Evidence for Multiple Introductions and Clonality in Spanish Populations of *Fusarium circinatum*

M. Berbegal, A. Pérez-Sierra, J. Armengol, and N. J. Grünwald

First, second, and third authors: Instituto Agroforestal Mediterráneo, Universitat Politècnica de València, Camino de Vera s/n 46022 Valencia, Spain; and fourth author: Horticultural Crops Research Laboratory, United States Department of Agriculture–Agricultural Research Service, 3420 NW Orchard Ave., Corvallis, OR 97330.

Accepted for publication 5 March 2013.

ABSTRACT

Berbegal, M., Pérez-Sierra, A., Armengol, J., and Grünwald, N. J. 2013. Evidence for multiple introductions and clonality in Spanish populations of *Fusarium circinatum*. *Phytopathology* 103:851–861.

Fusarium circinatum is thought to have been moved around the world with pine planting stock consisting, most probably, of infected seed. In this effort, we investigate the genetic structure of *F. circinatum* in Spain and globally. In total, 223 isolates were studied from five regions in northern Spain and eight countries. Eight microsatellite markers revealed 66 multilocus genotypes (MLGs). Minimum spanning network analysis of MLGs by region within Spain as well as globally, discriminant analysis of principal components, and analysis of molecular variance revealed that

Spanish populations are significantly differentiated and structured into two distinct groups, each one including one of the dominant genotypes observed. This result suggests that two independent introductions occurred into Spain that subsequently underwent clonal divergence and admixture. This result is further supported by the linkage disequilibrium and clonality observed for *F. circinatum* populations in northern Spain. The maintenance of differentiation between the clusters could result from the lack of or rare sexual reproduction in Spain. Possible introduction pathways from other countries and subsequent routes of dispersion of *F. circinatum* in Spain are discussed.

Additional keywords: population genetic structure, reproductive system.

Population genetics has proven to be a useful approach for reconstructing routes of introduction of invasive organisms (20). Patterns of pathogen migration around the world and within regions can be inferred from studying the geographic distribution and population structure (30,32,51,53). Inferences obtained from population genetic analyses can be useful for management and mitigation of invasive pathogens by providing critical insights into the pathways of migration and demographic history (33). In addition, population genetic inference provides insights into whether populations are sexual or clonal, differentiated or admixed, and whether phenotypes are linked to genotypes. All of this knowledge is important for development of targeted disease management strategies and disease-resistant cultivars.

Pitch canker, caused by the ascomycete fungus *Fusarium circinatum*, is a well-known, recently emerged disease affecting pine plantations and nurseries in several countries worldwide (72). The disease can lead to reduced yields, tree mortality, and, consequently, severe economic losses (17). *F. circinatum* has been found to be pathogenic to or reported on >60 species of pine as well as *Pseudotsuga menziesii* (28,29,37).

The disease was first recorded in North Carolina, United States, in the 1940s (35) and, to date, is known to occur throughout the southeastern United States and the coastal regions of California (17,50,69). Pitch canker has also been reported in Haiti (36), South Africa (68), Japan (42), Korea (45), Mexico (7), Chile (73), and Uruguay (2). In Europe, *F. circinatum* expanded its geographical distribution considerably after its detection in Galicia

(49) and País Vasco (16), located in northern Spain; although the first published report was located in Asturias and Cantabria in 2005 (44), the disease has been detected since then in other regions (57). Soon thereafter, it was reported in France (18,19), Italy (12), and Portugal (5). Further spread of *F. circinatum* represents a significant threat to many countries where susceptible species occur naturally or are grown extensively in plantations (25).

F. circinatum is thought to have been moved around the world with pine planting stock consisting, most probably, of infected seed; however, direct evidence is still lacking (26,27). Introduced populations of *F. circinatum* have been well characterized in the United States and South Africa and, to a lesser extent, elsewhere on the globe (8,14,71). However, despite the importance of this pathogen to pine plantations, forests, and the industries relying on them, the history of introduction and pathways of migration remain poorly understood.

As with other heterothallic, outcrossing pathogens, the mode of reproduction in *F. circinatum* affects the population structure. Mixed reproductive systems that include both sexual and asexual cycles of reproduction result in recombination and clonal propagation (9,34,47). An understanding of the reproductive biology and the genetic structure of plant pathogen populations is important for predicting the potential occurrence of adaptive genotypes; for example, those genotypes compatible with specific resistance genes found in certain cultivars or genotypes resistant to specific fungicides (52).

The outcome of sexual reproduction among *F. circinatum* isolates is dependent on mating type compatibility, as determined by the *MAT* locus, as well as female fertility. Female fertile isolates are those capable of producing sexual fruiting structures upon fertilization (14,46). Therefore, the distribution of mating type and the sexual and fertility status can be used to characterize the genetic structure and dynamics of filamentous fungi (47). This type of analysis has been conducted to determine the effective

Corresponding author: M. Berbegal; E-mail address: mobermar@etsia.upv.es

*The e-Xtra logo stands for “electronic extra” and indicates that the online version contains two supplemental tables. Figure 1 appears in color online.

<http://dx.doi.org/10.1094/PHYTO-11-12-0281-R>
© 2013 The American Phytopathological Society

population size and evolution in a South African population of *F. circinatum* (9).

Following Tibayrenc et al. (66), populations are considered predominantly clonal when they exhibit (i) excess frequencies of one or a few multilocus haplotypes, especially when these haplotypes are sampled in multiple locations or over different times; (ii) absence of recombinant types; (iii) linkage disequilibrium; and (iv) correlation among independent sets of different genetic markers. An additional criterion that can be used is the absence of sexual structures, although absence of visible sexual structures is not necessarily indicative of absence of sex because structures can be ephemeral and hard to find.

