*P. sylvestris*	Karhula	2004	Eu/Biotype A	FN663577	n.d.	n.d.
Ka04-11	*P. sylvestris*	Karhula	2004	Eu/Biotype A	FN663561	n.d.	n.d.
Ka04-22	*P. sylvestris*	Karhula	2004	Eu/Biotype A	FN663578	n.d.	n.d.
Ka04-24	*P. sylvestris*	Karhula	2004	Eu/Biotype A	FN663562	n.d.	n.d.
Ka04-30	*P. sylvestris*	Karhula	2004	Eu/Biotype A	FN663563	n.d.	n.d.
Ka04-32	*P. sylvestris*	Karhula	2004	Eu/Biotype A	FN663579	n.d.	n.d.
Ka04-34	*P. sylvestris*	Karhula	2004	Eu/Biotype A	FN663564	n.d.	n.d.
Ka04-35	*P. sylvestris*	Karhula	2004	Eu/Biotype A	FN663565	n.d.	n.d.
Ka04-36	*P. sylvestris*	Karhula	2004	Eu/Biotype A	FN663580	FN663246	FN663347
Ka04-37	*P. sylvestris*	Karhula	2004	Eu/Biotype A	FN663566	n.d.	n.d.
Ka04-38	*P. sylvestris*	Karhula	2004	Eu/Biotype A	FN663567	FN663247	FN663348
Ka04-39	*P. sylvestris*	Karhula	2004	Eu/Biotype A	FN663568	n.d.	n.d.
Ka04-43	*P. sylvestris*	Karhula	2004	Eu/Biotype A	FN663569	n.d.	n.d.
Ka04-44	*P. sylvestris*	Karhula	2004	Eu/Biotype A	FN663570	n.d.	n.d.
Ka04-45	*P. sylvestris*	Karhula	2004	Eu/Biotype A	FN663571	n.d.	n.d.
Ka04-46	*P. sylvestris*	Karhula	2004	Eu/Biotype A	FN663572	n.d.	n.d.
Ka04-54	*P. sylvestris*	Karhula	2004	Eu/Biotype A	FN663573	n.d.	n.d.
Ka04-55	*P. sylvestris*	Karhula	2004	Eu/Biotype A	FN663574	FN663248	FN663349
Ka04-65	*P. sylvestris*	Karhula	2004	Eu/Biotype A	FN663575	n.d.	n.d.
Ka04-66	*P. sylvestris*	Karhula	2004	Eu/Biotype A	FN663576	n.d.	n.d.
K2	*P. sylvestris*	Keräkankare	2003	Eu/Biotype A	FN663548	n.d.	n.d.
K2V	*P. sylvestris*	Keräkankare	2003	Eu/Biotype A	FN663549	FN663234	FN663335
K13	*P. sylvestris*	Keräkankare	2003	Eu/Biotype A	FN663550	FN663235	FN663336
K17	*P. sylvestris*	Keräkankare	2003	Eu/Biotype A	FN663551	FN663236	FN663337
K20	*P. sylvestris*	Keräkankare	2003	Eu/Biotype A	FN663552	FN663237	FN663338
K24	*P. sylvestris*	Keräkankare	2003	Eu/Biotype A	FN663553	FN663238	FN663339
K30	*P. sylvestris*	Keräkankare	2003	Eu/Biotype A	FN663554	FN663239	FN663340
K31	*P. sylvestris*	Keräkankare	2003	Eu/Biotype A	FN663555	FN663240	FN663341
K35	*P. sylvestris*	Keräkankare	2003	Eu/Biotype A	FN663556	FN663241	FN663342
K38	*P. sylvestris*	Keräkankare	2003	Eu/Biotype A	FN663557	FN663242	FN663343
