A genetically homogenous population of *Fusarium circinatum* causes pitch canker of *Pinus radiata* in the Basque Country, Spain

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Abstract

Pitch canker, caused by *Fusarium circinatum*, is a destructive disease of *Pinus* species and has recently been shown to represent a substantial threat to natural and commercial forests in northern Spain. The genetic diversity of *F. circinatum* in the Basque Country of Spain was assessed by characterising 96 isolates based on vegetative compatibility groups (VCGs), mating type assays, polymorphic DNA-markers and amplified fragment length polymorphism (AFLP) analyses. For this purpose, *F. circinatum* isolates were collected from diseased *Pinus radiata* as well as from insects associated with this host. Overall, a low level of diversity was detected in the population. The isolates represented only two VCGs and they were all of the same mating type. AFLP analyses revealed three genotypes and polymorphic DNA-markers specific for *F. circinatum* showed nine genotypes. The most common genotypes represented 97 % of all isolates for AFLP analysis and 68 % of isolates for the polymorphic DNA-marker sets. Over all, this indicates that pitch canker in the Basque Country of Spain is caused by a clonally propagating population of *F. circinatum*, typical of a recently introduced pathogen.

Introduction

Pitch canker, caused by *Fusarium circinatum* (Nirenberg & O'Donnell 1998) (teleomorph = *Gibberella circinata*), is a destructive disease of *Pinus* species. The disease is characterised by heavy exudation of resin at the site of infection (Wingfield *et al*. 2008). In addition to negatively impacting growth, this pathogen is capable of killing both mature trees and seedlings.

The first report of pitch canker was in North Carolina, USA in the 1940s (Hepting & Roth 1946) and the pathogen is now known to be present across the southeastern United States, as well as in California (Dwinell *et al*. 1985; McCain *et al*. 1987; Wingfield *et al*. 2008). The disease has also been recorded in numerous countries worldwide, including Haiti (Hepting & Roth 1953), Chile (Wingfield *et al*. 2002), South Africa (Viljoen *et al*. 1994), Japan (Kobayashi & Muramoto 1989), Korea (Lee *et al*. 2000), Mexico (Britz *et al*. 2001), Italy (Carlucci *et al*. 2007), Portugal (Braganca

et al. 2009) and Spain (Landeras *et al*. 2005). *Fusarium circinatum* has been found to be pathogenic to, or reported on, over 60 species of pine (Hodge & Dvorak 2000; Gordon 2006), and one non-pine host, *Pseudotsuga menziesii* (Douglas-fir) (Storer *et al*. 1997). Of these, *Pinus radiata* is considered to be one of the most susceptible (Gordon *et al*. 2001). This pathogen represents a significant threat to regions where *Pinus* species or *P. menziesii* occur naturally or where native or non-native species are commercially grown.

Successful pitch canker infections require wounds or openings on the tree as intact tissue is not vulnerable to invasion by the fungus (Gordon *et al*. 1998a). In general, pitch canker is associated with wounds created by insects, weather or mechanical damage, and spores can be disseminated by wind, rain, animals, insects or soil. The importance and association of vectors and wounding agents can vary between locations where pitch canker occurs. For instance, in the southeastern United States, infection courts are thought to be created primarily by weather and mechanical damage (Dwinell *et al*. 1985), whereas in California pitch canker infections are associated mainly with wounding caused by insects (Gordon *et al*. 2001). The pathogen can also be transmitted via seed. Infected seed often display no symptoms until the seed germinates, although in some cases infected seed can germinate and produce symptomless seedlings from which the fungus can be isolated (Storer *et al*. 1998).

Fusarium circinatum, which is thought to spread primarily by conidia produced as a result of asexual reproduction, is a heterothallic fungus in which sexual compatibility is determined by a single mating type locus harbouring one of two alternative idiomorphs: MAT-1 or MAT-2 (Kerenyi *et al*. 1999). The sexual stage (*G. circinata*) has not been observed in nature but has been produced under controlled conditions (Britz *et al*. 2002a; Viljoen *et al*. 1997a). Numerous studies have sought to assess the relative importance of sexual and asexual reproduction in natural populations of *F. circinatum* by characterising vegetative compatibility group (VCG) diversity, determining the relative frequencies of the two mating type idiomorphs or using DNA based polymorphic markers to assess genotypic diversity (Britz *et al*. 2005). Based on these studies, it appears that clonal propagation dominates in most populations, but recent outcrossing cannot be excluded in all cases (Correll *et al*. 1992; Gordon *et al*. 1996; Viljoen *et al*. 1997b; Wikler & Gordon 2000).