Recent work on *F. circinatum* in the País Vasco region in Northern Spain provided evidence for clonal populations of the pathogen using vegetative compatibility groups (VCGs), polymorphic DNA markers, and amplified fragment length polymorphism (AFLP) (38). A broader isolate collection representing all the affected regions in Northern Spain remains to be studied to understand the evolutionary history of the Spanish populations. The populations in the regions neighboring País Vasco, including Asturias, Cantabria, and Galicia, might be different because both mating types are present and populations could potentially be sexual in nature (57). It is not known whether pitch canker infestations in País Vasco and nearby regions have separate origins or resulted from dispersal following a single introduction into Northern Spain (38). Insights into these and other questions might be gained by studying a geographically broader *F. circinatum* collection in Spain.

TABLE 1. *Fusarium circinatum* isolates obtained from *Pinus* spp. in Spain and other countries worldwide included in this study^a

Country, province	Origin		Number of isolates
	Forest	Nursery	
Spain			
All	70	61	131
Asturias	12	36	48
Cantabria	27	5	32
Castilla León	5	8	13
Galicia	11	8	19
País Vasco	15	4	19
Chile	...	...	10
France	...	...	3
Japan	...	...	8
Mexico	...	...	6
Portugal	...	...	8
South Africa	...	...	11
Uruguay	...	...	4
United States ^b	...	...	42
Total	70	61	223

^a Isolates from Chile were provided by E. R. Chávez (Unidad de Fito-patología, Servicio Agrícola y Ganadero, Departamento Laboratorio y Estaciones Cuarentenarias, Santiago, Chile). French isolates were provided by R. Ios (Station de Mycologie, Laboratoire National de la Protection des Végétaux, Malzeville, France). Japanese isolates were provided by NIAS Genebank, National Institute of Agrobiological Sciences, Ibaraki, Japan. Mexican isolates were studied by Wikler and Gordon (71) and were provided by T. R. Gordon (Department of Plant Pathology, University of California, Davis). Isolates from Portugal were provided by E. Diogo (Laboratório de Micologia, Instituto Nacional de Recursos Biológicos, IP/L-INIA Unidade de Investigação de Protecção de Plantas, Lisbon, Portugal). Isolates from South Africa were provided by B. D. Wingfield (Department of Genetics, FABI, University of Pretoria, Pretoria, South Africa). Isolates from Uruguay were provided by R. Alonso (Laboratorio de Micología, Facultad de Ciencias-Facultad de Ingeniería, Universidad de la República, Montevideo, Uruguay).

^b Of the 42 isolates from the United States, 20 were those studied by Winkler and Gordon (71) and were provided by T. R. Gordon (Department of Plant Pathology, University of California, Davis), while the rest were provided by D. Geiser (Fusarium Research Centre, Pennsylvania State University, University Park) and the NIAS Genebank, National Institute of Agrobiological Sciences, Ibaraki, Japan.

The diversity of *F. circinatum* populations in the United States has been studied mostly by assessing phenotypic diversity based on VCGs (14,27,38,67,71). Britz et al. (8) analyzed two different populations of *F. circinatum* from South Africa using molecular markers developed for *F. circinatum* using the internal short-sequence repeats polymerase chain reaction (ISSR-PCR) technique (10). On a global scale, little is known about *F. circinatum* population differentiation. In a preliminary study, populations from Japan, Mexico, South Africa, and the United States (California and the southeastern United States) were compared using restriction-enzyme-based polymorphic DNA markers (71). Patterns of relatedness indicated that the Californian and Japanese populations of the fungus share lineages with the southeastern U.S. population. The highest genetic diversity was observed in Mexico, implying that it was the putative center of origin for *F. circinatum*, being also the center of diversity of *Pinus* spp. (58,71).

Microsatellite or simple-sequence repeat (SSR) markers have become very valuable for population genetic analyses due to their faster rate of evolution that make them more sensitive to recent phylogeographic and demographic events, often revealing clonal divergence in recently established populations (31,48,64). In 2009, Santana et al. (61) developed new SSR markers for *F. circinatum* by deep sequencing of SSR-enriched genomic libraries based on one isolate of *F. circinatum*. To date, none of these potentially useful SSR markers have been applied to analyze the population structure of *F. circinatum*.

The main objectives of this study were to (i) determine the genetic structure of *F. circinatum* in Spain at a regional scale relative to the global populations and (ii) infer whether populations show evidence of sexual reproduction in Spain using the novel SSR markers. This information will provide critical insights into the possible introduction pathways and subsequent routes of dispersion of *F. circinatum*.

MATERIALS AND METHODS

Isolates. A two-tiered sampling strategy was used, consisting of intensive sampling in Spain combined with a global reference collection obtained from research groups working with *F. circinatum* around the world (Table 1). In total, 223 *F. circinatum* isolates were genotyped. The collection consisted of 142 isolates from Europe, 42 from North America, 20 from South America (including Mexico), 11 from South Africa, and 8 from Japan. European isolates included 131 from Spain, 8 from Portugal, and 3 from France. South American isolates included 10 from Chile and 4 from Uruguay; 6 isolates from Mexico were also included in this group.

