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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,