In the northern regions of Spain, pitch canker poses a serious threat to commercial plantations of non-native *P. radiata* and *P. menziesii* as well as native populations of *Pinus pinaster* and *Pinus sylvestris* (Landeras *et al*. 2005). Perez-Sierra *et al*. (2007) sampled populations of *F. circinatum* from *P. radiata*, *Pinus nigra*, *P. pinaster* and *P. sylvestris* in three adjacent, autonomous communities (Galicia, Asturias and Cantabria) on the north coast of Spain, where they found both mating types of the fungus to be present. This implies that the pathogen has the capacity for sexual reproduction, although additional evidence is required to demonstrate that out-crossing actually occurs in these populations.

In the Basque Country of Spain, *P. radiata* is considered to be the most important plantation species. This species accounts for almost 60 % of the forested area in Bizkaia, a province within the Basque Country (Gonzalez-Ariasa *et al*. 2006). The presence of pitch canker in the Basque Country and its association with bark beetles is a serious concern to its forest industry (Romon *et al*. 2007). The objective of this study was to characterise the genetic diversity of *F. circinatum* in the Basque Country using mating type assays, VCG tests, DNA-based polymorphic markers, and amplified fragment length polymorphism (AFLP) analyses. This information is expected to provide insights into the predominant mode of reproduction in this population and how recently it may have become established in the Basque Country.

Materials and methods

Collection and isolation of *Fusarium circinatum*

In 2004, two sets of collections of *Fusarium circinatum* were obtained from *Pinus radiata* displaying symptoms resembling those of pitch canker (i.e., stems exuding resin, flagged branches with apparent cankers) in the Basque Country of Spain. The first set of collections (Collection 1) consisted of isolates collected from *P. radiata* across the provinces of Bizkaia, Gipuzkoa and the northern region of Araba. One individual stemor branch canker was sampled fromeach tree. The second set of collections of isolates (Collection 2) was obtained from three *P. radiata* plantations in Durango (P1) and Laukiz (P2) in the province of Bizkaia, and Onyi (P3) in the province of Gipuzkoa, which subsequently have been destroyed. For each plantation, the sampling strategy involved collecting one individual stem or branch canker from each of 20 symptomatic trees and 10 samples (individual stem or branch cankers) from a single, heavily infected, symptomatic tree. Any insects found in association with the trees sampled in plantation P3 were also collected. This included insects found on the bark of the trees or insects found inside the tree where cankers were sampled.

To isolate the pitch canker fungus, stem or branch canker samples were surface sterilised with 1 % sodium hypochlorite and plated onto *Fusarium* selective medium(FSM), as described by Aegerter & Gordon (2006), and 4 % (w/v) potato dextrose agar (PDA), while insects were plated directly onto FSM. Plates were sealed with parafilm and incubated at 22 C in the dark. Any resulting fungal colonies resembling *F. circinatum* in culture were isolated by transferring a germinated single conidium to fresh PDA medium. All of the subsequent single-conidium isolates (CMW28934-CMW29025, CMW32945-CMW32948) used in this study are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI),University of Pretoria, South Africa. A representative strain (CECT 20759) has been deposited in the Spanish Type Culture Collection, Coleccion Espanola de Cultivos Tipo (CECT), at the University of Valencia, Spain.

DNA extraction

All isolates were grown on PDA for 7e10 d at 25 C and mycelium was then scraped from the surface of the growth medium and transferred to sterile tubes. DNA for amplification of specific gene or genomic regions was extracted using either a DNeasy Plant Mini Kit (Qiagen, California, USA) or a Nucleo-Spin® Plant kit (Macherey-Nagel, Duren, Germany). For the AFLP analyses, DNA was prepared by homogenising the mycelium harvested from a single plate in 500 mL TES-ProtK buffer [100mM Tris-HCL (pH 8); 10mM EDTA (ethylene diamine tetraacetate, pH 8.0), 2 % (w/v) SDS (Sodium dodecyl sulphate); 0.2 mg/mL Proteinase K (Roche, Indiana, USA)], followed by freezing the extraction mixtures at –20 C and then incubating them at 60 C for 60min. To each of these, 320 mL CTAB-NaCl buffer [2.5 % (w/v) CTAB (N-cetyl-N,N,N-trimethyl-ammonium bromide), 3.75M NaCl] was added, followed by incubation at 65 C for 10 min and standard phenol-chloroform (1:1) extractions (Sambrook *et al*. 1989) for DNA purification. The DNA was then precipitated with 0.6 volume of 2-propanol and overnight incubation at –20 C, and harvested by centrifugation at 15 700g for 30min at 4 C, washed with 70 % ethanol, air-dried, and resuspended in deionised water.