K43	*P. sylvestris*	Keräkankare	2003	Eu/Biotype A	FN663558	FN663243	FN663344
K48	*P. sylvestris*	Keräkankare	2003	Eu/Biotype A	FN663559	FN663244	FN663345
K50	*P. sylvestris*	Keräkankare	2003	Eu/Biotype A	FN663560	FN663245	FN663346
So4	*P. sylvestris*	Somero	2003	Eu/Biotype A	FN663596	FN663265	FN663366
So12	*P. sylvestris*	Somero	2003	Eu/Biotype A	FN663602	FN663266	FN663367
So28	*P. sylvestris*	Somero	2003	Eu/Biotype A	FN663603	FN663267	FN663368
So40	*P. sylvestris*	Somero	2003	Eu/Biotype A	FN663604	FN663268	FN663369
So46	*P. sylvestris*	Somero	2003	Eu/Biotype A	FN663605	FN663269	FN663370
So52	*P. sylvestris*	Somero	2003	Eu/Biotype A	FN663606	FN663270	FN663371
So04-32	*P. sylvestris*	Somero	2004	Eu/Biotype A	FN663597	n.d.	n.d.
So04-40	*P. sylvestris*	Somero	2004	Eu/Biotype A	FN663598	n.d.	n.d.
So04-46	*P. sylvestris*	Somero	2004	Eu/Biotype A	FN663599	n.d.	n.d.
So04-48	*P. sylvestris*	Somero	2004	Eu/Biotype A	FN663600	n.d.	n.d.
So04-55	*P. sylvestris*	Somero	2004	Eu/Biotype A	FN663601	n.d.	n.d.
B9	*P. sylvestris*	Muonio	1994	Eu/Biotype A	FN663532	n.d.	n.d.
GG3	*P. sylvestris*	Perttaus	1995	Eu/Biotype A	FN663547	FN663233	FN663334
AA2	*P. sylvestris*	Värriö	1994	Eu/Biotype B	FN663470	n.d.	n.d.
AA3	*P. sylvestris*	Värriö	1994	Eu/Biotype B	FN663471	n.d.	n.d.
AA4	*P. sylvestris*	Värriö	1994	Eu/Biotype B	FN663472	n.d.	n.d.
AU15.1	*P. contorta*	Värriö	n.s.	Eu/Biotype B	FN663473	n.d.	n.d.
AU26	*P. contorta*	Värriö	n.s.	Eu/Biotype B	FN663474	n.d.	n.d.
AU58	*P. contorta*	Värriö	n.s.	Eu/Biotype B	FN663475	n.d.	n.d.
DD2	*P. sylvestris*	Sodankylä	1995	Eu/Biotype B	FN663476	FN663188	FN663288
DD3	*P. sylvestris*	Sodankylä	1995	Eu/Biotype B	FN663477	n.d.	n.d.
DD5	*P. sylvestris*	Sodankylä	1995	Eu/Biotype B	FN663478	n.d.	n.d.
DD6	*P. sylvestris*	Sodankylä	1995	Eu/Biotype B	FN663479	FN663189	FN663289
DD7	*P. sylvestris*	Sodankylä	1995	Eu/Biotype B	FN663480	FN663187	FN663290
DD8	*P. sylvestris*	Sodankylä	1995	Eu/Biotype B	FN663481	n.d.	n.d.
DD9	*P. sylvestris*	Sodankylä	1995	Eu/Biotype B	FN663482	FN663190	FN663291
EE2	*P. abies*	Kolari	1995	Eu/Biotype B	FN663485	n.d.	n.d.
EE3	*P. abies*	Kolari	1995	Eu/Biotype B	FN663492	n.d.	n.d.
E41	*P. contorta*	Rovaniemi	1994	Eu/Biotype B	FN663483	FN663191	FN663292