Spanish populations were sampled from forest ($n = 70$) and nursery ($n = 61$) environments. Forest isolates were collected between 2004 and 2011 in 43 affected pine plantations from five regions in Northern Spain (Asturias, Cantabria, Castilla León, Galicia, and País Vasco) (Fig. 1; Table 1). Additionally, 61 isolates were collected from nurseries in the same regions and are considered to be representative of the overall nursery populations in Spain. The nursery population was defined as one single population regardless of geographical origin. Isolations were made from symptomatic pine trees, pine seedlings, or pine seed onto Komada medium (43) or onto potato dextrose (PD) agar supplemented with streptomycin sulfate at 0.5 mg ml⁻¹. For mature trees, the cankers were washed thoroughly with water, surface sterilized for 1 min in a 1.5% sodium hypochlorite solution, and rinsed twice with sterile distilled water. Isolations were made from the edge of lesions found beneath the affected bark and on seedlings from roots and stem bases. Some isolates from Spain were studied previously by Pérez-Sierra et al. (57).

Pure cultures were transferred separately to a flask containing 100 ml of PD broth medium and incubated at room temperature

for 7 to 15 days. Liquid cultures were vacuum-filtered and conidia and mycelia were stored at -80°C .

DNA isolation and quantification. Freeze-dried fungal tissue was ground to a fine powder under liquid nitrogen using a mortar and pestle. Total DNA was extracted using the E.Z.N.A. Plant Miniprep Kit (Omega Biotek, Doraville, GA) following the manufacturer's instructions. DNA was quantified using a spectrophotometer (ND-2000, NanoDrop Technologies, Wilmington, DE) and adjusted to a final concentration of genomic DNA at $15\text{ ng }\mu\text{l}^{-1}$ for PCR reaction.

Mating type determination. The mating type of each isolate was determined with a multiplex PCR assay using primers GcHMG1, GcHMG2, MAT1p1, and MAT1p3, as described by Wallace and Covert (70) and Schweigkofler et al. (63). Each $25\text{ }\mu\text{l}$ of the PCR mixture contained $1\times$ PCR buffer, 2.5 mM MgCl_2 , 0.2 mM dNTPs , 0.4 mM each primer, 1 U of MBL DNA Taq polymerase (Dominion MBL, Cordoba, Spain), and $1\text{ }\mu\text{l}$ of DNA template. PCR amplifications were performed on a Peltier Thermal Cycler-200 (MJ Research, Waltham, MA). The PCR program consisted of an initial step of 3 min at 94°C followed by 30 cycles of denaturation at 94°C for 30 s , annealing at 61°C for 50 s , and elongation at 72°C for 50 s . A final extension was performed at 72°C for 12 min . PCR-amplified products were separated by electrophoresis in 1.5% agarose gels (agarose D-1 Low EEO; Conda, Madrid) and stained with ethidium bromide. A 100-bp ladder was used as a molecular weight marker (Molecular Biology Laboratory, Cordoba, Spain).

Haplotype identification. Primers for 13 previously characterized SSR loci (61) were tested for polymorphism with a selec-

tion of 11 *F. circinatum* isolates representing different origins (France, Japan, South Africa, Spain, and United States). Primers were synthesized by Invitrogen (Carlsbad, CA). Each PCR reaction ($20\text{ }\mu\text{l}$) contained $1\times$ GenScript PCR buffer (10 mM Tris-HCl , 50 mM KCl , 1.5 mM MgCl_2 , and 0.1% Triton X-100 buffer), 0.2 mM dNTPs , $0.2\text{ }\mu\text{M}$ each primer, 0.05 U of GenScript Taq polymerase (GenScript Corporation, Piscataway, NJ), and $1\text{ }\mu\text{l}$ of DNA template. PCR amplifications were performed on a Veriti Thermal Cycler (Applied Biosystems, Foster City, CA). The PCR program consisted of an initial step of 4 min at 94°C followed by 40 cycles of denaturation at 94°C for 1 min , annealing at $45\text{ to }60^{\circ}\text{C}$ (61) for 1 min , and elongation at 72°C for 1 min . A final extension was performed at 72°C for 6 min . Ten SSR loci showed polymorphism, specific amplification, and reproducible allele calls: FCM-2, FCM-3, FCM-4, FCM-6, FCM-7, FCM-9, FCM-19, FCM-24, FCM-25, and FCM-26. Variation in the number of repeats for SSR was assessed for the 11 representative isolates selected by sequencing the amplified loci. Amplification products were purified (ExoSap, USB-Affymetrix, Santa Clara, CA) and validated by sequencing using a 3730 Genetic Analyzer (Applied Biosystems) at the Center for Genome Research and Biocomputing, Oregon State University.

Fluorescent labeling of PCR fragments was performed for each SSR locus in one reaction using three primers: SSR forward primer with M13 tail (TGTAACGACGGCCAGT) at its $5'$ end (Invitrogen), SSR reverse primer, and the fluorochrome 6-carboxy-fluorescein (FAM)-labeled M13 primer (TGTAACGACGGCCAGT) (Invitrogen), as described by Schuelke (62). PCR reactions ($10\text{ }\mu\text{l}$) were performed with the following final con-

Fig. 1. Geographic location of samples from the Spanish population of *Fusarium circinatum* analyzed in this study. Shown are mating type frequencies (pie charts) considering isolates obtained from natural forests or plantations (circles) for each location and isolates obtained from nurseries combined (diamonds). The probability that the χ^2 value observed for mating type frequencies differs from an expected ratio of 1:1 with 1 degree of freedom is also indicated for each region, except for País Vasco and Cantabria, in which all isolates studied were MAT-2.

centrations: 1× GenScript PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, and 0.1% Triton X-100 buffer), 0.2 mM dNTPs, 0.04 μM M13-SSR primer, 0.16 μM SSR primer, 0.16 μM FAM-M13 primer, 0.05 U of GenScript Taq polymerase, and 1 μl (≈15 ng) of DNA template. The PCR program consisted of an initial step of 2 min at 94°C followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and elongation at 72°C for 30 s. A final extension was performed at 72°C for 6 min. Only eight of the initially selected SSR loci showed specific amplification for the annealing temperature (56°C) recommended in the protocol (62) and, therefore, were included for data analysis. Amplifications were conducted in 96-well plates, and a negative control was included in each plate.