Fusarium circinatum identification

Isolates were provisionally identified as *Fusarium circinatum* based on morphology, followed by subsequent confirmations based on molecular criteria. For identifications based on morphology, cultures were incubated on PDA, carnation leaf agar (Fisher *et al*. 1982) and synthetic low nutrient agar (Nirenberg 1976) for 10 d at 25 C under near-ultraviolet light. Isolates were then examined

microscopically to evaluate diagnostic characters reported by Nirenberg & O'Donnell (1998). To determine the identity of cultures using DNA-based methods, isolates were subjected to the histone H3 PCR-RFLP (restriction fragment length polymorphism) diagnostic technique for *F. circinatum* using the primers, PCR conditions and digestion protocol described previously (Steenkamp *et al*. 1999). Isolates that produced the expected 232 and 250 base pair (bp) fragments that appeared as a doublet on 2 % agarose gels after electrophoresis were considered as *F. circinatum*. A portion of the gene encoding translation elongation factor 1-alpha (EF-1a) was also sequenced, in both the forward and reverse directions, for seven isolates as described previously (Geiser *et al*. 2004) using primers EF1 and EF2 (O'Donnell *et al*. 1998), the ABI PRISM BigDye Terminator v3.0 Cycle Sequencing Kit (Applied Biosystems, California, USA) and a 3730 DNA Analyser (Applied Biosystems). Sequences generated were compared to those in the *Fusarium* identification database (Geiser *et al*. 2004) (http://fusarium.cbio.psu.edu/) using the BLAST search tool (Altschul *et al*. 1990).

Vegetative compatibility groups (VCGs) and mating type

VCGs were determined for a selection of isolates based on the protocol described by Correll *et al*. (1987). Isolates from both collections (Collections 1 and 2) were plated onto PDA with chlorate to obtain nitrate non-utilising (*nit*) mutants. The resulting nit mutants were subcultured and assigned to phenotypes (*nit*1, *nit*3 or NitM) (Correll *et al*. 1987) based on their ability to manifest wild type growth on minimal medium supplemented with different nitrogen sources (nitrate, nitrite or hypoxanthine, respectively). Pairings between *nit* mutants derived from different isolates and control pairings (different *nit* mutants from the same isolate) were performed on minimal medium with nitrate. Isolates were assigned to the same VCG when wild type growth developed along the line of contact between the isolate pairs being tested.

Mating type was assayed by amplifying *MAT*-1 and *MAT*-2-specific fragments using primer pairs GFmat1a and GFmat1b, and GFmat2c and GFmat2d, respectively (Steenkamp *et al*. 2000). The *MAT*-2-specific PCR product was sequenced from two isolates (representing different VCGs) to confirm that the amplified fragments represented the *MAT*-2 region of the MAT-locus.

DNA-based polymorphic marker analyses

Two sets of polymorphic DNA-markers were used to analyse the *Fusarium circinatum* isolates in this study. The first set included FC5 and FC9 (amplified with primers HB18þ HB19 and HB34þ HB35, respectively) developed by Britz *et al*. (2002b), and the second included G18, G60, G98 and G220 (amplified with primers 18Fþ 18R, 60Fþ 60R, 98Fþ 98R, and 220Fþ 220R, respectively) developed by Wikler & Gordon (2000). The markers G18, G60, G98 and G220 were scored based on the size of amplicons, which were visualised using 1 % (w/v) agarose gel electrophoresis (Wikler & Gordon 2000). For the two markers developed by Britz *et al*. (2002b), one primer from each set was labelled with a phosphoramidite fluorescent dye (MWG). For FC5, primer HB18 was labelled with PET and for primer set FC9, HB34 was labelled with FAM. The resulting FAM- and PET-labelled PCR products for each isolate were pooled and mixed with the GeneScan™-500 LIZ™ size standard (Applied Biosystems) and loading buffer, after which samples were denatured at 95 C for 15 min and run on an ABI Prism 377 DNA sequencer (Applied Biosystems). PCR product size was determined using GeneScan® 2.1 analysis software (Applied Biosystems) and GeneMapper® 3.0 (Applied Biosystems).