Table 1 – (continued)

Isolate code	Host tree	Origin[a]	Year[b]	Race/biotype[c]	Accession numbers for EMBL		
					GAAA1000	GAAA800	ACA900
E45	*P. contorta*	Rovaniemi	1994	Eu/Biotype B	FN663484	FN663192	FN663293
E47	*P. contorta*	Rovaniemi	1994	Eu/Biotype B	FN663486	FN663193	FN663294
E48	*P. contorta*	Rovaniemi	1994	Eu/Biotype B	FN663487	FN663194	FN663295
E49	*P. contorta*	Rovaniemi	1994	Eu/Biotype B	FN663488	FN663195	FN663296
E50	*P. contorta*	Rovaniemi	1994	Eu/Biotype B	FN663489	n.d.	n.d.
E52	*P. contorta*	Rovaniemi	1994	Eu/Biotype B	FN663490	n.d.	n.d.
GG1	*P. sylvestris*	Perttaus	1995	Eu/Biotype B	FN663500	FN663201	FN663302
GG2	*P. sylvestris*	Perttaus	1995	Eu/Biotype B	FN663501	n.d.	n.d.
G42	*P. sylvestris*	Olenegorsk, Russia	1994	Eu/Biotype B	FN663493	FN663196	FN663297
G43	*P. sylvestris*	Olenegorsk, Russia	1994	Eu/Biotype B	FN663494	FN663197	FN663298
G45	*P. sylvestris*	Olenegorsk, Russia	1994	Eu/Biotype B	FN663495	FN663198	FN663299
G46	*P. sylvestris*	Olenegorsk, Russia	1994	Eu/Biotype B	FN663496	FN663199	FN663300
G48	*P. sylvestris*	Olenegorsk, Russia	1994	Eu/Biotype B	FN663497	n.d.	n.d.
G49	*P. sylvestris*	Olenegorsk, Russia	1994	Eu/Biotype B	FN663498	FN663200	FN663301
G50	*P. sylvestris*	Olenegorsk, Russia	1994	Eu/Biotype B	FN663499	n.d.	n.d.
HH1	*P. sylvestris*	Juomukuru	1995	Eu/Biotype B	FN663509	n.d.	n.d.
HH3	*P. sylvestris*	Juomukuru	1995	Eu/Biotype B	FN663510	FN663205	FN663306
HH4	*P. sylvestris*	Juomukuru	1995	Eu/Biotype B	FN663511	n.d.	n.d.
HH5	*P. sylvestris*	Juomukuru	1995	Eu/Biotype B	FN663507	FN663206	FN663307
H42	*P. sylvestris*	Kemijärvi	1994	Eu/Biotype B	FN663502	n.d.	n.d.
H45	*P. sylvestris*	Kemijärvi	1994	Eu/Biotype B	FN663503		n.d.
H46	*P. sylvestris*	Kemijärvi	1994	Eu/Biotype B	FN663504	FN663202	FN663303
H47	*P. sylvestris*	Kemijärvi	1994	Eu/Biotype B	FN663505	n.d.	n.d.
H49	*P. sylvestris*	Kemijärvi	1994	Eu/Biotype B	FN663506	FN663203	FN663304
H50	*P. sylvestris*	Kemijärvi	1994	Eu/Biotype B	FN663508	FN663204	FN663305
KAI1-7	*P. sylvestris*	Rovaniemi mlk	n.s.	Eu/Biotype B	FN663512	n.d.	n.d.
O2	*P. sylvestris*	Liesijärvi	1994	Eu/Biotype B	FN663513	n.d.	n.d.
O3	*P. sylvestris*	Liesijärvi	1994	Eu/Biotype B	FN663514	FN663207	FN663308
O4	*P. sylvestris*	Liesijärvi	1994	Eu/Biotype B	FN663515	n.d.	n.d.
O5	*P. sylvestris*	Liesijärvi	1994	Eu/Biotype B	FN663516	n.d.	n.d.
O6	*P. sylvestris*	Liesijärvi	1994	Eu/Biotype B	FN663517	FN663208	FN663309
O8	*P. sylvestris*	Liesijärvi	1994	Eu/Biotype B	FN663518	n.d.	n.d.
O9-u	*P. sylvestris*	Liesijärvi	1994	Eu/Biotype B	FN663519	n.d.	n.d.
RL10	*P. sylvestris*	Rovaniemi	n.s.	Eu/Biotype B	FN663520	n.d.	n.d.
S1	*P. sylvestris*	Ruosselkä	1994	Eu/Biotype B	FN663521	FN663209	FN663310
S3	*P. sylvestris*	Ruosselkä	1994	Eu/Biotype B	FN663522	FN663210	FN663311
S4	*P. sylvestris*	Ruosselkä	1994	Eu/Biotype B	FN663523	FN663211	FN663312
S5	*P. sylvestris*	Ruosselkä	1994	Eu/Biotype B	FN663524	FN663212	FN663313
S7	*P. sylvestris*	Ruosselkä	1994	Eu/Biotype B	FN663525	FN663213	FN663314
S8	*P. sylvestris*	Ruosselkä	1994	Eu/Biotype B	FN663526	FN663214	FN663315
U3	*P. contorta*	Ruosselkä	1994	Eu/Biotype B	FN663527	FN663215	FN663316
U4	*P. contorta*	Ruosselkä	1994	Eu/Biotype B	FN663528	FN663216	FN663317
U5	*P. contorta*	Ruosselkä	1994	Eu/Biotype B	FN663529	n.d.	n.d.
U7	*P. contorta*	Ruosselkä	1994	Eu/Biotype B	FN663530	FN663217	FN663318
U9	*P. contorta*	Ruosselkä	1994	Eu/Biotype B	FN663531	FN663218	FN663319
Samples from Switzerland							
G6-2	*P. mugo*	Davos, Stillberg	n.s.	Eu/Alpine	FN663464	FN663170	FN663272
G9-4	*P. mugo*	Davos, Stillberg	n.s.	Eu/Alpine	FN663465	FN663172	FN663273
G14-6	*P. mugo*	Davos, Luxalp	n.s.	Eu/Alpine	FN663466	FN663171	FN663271
G17-2	*P. mugo*	Davos, Luxalp	n.s.	Eu/Alpine	FN663467	FN663173	FN663274
G22-8	*P. mugo*	Avers, Bleikewald	n.s.	Eu/Alpine	FN663468	FN663174	FN663275
G26-4	*P. mugo*	Avers, Bleikewald	n.s.	Eu/Alpine	FN663469	FN663175	FN663276