PCR products were sized using capillary electrophoresis on a 3100 Avant Genetic Analyzer (Applied Biosystems) using the internal size-standard LIZ 500 (Applied Biosystems). Allele calls were made using the GeneMapper 3.7 software package (Applied Biosystems). Analysis was replicated for a subset of isolates, with independent DNA extractions, PCR, and sizing of fragments to confirm reproducibility of results.

Data analysis. For each population defined by country of origin or subpopulations defined by region or nursery within Spain (nursery is a single population separate from forest subpopulations by regions), the total number of alleles and allele frequencies at each SSR locus were estimated. A multilocus genotype (MLG) was constructed for each isolate by combining data for single SSR alleles.

Given the clonality observed, analyses were conducted on a global data set including all individuals and a clone-corrected data set including one member of each MLG for each mating type and geographical origin by population. For some of the analyses, only clone-corrected data were used as indicated.

To assess the possible evolutionary relationships among MLGs, minimum spanning networks were constructed, first from genotypes of different regions in Spain and also from all genotypes together. Genetic distances were calculated using the method described by Bruvo et al. (11), which takes repeat number into account. In this method, a distance of 0.1 is equivalent to one mutational step (one repeat) but larger distances do not strictly

correspond to a given number of mutational steps. Bruvo distance matrices were calculated using the procedure available in the package POLYSAT (13) for R version 2.7.2 (The R Foundation for Statistical Computing). MINSPNET (22) was used to generate minimum spanning networks from the matrices. All tied links were included in the network, which was visualized using the HAPSTAR software (65).

Discriminant analysis of principal components (DAPC) (41), a multivariate method implemented in the R package ADEGENET (40), was used to infer the most likely number of populations that could be differentiated from the clone-corrected data set without considering geographical criteria. Populations with a low number of individuals (France, Japan, and Uruguay) were not considered for the analysis. The DAPC approach is based on a principal component analysis of allelic diversity among isolates and a sequential K-means clustering procedure using the Bayesian information criterion to infer the optimal number of groups (41). A discriminant analysis is subsequently applied to visualize genetic differentiation among groups. Analysis with DAPC maximizes variation between the groups, minimizing the within-group genetic variability, and does not require assumptions regarding a particular genetic model (41).

The genetic richness (R), the evenness index adapted from the Simpson index of genotypic diversity (ED*), and the complement of the Pareto distribution of clonal membership were calculated for each population using GENCLONE (3). The Pareto coefficient increases as the diversity and evenness in the sample increase (4). These indices were recommended by Arnaud-Haond et al. (4) as the most parsimonious set of nonredundant indices of clonal diversity. Genotypic diversity (D) was calculated using $D = n/(n - 1)(1 - \sum p_i^2)$ as implemented in MULTILOCUS (1), where p_i is the frequency of the i th genotype and n is the number of individuals sampled. D represents the probability that two individuals sampled at random with replacement have the same genotype, and is similar to the diversity measure of Pielou (59).

To study the contribution of possible sexual reproduction to the genotypic diversity observed for each population, the probability that a genotype was obtained by chance throughout a sexual event (P_{gen}) was calculated using GENCLONE (3), as well as the

TABLE 2. Genetic diversity of the Spanish populations of *Fusarium circinatum* contrasting regional forest populations and the overall nursery population^a

Parameters ^b	Asturias		Cantabria		Castilla León		Galicia		País Vasco		Nursery	
	All	Corr	All	Corr	All	Corr	All	Corr	All	Corr	All	Corr
Sample size (N)	12	7	27	17	5	3	11	9	15	8	61	39
Genotypes (G)	3	3	1	1	3	3	6	6	1	1	12	12
Genotypic richness (R)	0.18	0.33	0	0	0.50	1	0.50	0.63	0	0	0.18	0.29
Evenness (ED*)	0.84	0.87	nc	nc	0.96	1	0.93	0.94	nc	nc	0.73	0.77
c (pareto)	nc	nc	nc	nc	nc	nc	1.87	nc	nc	nc	1.27	1.34
Diversity (D)	0.62**	0.67**	0	0	0.8**	1	0.87**	0.89**	0	0	0.78**	0.81**

^a The set of nonredundant indices of genotypic diversity recommended by Arnaud-Haond et al. (4) was calculated for each population on the total (All) and clone-corrected (Corr) data set; nc, cannot be calculated.

^b G, number of distinct multilocus genotypes discriminated; R, genotypic richness, $R = (G - 1)/(N - 1)$; ED*, evenness index adapted from Simpson diversity; c (pareto), the slope of the Pareto distribution of clonal membership; D, genotypic diversity, ** = $P < 0.01$.