Negative controls and positive controls were used in all PCR amplifications. The positive controls used were *F. circinatum* isolates 2494, 2481 and 2248 from the FABI *Fusarium* culture collection. These isolates had been used in either Britz van Heerden (2002) or Wikler & Gordon (2000). PCR amplifications were repeated at least twice for any isolates for which no amplicon was obtained to

confirm the absence of a product.

AFLP analysis

For AFLP analysis, the procedure described by Vos *et al*. (1995) was utilised, where genomic DNA was digested with restriction enzymes *Eco*RI (Roche) and *Mse*I (New England Biolabs, Massachusetts, USA) and ligated to corresponding enzyme-specific oligonucleotide adapters. However, preselective and selective amplifications utilised zero-base-addition *Eco*RI and *Mse*I adapter-specific primers and two-base-addition *Eco*RI and *Mse*I adapter-specific primers, following the reaction and cycling conditions described previously (De Vos *et al*. 2008). A set of four two-base-addition *Eco*RI and *Mse*I adopter-specific selective primers (*Eco*RI-AC + *Mse*I-TT, *Eco*RI-TT + *Mse*I-TT, *Eco*RIAC + *Mse*I-AT, and *Eco*RI-AT + *Mse*I-AT) were used. In each case, the *Eco*R1 selective primer was labelled with either an IRDye™ 700 or IRDye™ 800 infrared dye (LI-COR#, Nebraska, USA). The resulting AFLP fragments and 50e700 bp size standard (LI-COR#) were separated using the 4200 LI-COR® automated DNA sequencer and analysed with QUANTAR Version 1.0 (KeyGene Products B.V., Wageningen, The Netherlands) as described previously (Myburg *et al*. 2001). Only clear and well-resolved bands were scored manually as present or absent (faint or very intense bands were not considered). To ensure that the AFLP patterns observed in this study were reproducible, the AFLP procedure was repeated using DNA from new extractions.

Genetic diversity and population structure

Genetic diversity was assessed using a combination of data obtained from VCGs, mating type, AFLP analysis and the polymorphic DNA-markers. The allele frequency at each locus and average genetic diversity (*H*) for loci derived from the polymorphic DNA-markers and AFLPs were determined using GenAlEx 6 (Peakall & Smouse 2005).

Results

Collection and identification of *Fusarium circinatum*

Fusarium circinatum was isolated from 35 *Pinus radiata* trees displaying pitch canker-like symptoms sampled from across the Basque Country of Spain (Collection 1). The pitch canker fungus was also isolated from all trees sampled from three *P. radiata* plantations in the provinces Bizkaia and Gipuzkoa in the Basque Country (Collection 2). Ninety isolates were collected from the three *P. radiata* plantations and 16 isolates were collected from three insect species, *Pissodes costaneus*, *Pityophthorus pulsceus* and *Tomicus piniperda*, associated with plantation P3.

The isolates collected had coiled, sterile hyphae in the aerial mycelium, lunate macroconidia, polyphialides, and no chlamydospores. They also displayed typical *F. circinatum* H3 PCR-RFLP fingerprints. Comparison of EF-1a sequences for seven of the isolates with those in the *Fusarium* identification database also confirmed the identity of these isolates as *F. circinatum*, as the sequences for all seven were the same as that for isolate NRRL 25331 in the database (Geiser *et al*. 2004). The only exception was one isolate (CMW28967) that differed at one nucleotide position from NRRL 25331.

Ninety-six isolates were selected from the 141 isolates of *F. circinatum* collected in this study for further genetic analyses, as described below. This included all 35 isolates from Collection 1 and 61 isolates from Collection 2. The latter included all 30 isolates from plantation P3, 13 isolates each from plantations P1 and P2, and five isolates from insects collected in plantation P3.