a Original locality where *Gremmeniella abietina* isolates were found.
b Year of isolation.
c Race and biotype described for every isolate; n.d., not described, n.s., not specified.

and ACA900) of extension at 72 °C. A final extension step was performed for 7 min at 72 °C.

Amplified DNA products were separated by electrophoresis in 1 % agarose gels (FMC BioProducts, Rockland, ME, USA) with 1 % SynerGel (Diversified Biotech, Boston, MA, USA), 1× TAE (40 mM Tris–acetate pH 8.0, 1 mM EDTA) and 10 μl of ethidium bromide. Results were visualized under UV light after 1 h (GAAA1000, GAAA800, ACA900) to 3 h (CCA and CGA), in 1 % TAE-buffer solution at 120 V. A Gene Ruler™ 100 bp DNA Ladder Plus (Fermentas) was used as a length marker. Finally, High Pure PCR Product

Purification Kit (Roche Diagnostics GmbH, Mannheim, Germany) was used to purify PCR products and the template DNA concentrations were determined by visual comparison in electrophoresis gel among the samples and a series of standard concentrations of λ-DNA (5, 10, 20, 40, 80 and 160 ng μl^{-1}).

Sequencing and analysis of data

The purified and quantified PCR products were sequenced directly in an automated sequencing machine Li-Cor Global Edition IR^2 system (Li-Cor Inc., Lincoln, NE, U.S.A.) and the SequiTherm EXCEL™ II DNA sequencing kit-LC (Epicentre®, Madison, WI, U.S.A.) as described by the manufacturer.

The resulting sequences were compiled and aligned using Vector NTI Advance 10 (Invitrogen Corp. U.S.A.) software package and MEGA 4.0.1 (Tamura *et al.* 2007).

DNA divergence among populations, and the average level of gene flow was measured using Dna SP ver. 5.0 (Librado & Rozas 2009). The number of haplotypes (*h*), number of polymorphic/indel/missing sites (*S*), haplotype diversity (Hd) and the average number of nucleotides differences (*K*) determined genetic diversity for each individual population and for total data. Genetic differentiation between populations for each locus was estimated using database containing haploid genomic information for each organism. From nucleotide data information, Wright's *F*-statistics (F_{ST}) and effective number of migrants per population and generation (Nm) were derived from equation 3 of Hudson *et al.* (1992). From haplotype data information, *G*-statistics (G_{ST}) (Nei, 1973), an extension of Wright's F_{ST} to the case of multiple alleles, and Nm values were computed following equations 5 and 6 from Hudson *et al.* (1992). In this analysis, sites with alignment gaps were considered as the fifth state, i.e. a different nucleotide variant. Conversely, R_{ST} statistics (Slatkin 1995) analogous to F_{ST} and specific for microsatellite markers was not calculated due to its high sensitivity with assumptions underlying microsatellite evolution (Gaggiotti *et al.* 1999). In *G. abietina* microsatellite evolution is unknown. Tamura and Nei Model 21 (Tamura & Nei 1993) was selected as a substitution model for the construction of the neighbour-joining (NJ) tree using the MODELTEST-program (Posada & Crandall 1998). MEGA 4.0.1 (Tamura *et al.* 2007) was used to construct optimal NJ tree for GAAA1000 and ACA900 markers (Saitou & Nei 1987); and Interior Branch Test (500 replicates, seed = 64 238 for GAAA1000; 500 replicates, seed = 17 099 for GAAA800 and finally, 500 replicates, seed = 17 099 for ACA900) was used as a phylogeny test.