TABLE 3. Genetic diversity of the global populations of *Fusarium circinatum* studied^a

Param. ^b	Spain		Chile		France		Japan		Mexico		Portugal		South Africa		Uruguay		United States		Total	
	A	C	A	C	A	C	A	C	A	C	A	C	A	C	A	C	A	C	A	C
N	131	83	10	6	3	3	8	4	6	6	8	8	11	10	4	4	42	36	223	160
G	15	15	2	2	2	2	2	2	6	6	6	6	9	9	4	4	32	32	66	66
R	0.1	0.1	0.1	0.2	0.5	0.5	0.1	0.3	1	1	0.7	0.7	0.8	0.9	1	1	0.8	0.9	0.3	0.4
ED*	0.5	0.6	0.5	0.6	0.9	0.9	0.9	1	1	1	0.9	0.9	0.9	0.9	1	1	0.9	0.9	0.7	0.8
c	1.1	1.1	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc	2.6	3.2	1.2	1.3
D	0.6**	0.7**	0.2	0.3	0.6**	0.6**	0.5	0.6	1	1	0.93	0.93	0.95**	0.98	1	1	0.98**	0.99**	0.86**	0.9**

^a The set of nonredundant indices of diversity recommended by Arnaud-Haond et al. (4) was calculated for each population on the total (A) and clone-corrected (C) data; nc, cannot be calculated.

^b Parameters: N, sample size; G, number of distinct multilocus genotypes discriminated; R, genotypic richness, $R = (G - 1)/(N - 1)$; ED*, evenness; c (pareto), the slope of the Pareto distribution of clonal membership; D, genotypic diversity, ** = $P < 0.01$.

probability that an isolate shared the same MLG as another in the sampled population (P_{sex}), assuming that the two isolates derive from sexual reproduction (4,6,56). Associations among SSR loci were tested by the index of multilocus linkage disequilibrium ($\bar{r}_d$) implemented using MULTILOCUS (1). Multilocus tests were not performed on sample sizes of <10 due to lack of statistical power (24). The index has an expected value of zero if there is no association of alleles at unlinked loci, as expected in randomly mating population. The significance of $\bar{r}_d$ was tested by comparing the observed variance with the distribution of the expected variance under the null hypothesis of random mating, as determined from 1,000 randomizations of the genotype data.

A different approach to evaluate the hypothesis of random mating was performed only for Spanish isolates by a χ^2 test with one degree of freedom to test mating type ratios on the clone-corrected data set. Mating type ratios are expected to be 1:1 for a randomly mating population (52) and were considered significantly different from 1:1 at $P < 0.01$.

Population genetic structure was analyzed by conducting an analysis of molecular variance (AMOVA) (23) on clone-corrected data using ARLEQUIN version 3.5 (21). The fixation index (F_{ST}) was also calculated using ARLEQUIN. Significance of F_{ST} was tested using 1,023 permutations. Due to low sample sizes, populations were defined by continent and Japan was excluded from the analysis. Therefore, four global populations were analyzed: Europe (Spain, Portugal, and France), South America (Chile, Mexico, and Uruguay), North America, and South Africa.

RESULTS

Genotypic diversity. Spanish subpopulations showed low genotypic diversity and clonality. Six of the eight loci were polymorphic for the 131 Spanish isolates and exhibited two (FCM4, FCM7, and FCM19), three (FCM26), four (FCM2), or eight (FCM25) alleles (Supplemental Table 1), resulting in a total of 15 MLGs. The most frequent MLG was MLG32 (56.5%), which was present in subpopulations from all the regions studied as well as the nursery subpopulation. MLG59 was also frequent (21.4%) and was detected in the nursery subpopulation and all the regions except for Cantabria and País Vasco. Of the 15 MLGs, 6 were observed once and 4 were restricted to the nursery subpopulation (70% of the 10 MLGs).

In the global population consisting of 223 *F. circinatum* isolates, the eight SSR loci exhibited between 2 (FCM19) and 14 alleles (FCM7) (Supplemental Table 2), resulting in a total of 66 MLGs. The most frequent MLGs were MLG32, detected in Spain (33.2%) and France (0.9%), and MLG59, detected in Spain (12.6%) and Portugal (0.5%). Spain shared many MLGs with Portugal (MLG60, MLG62, MLG64, and MLG66) and only MLG53 with Uruguay. Some MLGs were shared among the United States and other countries such as Spain (MLG43), France (MLG24), Uruguay (MLG31), and Japan (MLG39). MLG9 was detected in Mexico (0.5%) and Chile (4%) but 54 MLGs (47 of which were only observed once) were restricted to single populations.

In general, indices of diversity did not differ among global or Spanish populations for total or clone-corrected data (Tables 2 and 3). R varied across subpopulations defined by different regions in Spain, from very high in Castilla León or Galicia (0.5 to 0.6), to high in Asturias or the nursery subpopulation (0.3) (Table 2). Only one genotype was detected in subpopulations from Cantabria and País Vasco. ED^* values were higher in the nursery subpopulation compared with the regional populations in Spain. In Spain, D values varied from very high (0.8 to 1) in subpopulations from Galicia, Castilla León, and nursery subpopulations, where nearly all individuals were a distinct genotype; to high in Asturias (0.6); to zero for Cantabria and País Vasco, where only one genotype was detected.

When considering populations defined by country of origin, R varied from 0.1 to 0.5 in populations from Spain, Chile, France, or Japan to 0.7 to 1 in those from Mexico, Portugal, South Africa, Uruguay, or the United States (Table 3). ED^* values were also very variable among the populations, and the highest were observed for those from Japan and the United States. D values on global populations varied from very high (0.9 to 1) in populations from Mexico, Portugal, South Africa, Uruguay, or United States and high (0.5 to 0.7) in those from Spain, France, or Japan to low (0.3) in Chile.

Relationships among genotypes. The minimum spanning network for the global population revealed two main groups of Spanish isolates (Fig. 2A, red). Each group included one of the two most frequent MLGs in Spain, MLG59 and MLG32 (Fig. 2B). The global MLG network showed a tight cluster around MLG39, which was the most frequent MLG in the United States, although MLGs identified in the United States were spread all across the network. Most of the singleton MLGs from South Africa were co-located on the network (Fig. 2A).