Vegetative compatibility groups (VCG) and mating type

VCGs were determined for a selection of 58 Basque Country *Fusarium circinatum* isolates from both collections. Two VCGs, designated A and B, were identified among these isolates. Of the 32 isolates tested from Collection 1, 14 were associated with VCG A and 18 with VCG B. Both VCGs were geographically distributed across the collection range (Fig 1C). All 26 isolates tested from Collection 2 (P1e3) belonged to VCG B.

PCR-based identification of mating type revealed that all 96 isolates of *F. circinatum* carry the MAT-2 idiomorph. The typical 800 bp fragment was amplified from all the isolates tested. Analyses of the sequences of the selected MAT-2 PCR products (GenBank accession numbers GU369704 and GU369705) also showed that these fragments encoded the expected portion of the conserved HMG (high-mobility-group) domain of the MAT-2 idiomorph of *F. circinatum* (Steenkamp *et al*. 2000).

Analyses using the polymorphic DNA-markers and AFLPs

Application of the two sets of polymorphic DNA markers to the selected isolates of *Fusarium circinatum* revealed few polymorphisms. The primer set for FC9 amplified a 244 bp fragment for all but one isolate, which amplified a 241 bp fragment (Table 1). Primer set for FC5 amplified a 450 bp fragment from all isolates tested (Table 1). The primer set for markers G18, G60, G98 and G220 amplified 510, 800, 950 and 400 bp fragments, respectively. Fragments corresponding to the expected size were produced in all the positive controls. However, for many of the isolates, the G markers did not amplify any product, despite at least two additional attempts during which more diluted and/or concentrated DNA were used as template (Table 1). This was probably due to the lack of suitable primer binding sites in these isolates to allow proper amplification.

Using AFLPs, we identified 103 loci represented by fragments ranging between 50 and 500 bp in size. Of the four selective primer sets used, *Eco*RI-AC + *Mse*I-AT revealed nine polymorphisms, while *Eco*RI-AT + *Mse*I-AT, *Eco*RI-AC + *Mse*ITT and *Eco*RI-TT + *Mse*I-TT revealed four, three and one polymorphism, respectively. However, all 17 of these polymorphisms were associated with only three isolates. The remaining 93 isolates had identical profiles for all four AFLP primer sets used. As expected (Myburg *et al*. 2001), identical profiles were generated when the AFLP procedure was repeated on the selected isolates.

Genetic diversity and population structure

The genetic diversity for the Basque *Fusarium circinatum* population using both sets of polymorphic DNA-markers (FC5, FC9, G18, G60, G98 and G220) was H = 0.169, and H = 0.248 when analysing the four DNA-markers (G18, G60, G98 and G220) (Table 1). In these analyses, the absence of a PCR product was interpreted as a null allele at the specific locus. As the possibility that each of these nulls actually represent distinct alleles could not be ruled out, we also subjected the isolates to AFLP analysis, which confirmed the overall low diversity observed with the G and FC markers. For the 17 polymorphic AFLP loci, the genetic diversity was H = 0.010.

Within the population, nine genotypes (M1-9) were distinguished using the five polymorphic DNA-markers (i.e., FC9 and G18, G60, G98 and G220) (Table 2, Fig 1A). Three genotypes (A1-3) were distinguished among the isolates using AFLPs (Table 2, Fig 1B). The most abundant genotypes, M1, M2 and A1, were associated with both VCG A and VCG B. The less frequently identified genotypes based on the polymorphic DNA markers (M3-6 and M8-9) and the AFLP genotypes (A2-3) were associated with VCG B only (Table 2). The VCG of the isolate with genotype M7 is unknown. All 10 isolates obtained from the single P3 tree and all isolates obtained from insects (Collection 2) were associated with the most common genotypes M1 (polymorphic DNA-markers)

and A1 (ALFPs). Only one isolate, which represented genotypes M9 and A3, was distinguishable using both the AFLP and polymorphic DNA markers.

Table 1. Genetic diversity of *Fusarium circinatum* isolates based on polymorphic DNA-markers.