Results

Genetic diversity within populations

Consistent with the banding pattern obtained from CCA or CGA markers, Spanish isolates were confirmed to be *Gremmeniella abietina* (Hantula & Müller 1997). The hypervariable sequence characterized region (SCAR) GAAA1000 (Uotila *et al.* 2006) of all DNA samples was amplified. PCR products of all isolates had a length of approximately 700 bp. Respective sequences were obtained and a total of 235 sequences were compiled and analyzed (Table 2). Overall, 92 Spanish isolates were compared to six Swiss isolates of Alpine biotype, 76 Finnish biotype A isolates, 54 Finnish biotype B isolates and seven Russian isolates of biotype B. Sequence alignments showed different nucleotide compositions and lengths of the region GAAA1000 depending on the biotype. According to (i) the number and diversity of haplotypes, (ii) number of polymorphic sites and (iii) number of nucleotides differences found in each population, the Spanish population showed the lowest genetic variability with only one polymorphic site in a total length of 525 bp of sequence. The rest of the biotypes had similar diversity, with A type showing the highest diversity (Table 2).

Table 2 – Genetic diversity within populations.

		GAAA1000	GAAA800	ACA900
	Total number of sequences	235	101	101
	Total number of sites	576	371	368
N	A	76	52	52
	B	61	32	32
	Alpine	6	6	6
	Spanish	92	11	11
	Total data estimates	33	7	3
h	A	16	3	2
	B	12	3	2
	Alpine	5	2	2
	Spanish	2	1	1
	Total data estimates	109	24	2
S	A	88	6	1
	B	29	24	1
	Alpine	17	9	1
	Spanish	1	0	0
	Total	0.81	0.67	0.52
Hd	A	0.72	0.11	0.38
	B	0.78	0.28	0.51
	Alpine	0.93	0.33	0.53
	Spanish	0.10	0	0
	Total data estimates	27.32	4.03	0.54
K	A	8.98	0.45	0.38
	B	5.52	3.95	0.51
	Alpine	7.93	3.00	0.53
	Spanish	0.10	0	0

N, Sequences studied per population. *h*, Number of haplotypes. *S*, Number of polymorphic/indel/missing sites. Hd, Haplotype diversity. *K*, Average number of nucleotides differences.

Other two SCAR markers, GAAA800 and ACA900, were amplified and sequenced with the purpose of further analyses of population genetic structure. Altogether, 102 isolates for each marker were amplified and sequenced (Table 2). PCR products of GAAA800 and ACA900 had lengths of approximately 450 bp, and their sequence alignments showed less genetic variability among populations than GAAA1000, particularly ACA900. In both cases, the Spanish sequences were least variable and B type most variable (Table 2).

The allele frequency data of GAAA1000, GAAA800 and ACA900 appear in Tables 3–5, respectively. Almost all alleles in GAAA1000 are unique to different populations, with the exception of most common allele in the Spanish population, which is also observed in one isolate of A type. Also in

Table 3 – Allele frequencies data for marker GAAA1000.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
A type	0.01	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.51	0.09	0.04	0.01	0.01	0.09	0.07	0.03	0.01	0.01	0.03	0.05	0.01	0	0	0.01
B type	0	0.23	0.39	0.02	0.08	0.03	0.02	0.02	0.03	0.1	0.05	0	0	0	0	0	0.02	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.02	0
Alpine	0	0	0	0	0	0	0	0	0	0	0	0.17	0.33	0.17	0.17	0.17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Spain	0.95	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.05	0	0

Table 4 – Allele frequencies data for marker GAAA800.

	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5	Allele 6	Allele 7
A type	0.04	0.94	0.02	0	0	0	0
B type	0	0	0.84	0.13	0	0.03	0
Alpine	0	0	0	0	0.83	0	0.17
Spain	1	0	0	0	0	0	0

GAAA800 most of the alleles are unique to different populations, with the exception of the fixed allele in the Spanish population, which is also found in low frequency in A type. The most common allele in B type occurs in low frequency in A type. In ACA900 the most common allele found in all populations: it is fixed in the Spanish type, and observed also as the most common one in B and Alpine types, and as a minor variant in A type. The second most common allele is the most common in A type and also observed in B type. The third allele in this locus is unique to the Alpine population.