The sequential K-means clustering procedure based on the Bayesian information criterion (BIC) indicated a range of clusters (5 to 20 clusters) that minimized diversity within clusters, and an optimum number (5 clusters) was selected for the analysis, given that additional clusters led to only negligible changes in BIC. All five clusters included 4 to 11 MLGs found in the United States (Fig. 3). The first axis (horizontal) clearly identified two groups of genetic clusters: all MLGs found in Spain and Portugal had positive coordinates (clusters 1, 3, and 4) and all MLGs found in South America (cluster 2) and South Africa (clusters 2 and 5) had negative coordinates (Fig. 3). The second axis separated MLGs into two groups: cluster 1 had negative coordinates and the rest had positive coordinates. Cluster 1 grouped nearly all MLGs found in Spain, including MLG59, which was one of the most frequent, and all MLGs from Portugal (Fig. 3). Cluster 4 included MLG41 and MLG43 from Spain and 11 MLGs found in the United States while cluster 3 included the most frequent MLGs found in Spain (MLG32) and 8 MLGs found in the United States. In the discriminant analysis of principal components (Fig. 3), the second axis clearly separated two groups of MLGs found in Spain that corresponded to the two clusters of Spanish MLGs identified in the minimum spanning networks (Fig. 2).

Selfing and clonality in populations. The multilocus linkage disequilibrium test ($\bar{r}_d$) on all individuals and the clone-corrected data in regional populations in Spain rejected the null hypothesis of recombination ($P < 0.01$) (Table 4). When populations defined by country of origin were analyzed, the $\bar{r}_d$ test rejected the hypothesis of recombination for Spanish and U.S. populations ($P < 0.01$) but could not do so for the Chilean ($P = 0.07$ and 0.21 for global and clone-corrected data set, respectively) and South African ($P = 0.04$ and 0.1 for global and clone-corrected data set,

TABLE 4. Multilocus linkage disequilibrium ($\bar{r}_d$) within Spanish subpopulations and global populations^a

Populations	All isolates		Clone-corrected	
	$\bar{r}_d$	P value	$\bar{r}_d$	P value
Asturias	0.7	<0.01	0.57	<0.01
Cantabria	nc	nc	nc	nc
Galicia	0.63	<0.01	0.63	<0.01
País Vasco	nc	nc	nc	nc
Nursery	0.69	<0.01	0.68	<0.01
Spain	0.75	<0.01	0.71	<0.01
Chile	1	0.07	1	0.21
South Africa	0.11	0.04	0.07	0.1
United States	0.08	<0.01	0.07	<0.01
Total	0.27	<0.01	0.24	<0.01

^a Tests were performed on all and clone-corrected data but excluded sample sizes of <10 individuals. Values that differ significantly from 0 indicate a departure from linkage disequilibrium; nc, cannot be calculated.

Fig. 2. Minimum spanning network showing the relationships among the individual multilocus genotypes observed in **A**, the global sample and **B**, the Spanish population of *Fusarium circinatum*. Distances between nodes are proportional to Bruvo's distance (11). Two apparent clusters of Spanish multilocus genotypes (MLGs) are separated by a red line. The two most common Spanish MLGs (59 and 32) are shown in solid red dots. Black dots represent inferred missing genotypes.

respectively) populations (Table 4). Regarding the distribution of mating types within the populations in Spain, only isolates from *MAT*-2 were identified in the Cantabria and País Vasco regions. There were ratios of *MAT*-1 to *MAT*-2 of 5:2, 2:1, and 6:3 in Asturias, Cantabria, and Galicia, respectively, which were not significantly different from a 1:1 ratio (Fig. 1), as would be expected from random mating, although the $\bar{r}_d$ index suggested a more clonal structure.

The probability that the most frequent genotypes detected in Spain can evolve by chance through sexual reproduction (P_{gen}) was very low (Table 5). Similarly, the probability that two isolates share these genotypes in the Spanish populations was low (P_{sex}) (Table 5). When considering global populations, the values of P_{gen} and P_{sex} were higher for Chile and Japan (Table 5).

Population differentiation. The AMOVA results based on Spanish populations (Table 6), showed 4% of the variation among forest populations considered together regardless of geographical origin and nursery populations, 34.5% among populations within forest and nursery environments, and 62% within populations.

The overall F_{ST} was 0.383 and the permutation P value was <0.00001 . The AMOVA results based on populations defined by isolates' continent of origin (Table 6) showed 75.5% of the variation within populations and 24.5% among populations. The overall F_{ST} was 0.245 and the permutation P value was <0.00001 .

DISCUSSION

Our analysis provides support for the hypothesis that *F. circinatum* was introduced at least twice into Spain. Minimum spanning network analysis, discriminant analysis of principal components, and AMOVA revealed that Spanish populations are significantly differentiated and structured into two distinct groups, each one including one of the two most common MLGs; namely, MLG59 and MLG32. This result is most parsimoniously explained by two independent introductions of each dominant genotype that subsequently underwent clonal divergence and admixture, and it is further supported by the clonality and lack of observed sexual structures.

Fig. 3. Scatterplot of the discriminant analysis of principal components on *Fusarium circinatum* multilocus genotypes. The first axis is the horizontal axis. Numbers in the boxes represent the five clusters found by the K-means method. At the bottom left, the Eigen values of the first four axes are represented. Shown is the proportion of genotypes (pie charts) from each population defined by geographical criteria observed in each of the five clusters, and numbers inside the sectors represent the number of isolates identified for each genotype.