Locus	Allele[a]	Frequency[b]	Genetic diversity (H)[c]
FC5	450	1	–
FC9	241	0.011	–
FC9	244	0.989	–
G18	510	0.738	–
G18	Null	0.262	–
G60	800	0.841	–
G60	Null	0.159	–
G98	950	0.852	–
G98	Null	0.148	–
G220	400	0.955	–
G220	Null	0.045	–
FC5, FC9	–	–	0.011 (±0.011)
G18, G60, G98, G220	–	–	0.248 (±0.062)
FC5, FC9, G18, G60, G98, G220	–	–	0.169 (±0.064)

a Alleles are characterised in terms of the size (indicated in base pairs) of the amplicons generated by the respective primer sets for the various markers or null in the absence of a PCR product.
b Frequency = number of isolates with genotype/total number of isolates (96).
c Average genetic diversity (±Standard Error) over all loci considered; see Materials and methods.

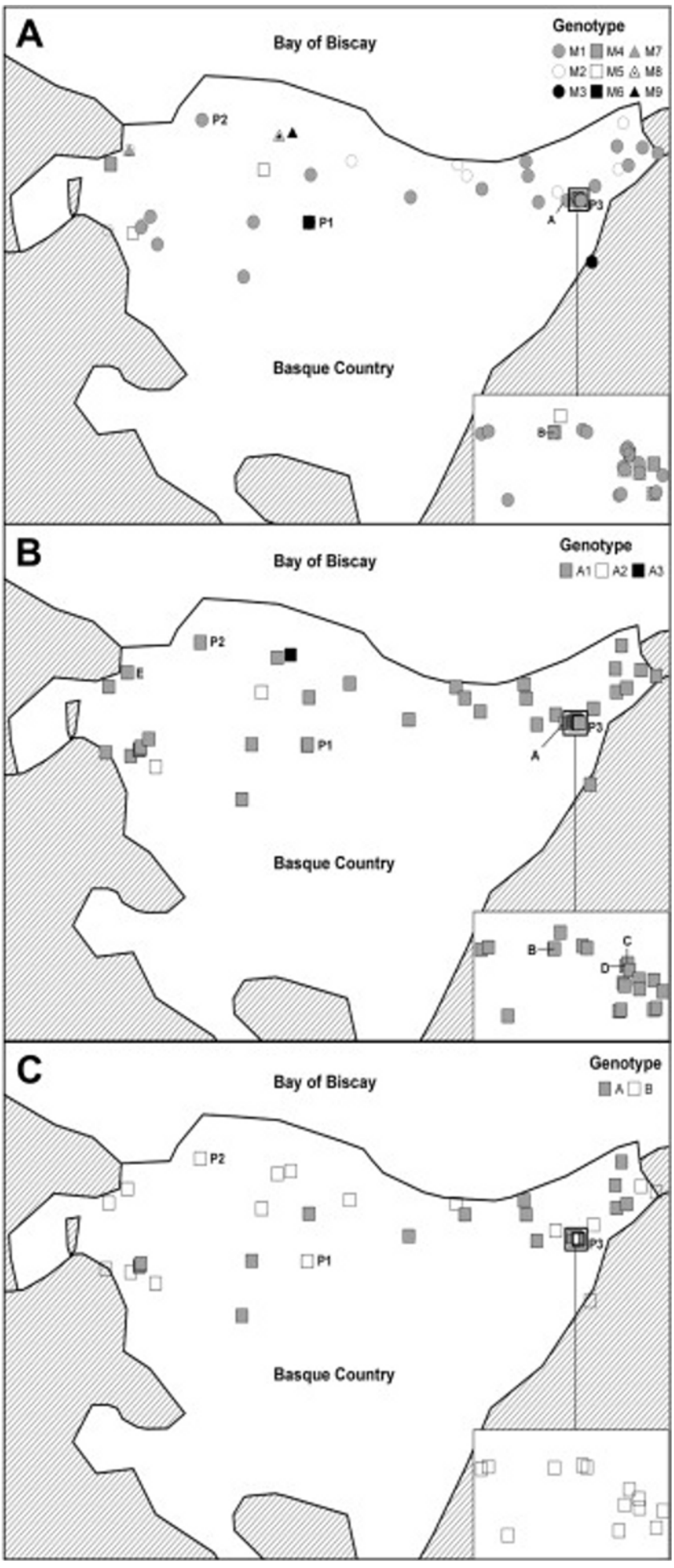
A
Bay of Biscay
Genotype
M1 M4 M7
M2 M5 M8
M3 M6 M9
P2
P1
P3
A
Basque Country
B
B
Bay of Biscay
Genotype
A1 A2 A3
P2
P1
P3
A
Basque Country
B
C
D
C
Bay of Biscay
Genotype
A B
P2
P1
P3
Basque Country