Genetic variability between populations

Four populations of European *Gremmeniella abietina* showed different allele distributions depending on the marker (GAAA1000, GAAA800, ACA900). Genetic differentiation values between populations are all expressed in Table 6. Based on this data, all studied populations showed high degree of polymorphism, with the exception of Spanish population, which was almost monomorphic.

Based on GAAA1000, genetic differentiation between populations was very high, as the F_{ST} value among all populations was 0.81. High genetic differentiation was confirmed by analyses of GAAA800 ($F_{ST} = 0.69$) and ACA900 ($F_{ST} = 0.45$) using smaller data set. Similar results were observed using G_{ST} values, with the highest and the lowest values observed at 0.75 and 0.26, respectively.

Pairwise comparisons were used to compare genetic relationships between populations. The Spanish population was highly differentiated from A, B and Alpine biotypes for all markers; smallest values were observed for marker ACA900 in respect to Alpine type ($F_{ST} = 0.20$; $G_{ST} = 0.14$). Between A-, B- and Alpine biotypes, a lower genetic differentiation was observed, although all of them had F_{ST} values higher than 0.13.

Finally, occurrence of linkage disequilibrium between mutations within each locus was tested. In all cases a statistically significant value of Chi-square (Nei 1987; Hudson *et al.* 1992, equation 1) was observed (p-value < 0.001). For GAAA1000 Chi-square was 677 812 and degrees of freedom (df) were 96. For GAAA800, Chi-square was 279 258 and df = 18, and finally, for ACA900 Chi-square was 59 453 and df = 6.

Table 5 – Allele frequencies data for marker ACA900.

	Allele 1	Allele 2	Allele 3
A type	0.23	0.77	0
B type	0.56	0.44	0
Alpine	0.67	0	0.33
Spain	1	0	0

Table 6 – F_{ST}, G_{ST} and Nm estimates between European populations of *Gremmeniella abietina*.

	GAAA1000		GAAA800		ACA900	
	F_{ST}	G_{ST}	F_{ST}	G_{ST}	F_{ST}	G_{ST}
Spanish *vs* A	0.91	0.43	0.92	0.74	0.75	0.34
Spanish *vs* B	0.94	0.42	0.55	0.61	0.42	0.16
Spanish *vs* Alpine	0.92	0.28	0.80	0.76	0.20	0.14
A *vs* B	0.28	0.14	0.66	0.68	0.16	0.09
A *vs* Alpine	0.30	0.06	0.63	0.52	0.58	0.17
B *vs* Alpine	0.13	0.06	0.63	0.40	0.32	0.07
Overall	0.81	0.39	0.69	0.75	0.45	0.26
Nm[a]	0.11	0.79	0.23	0.17	0.6	1.44

a Total estimation of effective number of migrants per population.

Analyses of variation in GAAA1000, GAAA800 and ACA900 SCARS

Final phylogenic NJ tree based on GAAA1000 (Fig 1) shows a closer position of Alpine type to Finnish B type than to Finnish A type. Moreover, Spanish isolates appeared clearly separated from other biotypes, with the exception of one sequence from A-type isolate Ka04-36, which was identical to Spanish isolates. Closer (detailed) observation of sequences revealed the 5′ end of GAAA1000 allele of AHT49 resemblance to the 5′ end of Spanish isolates, whereas the 3′ end was more typical of other A-type isolates.

In addition, deletions were detected inside the alignment; from positions 17 to 21, one deletion was shared by all sequences except one B-type isolate. Second deletion ranged from positions 134 to 142, and was shared by all Spanish, two A-type and three B-type isolates. The rest of samples had a deletion from 139 to 142, except three Alpine sequences of which one lacks it completely, and in other two, deletion ranges from positions 140 to 141. Spanish isolates as well as type A isolates AHT49 and Ka04-36 share a deletion ranging from 155 to 174. Also, the lengths of other sequences vary considerably in this area. From positions 283 to 287, deletion occurred in all sequences except Spanish Ka04-36 and AHT49. From positions 356 to 365 all sequences except one A-type isolate had the same deletion; which in the Spanish isolates and Ka04-36 is longer and extends to position 372. Additionally, four B-type and three A-type isolates coincide between positions 368 and 377. Finally, all Spanish sequences and Ka04-36 match at deletion ranging from 530 to 544, while four B-type and ten A-type isolates share 553– 554 deletion (Table 7).