In total, 66 *F. circinatum* MLGs were detected worldwide, of which 15 occur in Spain using six SSR loci. Although two genotypes were widely distributed in Spain, the majority of the other MLGs detected were less abundant and usually found at only one or two sites. Our results support a clonal population structure of *F. circinatum* in northern Spain based on several criteria tested following Tibayrenc et al. (66). (i) Several MLGs were observed with widespread geographical and temporal distribution across natural forest and plantation environments. MLG59 was found in Asturias, Castilla León, and Galicia; and MLG32 was the dominant genotype in Spain and was detected in all Spanish regions studied. Moreover, MLG32 was found 10 years apart: namely, in 1997 in the País Vasco region and again, widely distributed within northern Spain, in 2007. (ii) Evidence of linkage disequilibrium was observed in all Spanish populations, although low sample sizes may affect the results in the test performed (55). (iii) Finally,

sexual structures of *F. circinatum* have never been observed under natural conditions (72).

The high proportion of some MLGs and the P_{sex} values associated with these genotypes provide additional evidence for clonal reproduction in the Spanish populations studied. In addition, the asexual reproductive mode of *F. circinatum* in Spain and, thus, the absence of recombination among the isolates could explain the morphological differences observed between mating types described by Pérez-Sierra et al. (57).

Based on earlier population diversity studies of *F. circinatum* using the relative frequencies of mating types, VCG, AFLP, or DNA-based polymorphic markers, it appears that clonal propagation is observed in most populations worldwide (9,14,28,38, 67,71). In California, despite observations going back 20 years, pathogen populations show little genetic differentiation and are exclusively asexual in nature (26). Apparently, high susceptibility

TABLE 5. Probability of occurrence or a second encounter of simple-sequence repeat genotypes observed more than once in the regional populations studied in Spain and global populations for the total (all) and clone-corrected (C) data^a

Population	MLG	N _{obs} ^b		P _{gen} ^c		P _{sex} ^d	
		All	C	All	C	All	C
Asturias	32	6	2	0.0124	0.0007	2.1×10^{-7}	0.005
	59	5	4	0.0294	0.0791	0.0003	0.0136
	32	27	17	1	1	1	1
	32	15	8	1	1	1	1
	32	21	11	0.0052	0.0014	9.9×10^{-31}	1.8×10^{-20}
	43	3	1	0.0045	0.0023	0.0309	nc
	59	19	13	0.0493	0.0909	5×10^{-10}	0.0001
País Vasco	62	6	4	0.0247	0.0390	0.0173	0.1936
	64	2	1	0.0012	0.0024	0.0725	nc
	66	4	3	0.0099	0.0130	0.0226	0.0912
	66	4	3	0.0099	0.0130	0.0226	0.0912
Spain	32	74	42	0.0560	0.0250	1.3×10^{-55}	6.6×10^{-43}
	43	3	1	0.0020	0.0015	0.0291	nc
	51	2	1	0.0005	0.0015	0.0591	nc
	59	28	20	0.0070	0.0185	2.2×10^{-31}	9.6×10^{-16}
	60	2	2	0.0005	0.0008	0.0631	0.0645
	61	2	2	0.0005	0.0015	0.0591	0.1201
	62	8	5	0.0028	0.0077	1.1×10^{-7}	0.0040
	64	2	1	0.0002	0.0003	0.0257	nc
	66	4	3	0.0014	0.0015	0.0009	0.0074
	9	9	5	0.6944	0.5625	0.3681	0.4669
	32	2	2	0.0313	0.0313	0.0910	0.0910
	38	5	2	0.5	0.5	0.6367	0.9375
Chile	39	3	2	0.5	0.5	0.9648	0.9375
	39	3	2	0.5	0.5	0.9648	0.9375
France	4	3	2	0.0118	0.0038	0.0071	0.0375
	4	3	2	0.0118	0.0038	0.0071	0.0375
Japan	17	2	1	6.5×10^{-5}	7.4×10^{-5}	0.0027	nc
	19	2	1	1.4×10^{-5}	2.1×10^{-5}	0.0006	nc
	24	3	2	0.0002	6.4×10^{-5}	1.9×10^{-5}	0.0023
	39	4	3	0.0023	0.0020	0.0001	0.0023
	43	3	2	0.0014	0.0014	0.0017	0.0482
	45	2	1	6.2×10^{-5}	6.5×10^{-5}	0.0026	nc
	45	2	1	6.2×10^{-5}	6.5×10^{-5}	0.0026	nc
South Africa	4	3	2	0.0118	0.0038	0.0071	0.0375
	4	3	2	0.0118	0.0038	0.0071	0.0375
United States	17	2	1	6.5×10^{-5}	7.4×10^{-5}	0.0027	nc
	19	2	1	1.4×10^{-5}	2.1×10^{-5}	0.0006	nc

^a MLG, multilocus genotype and nc, cannot be calculated.

^b Number of times when the genotype was observed in the population.

^c Probability of occurrence of the microsatellite genotype.

^d Probability that two individuals share this genotype assuming random mating.

TABLE 6. Analysis of molecular variance (AMOVA) testing for genetic differentiation between regional populations of *Fusarium circinatum* in Spain and populations defined by continent of origin

Source of variation ^a	Variation (%)	F _{ST}	P ^b
Regional populations within Spain			
Among forest populations regardless the geographical origin and nursery population	4	...	...
Among populations within forest and nursery	34.5	...	...
Within populations	62	0.383	<0.00001
Global populations by continent of origin			
Among populations	24.5	...	...
Within populations	75.5	0.245	<0.00001

^a Genetic variation was partitioned within and among population at several hierarchical levels and groupings of populations, especially within the Spanish populations.