Figure 1. Genetic diversity and distribution of genotypes of *Fusarium circinatum* in the Basque Country, Spain. P1, P2 and P3 denote the location of the three *Pinus radiata* plantations sampled. Adjacent autonomous communities or countries (France) are thatched. (A) Nine genotypes (M1–9) obtained using six loci from the polymorphic DNA-markers sets (FC5, FC9, G18, G60, G98 & G220). P1 represents nine M1, one M2, one M3 and one M6 genotypes. P2 represents nine M1 and three M5 genotypes. A represents four M1 and B represents 10 M1 and one M4 genotypes. (B) Three genotypes (A1–3) obtained using 103 loci from AFLP analysis. P1 and P2 each represent 13 A1 genotypes. A represents four A1 genotypes, B represents 11 A1 genotypes, and C, D and E each represent two A1 genotypes. (C) Two vegetative compatibility groups (A and B). P1 represents four B genotypes and P2 represents 11 B genotypes.

Table 2. Genotype distribution of *Fusarium circinatum* isolates based on VCG, polymorphic DNA-marker and AFLP analyses.

	Genotype	Frequency[a]	VCG[b]	Collection	Polymorphism[c] [Locus (Allele) or AFLP set: Allele (1/0)]
Polymorphic DNA-marker	M1	0.68	A, B	1, 2	FC9 (244), G18 (510), G60 (800), G98 (950), G220 (400)
	M2	0.12	A, B	1, 2	FC9 (244), G18 (null), G60 (800), G98 (950), G220 (400)
	M3	0.09	B	1, 2	FC9 (244), G18 (510), G60 (null), G98 (950), G220 (null)
	M4	0.04	B	1, 2	FC9 (244), G18 (510), G60 (800), G98 (null), G220 (400)
	M5	0.02	B	1, 2	FC9 (244), G18 (510), G60 (null), G98 (950), G220 (400)
	M6	0.02	B	1	FC9 (244), G18 (null), G60 (800), G98 (null), G220 (400)
	M7	0.01	Unknown	1	FC9 (244), G18 (null), G60 (null), G98 (null), G220 (400)
	M8	0.01	B	2	FC9 (244), G18 (null), G60 (null), G98 (null), G220 (null)
	M9[d]	0.01	B	1	FC9 (241), G18 (null), G60 (null), G98 (null), G220 (null)
Total	9	1	*n/a*	*n/a*	*n/a*
AFLP	A1	0.97	A, B	1, 2	*Eco*RI-AC + *Mse*I-AT: 215(0), 204(0), 157(0), 150(0), 145(0), 143(0), 108(0), 107(0); *Eco*RI-AT + *Mse*I-AT: 145(1), 102(1), 90(1), 65(0); *Eco*RI-AC + *Mse*I-TT: 185(0), 140(0), 80(1); *Eco*RI-TT + *Mse*I-TT: 128(0)
	A2	0.02	B	1	*Eco*RI-AC + *Mse*I-AT: 215(1), 204(0), 157(1), 150(0), 145(1), 143(0), 108(1), 107(1); *Eco*RI-AT + *Mse*I-AT: 145(1), 102(1), 90(1), 65(1); *Eco*RI-AC + *Mse*I-TT: 185(1), 140(1), 80(1); *Eco*RI-TT + *Mse*I-TT: 128(0)
	A3[d]	0.01	B	1	*Eco*RI-AC + *Mse*I-AT: 215(0), 204(1), 157(0), 150(1), 145(0), 143(0), 108(0), 107(0); *Eco*RI-AT + *Mse*I-AT: 145(0), 102(0), 90(0), 65(0); *Eco*RI-AC + *Mse*I-TT: 185(0), 140(0), 80(0); *Eco*RI-TT + *Mse*I-TT: 128(0)
Total	3	1	*n/a*	*n/a*	*n/a*

a Frequency = number of isolates with genotype/total number of isolates (96).
b VCGs associated with each genotype.
c Polymorphic alleles are characterised in terms of the size (indicated in base pairs) of the amplicons generated by the respective primer sets for the various markers. Presence or absence of these fragments or alleles is indicated by (1) and (0), respectively.
d Genotypes common to both molecular analyses methods.