The phylogram based on ACA900 showed no clear differentiation linked to populations (not shown); and therefore did not provide information about relative links between different populations. It did not contain indels and variability was based only on two point mutations. At position 207 two Alpine type sequences differed from the rest and at position 222 two alleles were present: 40 A-type sequences and 14 B-type sequences shared one allele, whereas 12 A-type, 18 B-type, six Alpine and 11 Spanish sequences had the other in common.

Finally, the variation in GAAA800 was based only on indels; and therefore we did not construct a phylogram, as no solid evolutionary model is available. However, based on this indel

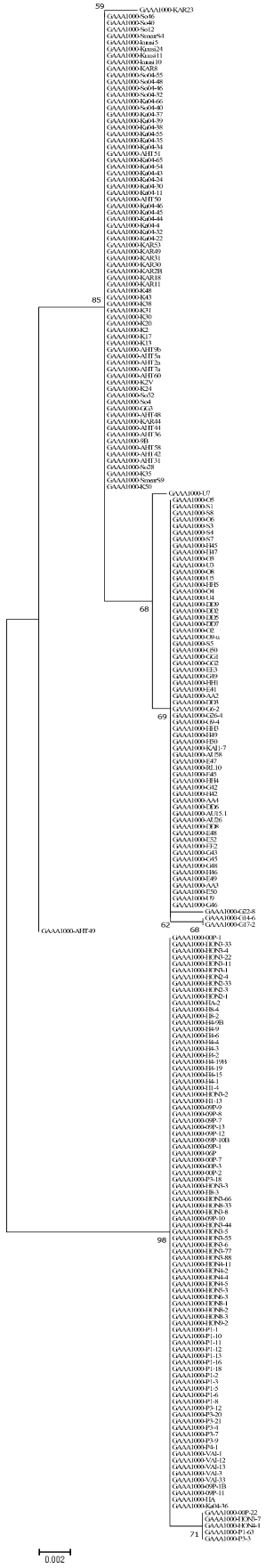

Fig 1 – Construction of the NJ tree based on GAAA1000 microsatellite.

Table 7 – Relation of the different positions of deletions in marker GAAA1000.

	Positions	A	B	Alpine	Spanish
Deletion 1	17–21	76	60	6	92
Deletion 2	134–142	2	3	0	92
Deletion 3	139–142	74	58	6	0
Deletion 4	140–141	0	0	2	0
Deletion 5	155–174	2	0	0	92
Deletion 6	283–287	74	61	6	0
Deletion 7	356–365	75	61	6	92
Deletion 8	356–372	1	0	0	92
Deletion 9	368–377	3	4	0	0
Deletion 10	530–544	1	0	0	92
Deletion 11	553–554	10	4	0	0

data all Spanish sequences were identical and shared the same sequence with two isolates of type A. Twenty-seven B-type sequences shared an indel with only one A-type isolate. The rest of A-type sequences were identical. Moreover, the sequences of four B-type isolates were identical, although one of them was 1 bp longer than the others. Finally, the Alpine type had two unique alleles composed of six and one isolates.

Discussion

Over the years, races, varieties, biotypes and species within *Gremmeniella* have been recognized through applications of different techniques, including conidial morphology (Petrini *et al.* 1989), serology (Dorworth 1974), soluble protein electrophoresis (Petrini *et al.* 1990; Lecours *et al.* 1994), FAST profiles (Müller & Uotila 1997) and DNA fingerprinting techniques, such as RAPD (Santamaría *et al.* 2005), ITS (Hamelin *et al.* 2000), STS (Dusabenyagasani *et al.* 2002) and RAMS (Hantula & Müller 1997; Kraj & Kowalski 2008). In this work, sequence analysis of the hypervariable SCAR marker GAAA1000 together with two other SCAR markers GAAA800 and ACA900 (Uotila *et al.* 2006) was used to determine the degree of genetic differentiation of Spanish isolates with regard to the rest of European biotypes. Simultaneously, we determined the phylogenic relationship of other biotypes based on sequence data of highly variable loci, which serve as a more accurate basis for taxonomy (Kasanen *et al.* 2004) than previously conducted analyses.