Discussion

The results of this study show the population of *Fusarium circinatum* in the Basque Country of Spain to be relatively homogeneous. Only two VCGs were identified among the isolates studied and both were associated with the same mating type. AFLPs and the FC markers (Britz *et al*. 2002b) also showed low levels of genetic diversity. Although the G marker set (Wikler & Gordon 2000) revealed somewhat more diversity, the extent of polymorphism in the Basque Country population was limited relative to that observed in a global sampling of *F. circinatum* populations (Wikler & Gordon 2000). In prior studies, both FC (Britz *et al*. 2002b) and G (Wikler & Gordon 2000) marker sets detected multiple alleles at each of the loci assayed, as compared to only one or two alleles per locus in the present study.

The most common genotype based on the FC and G marker sets (68 % of the sample) was associated with both VCGs, whereas the remaining genotypes were associated only with VCG B. Likewise the most common genotype based on AFLPs (97 %) was associated with VCGs A and B, and the remaining genotypes only with VCG B. This pattern would be consistent with a clonal relationship between isolates associated with different VCGs. In this hypothetical relationship VCG B would be the progenitor, with VCG A having been derived from VCG B through a mutation affecting somatic compatibility. Similar relationships have been described in the Florida and California populations of *F. circinatum* (Wikler & Gordon 2000) and experimental evidence suggests that 'novel' VCGs may originate spontaneously in the absence of outcrossing (Petersen & Gordon 2005).

Whether or not VCGs A and B share a clonal relationship, VCG diversity in the Basque Country population is clearly low, which is consistent with the recent establishment of this population through one or a limited number of introductions. Likewise identification of only five VCGs in the California population of *F. circinatum*, as compared to 45 VCGs in a well established Florida population, was taken as evidence in support of recent introduction of *F. circinatum* to California (Correll *et al*. 1992). It is not known if the pitch canker infestations in the Basque Country and in the nearby autonomous communities of Galicia, Asturias and Cantabria (Perez-Sierra et al. 2007) have separate origins, or resulted from dispersal following an introduction into one region. Insight into this question might be gained through a broader geographic assessment of diversity and relationships among *F. circinatum* populations in Spain, based on VCGs and/or informative molecular markers.

Fusarium circinatum isolates from the Basque Country were found to be associated exclusively with the MAT-2 mating type. However, both mating types have been reported to occur in other parts of Northern Spain (Perez-Sierra *et al*. 2007), suggesting that sexual reproduction could occur in this region. Although studies in California have shown that co-occurrence of two mating types does not preclude maintenance of a clonal population structure (Gordon *et al*. 1996, 2006b), the risk of sexual reproduction in Spain remains a concern. The possibility of selection for greater virulence to pine is suggested by the significant variation in virulence among *F. circinatum* isolates reported by Perez-Sierra *et al*. (2007). It was noted in this same study that MAT-1 isolates were more virulent than isolates associated with the MAT-2 idiomorph (Perez-Sierra *et al*. 2007), whereas Gordon *et al*. (1998b, 2006a) found the reverse to be true. This discrepancy indicates there is not a consistent association between mating type and virulence. Perez-Sierra *et al*. (2007) also reported that MAT-2 isolates lacked coiled, sterile hyphae, whereas MAT-2 isolates from the Basque Country all produced coiled, sterile hyphae characteristic of *F. circinatum*.

Fusarium circinatum has been associated with numerous insect species throughout its range. In California, engraver beetles (Fox *et al*. 1991) and twig beetles (Storer *et al*. 2004) are thought to play critical roles as vectors and wounding agents, whereas insects are accorded much less importance in the pitch canker disease cycle in the southeastern United States (Blakeslee & Oak 1979). Several bark beetle and weevil species are associated with *F. circinatum* in the Basque region of Spain (Romon *et al*. 2007). In this study, there was no difference in genetic diversity of *F. circinatum* isolates associated with insects versus other isolates from Collections 1 and 2. The association of *F. circinatum* with insects makes eradication of this pathogen in Spain difficult, as it can provide a method for dissemination beyond a zone of infestation. Eradication of the pathogen from Spain is also problematic because of its present distribution (Perez-Sierra *et al*. 2007) and the predicted climatic suitability for disease establishment in this region (Ganley *et al*. 2009).

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