Phylogenic relationships among A, B, Alpine and Spanish populations revealed a close relationship of Alpine and B types, whereas Spanish and A-type isolates appeared separated from any other biotypes. This conclusion is supported by the high F_{ST} and G_{ST} values between all populations, which indicate low gene flow between them. Moreover, high level of linkage disequilibrium observed in all populations is in accordance with the conclusion. Thus, based only on these analyses the Spanish population resembles another previously unknown biotype or species in Europe. This finding is in agreement with a previous study by Santamaría *et al.* (2005), who showed that Spanish isolates are different from North American, B- and Alpine-type isolates. The results suggested low variability within Spanish population and a closer association to the EU race in North America.

However, Ka04-36 isolate is particularly important, as it shares an identical GAAA1000 sequence with Spanish isolates. Ka04-36 sequence was determined in a Finnish laboratory long before any Spanish isolates had been analyzed; therefore the likelihood of contamination is improbable. Consequently, this isolate (together with isolate AHT49, which includes Spanish-type sequence) forms a link between the European A-type and Spanish isolates. As a result, Spanish isolates form a highly differentiated population of *Gremmeniella abietina*, which has gone through a genetic bottleneck during its history.

View of a severe genetic bottleneck in the history of Spanish isolates is supported by the extremely low degree of variation among them (Table 2). The bottleneck could have been caused by several factors. It could have resulted from a founder effect due to a small population dispersed in Spain. Or, it may have developed in a restricted region where isolates are currently found in an approximated area with diameter of 50–100 km. Lastly, it can be attributed to mild climatic conditions of the affected region, which are not favourable to production of apothecia (Kraj & Kowalski 2008; Kraj 2009), which may limit the population genetic structure (McDonald & Linde 2002) and consequently the number of gene combinations.

Taxonomic positions of other *Gremmeniella* populations in Europe are easier to interpret. Alpine- and B-type populations are members of the same species separated by geographic location into two distinct populations. They both share highly similar alleles and group together in clustering analysis. However, relatively high F_{ST} and G_{ST} values observed between these biotypes reflect a lengthy separation of the two populations since the last ice age.

Phylogenic proximity of Alpine and B types corresponds with population ecology, as both inhabit high altitudes with harsh climatic conditions and their associated hosts exhibit similar symptoms. Both pathogens cause cankers and produce pycnidia or apothecia on young trees covered with snow in winter (Uotila 1983; Karlman *et al.* 1994; Hamelin *et al.* 1996). Moreover, they share the characteristic of generating high amounts of apothecia, which is genetically determined (Uotila 1992).

Because of the fairly recent re-discovery of *G. abietina* in Spain, a small number of epidemiology studies have been published (Santamaría *et al.* 2003). Currently, Spanish *G. abietina* has not been detected in its sexual phase (Santamaría *et al.* 2003), resembling A biotype, which however, sporadically produces a limited number of apothecia in the field (Uotila 1983). However, based on geography, *G. abietina* turns up in north-west of Spain at 800 m above sea level in a Mediterranean continental climate, where temperatures below zero can occur but snow is rare. It infects trees of all ages and currently it has not been detected to reproduce sexually. Thus, in this respect it is similar to the A type, which infects the crown of both young and mature trees (Uotila 1983; Hellgren & Högberg 1995; Hamelin *et al.* 1996). As a difference between the two, type A produces a limited number of apothecia in the field (Uotila 1983), which have not been found in Spain (Santamaría *et al.* 2003). However, it cannot be ruled out that the lack of apothecia can be caused by unfavourable environmental conditions in Spain, i.e. lack of long periods of rain and snow (Kraj & Kowalski 2008; Kraj 2009; Thomsen 2009). Lastly, type A has been found in regions of Italy (Barbacovi *et al.* 1979) and Turkey (Spaulding 1961) comparable to Spanish latitudes.

As a conclusion, the Spanish population of *G. abietina* looks like a highly specialized population derived from biotype A. It lacks gene flow with other populations of *G. abietina* in Europe,

which may be either a result of geographical separation or already established speciation. As those two alternatives cannot be resolved by DNA analyses, the taxonomic status of Spanish *G. abietina* can only be tested by pairing experiments and analyses of possible offspring as previously conducted for A and B types (Uotila *et al.* 2006).

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