^b F_{ST}, fixation index; significance was determined by 1,023 permutations. Clone-corrected data were used for the AMOVA.

of the host (27) and long periods of favorable climatic conditions have facilitated the clonal spread of *F. circinatum*, resulting in a lack of genetic differentiation (26).

Our analyses revealed an unequal distribution of MLGs among the Spanish regions studied, despite the fact that short geographic distances separate these regions (Fig. 1) and that these regions lack physical barriers such as mountain ranges. MLG59, which is exclusively *MAT*-1, was detected in all regions except for País Vasco and Cantabria. In contrast, MLG32, which is exclusively *MAT*-2, was detected in all the regions studied and was the only one identified in País Vasco and Cantabria. This result corresponds with an earlier report that the *F. circinatum* population in the País Vasco region is genetically homogeneous and *MAT*-2, a fact that was considered as evidence of a recent establishment of *F. circinatum* (38). Founder effects can lead to populations with relatively little genetic variation (60). *Cryphonectria parasitica*, causal agent of chestnut blight, and *F. circinatum* are examples of plant pathogens that have limited genetic variation, based on genotypic and phenotypic markers, in their area of introduction compared with their native geographical range (27,54).

Regarding the possible paths of spread of *F. circinatum* in Spain, País Vasco was one of the regions where pitch canker was first observed in the 1990s (16) although the first official report was located in Asturias and Cantabria in 2005 (44). Some of the *F. circinatum* isolates included in the study identified as MLG32 were those detected in the first infected nursery in País Vasco in 1997, which were deposited in the *Fusarium* Research Center collection (Pennsylvania State University). Therefore, *F. circinatum* MLG32 might well have established first in País Vasco before spreading west and southward from there within Spain.

Most of the microsatellite allelic variation observed in Spain was detected in the nursery population. Of the 15 *F. circinatum* MLGs identified in the Spanish isolates studied, 13 were recovered from nurseries. Higher genotypic diversity in nurseries could be explained by the repeated exchange of pathogen genotypes through the trade of infected plant material, strong selection pressure affecting new genotypes created through mutation, a larger effective population size, or a combination of all of these mechanisms (39). In nurseries, cultural practices and chemical treatments can exert a selection pressure. Variation in microsatellite markers in the Spanish population may reflect recent mutations after introductions, because highly polymorphic microsatellite markers can undergo rapid change (74).

Since the first pitch canker infestation was detected in a single nursery (E. Iturrutxa, *personal communication*) in the 1990s in País Vasco, it is likely that the original source of inoculum consisted of imported seed used in the nursery. The first European directive regarding the introduction of forest reproductive material, including *P. radiata*, was not published until 1999 (<http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:1999L0105:20000115:ES:HTML>). In 2007, the European Union adopted emergency measures to prevent the introduction into and the spread within the European Community of *F. circinatum*, including specific import requirements for propagation materials in *P. menziesii* and the genus *Pinus* (Commission Decision 2007/433/EC).

Population differentiation analysis in this study revealed similarities among *F. circinatum* populations from Spain, France, Portugal, Uruguay, and the United States. In fact, all clusters identified by discriminant analysis of principal components included individuals from U.S. populations, indicating that it might be the common source for these introductions. A common founder source is also suggested by the AMOVA analyses, where most (75.5 %) genetic diversity was found within populations and only 24.5 % among populations. Additional genotype movements across the world should not be dismissed, because Wikler and Gordon (71) concluded that the South African population of *F. circinatum* probably originated in Mexico, which is in agreement

with previous suggestions that the disease was brought into South Africa on contaminated seed collected in Mexico (7). In the present study, a very low level of genetic differentiation among *F. circinatum* populations from South Africa and Mexico is in agreement with this hypothesis. This result is further supported by the fact that all MLGs from Mexico and many MLGs from South Africa grouped into a unique cluster in the discriminant analysis of principal components that also clearly separated Spanish and Portuguese MLGs from those identified in South Africa, Mexico, and Chile.

Many MLGs were found in Spain and Portugal (MLG62, MLG66, MLG64, MLG59, and MLG60) and MLG32 was observed in Spain and France. The results obtained indicate that Spain could be the source of some Portuguese and French genotypes. This is supported by the fact that there is geographic proximity among these countries but also that some of the shared genotypes (MLG32 and MLG59) showed very high frequencies in Spain, where the disease was well established before it was detected in France and Portugal.

This study is the first to use SSR markers to analyze the population structure of a diverse collection of *F. circinatum* isolates from different regions in Spain within the context of a global sample. The results obtained provide novel insights into the possible emergence of *F. circinatum* in Spain. Although pitch canker is widely distributed, there are many important pine-growing countries, such as Australia and New Zealand, where the disease has not yet established itself (28). Knowledge about the introduction and dispersion routes of a pathogen are essential to design management strategies for preventing new introductions; for example, by promoting heightened vigilance of potential source populations through monitoring and quarantine measures (20). Unfortunately, reliable records on the origins of introduced populations do not exist for most invasive species. Instead, inferences on patterns of emergence typically rely on post hoc surveys of molecular variation to identify regions that are likely to contain the source of a particular introduction (15). The correctness of inferences based on this approach depend on representative sampling strategies in introduced and potential source areas and on the resolution of the molecular markers used. The present study has demonstrated the potential of newly developed SSR markers to study the population structure of *F. circinatum* but further research is needed to answer the questions about global genotype movements and the resulting implications.

ACKNOWLEDGMENTS

M. Berbegal was a contract holder of the “Campus de Excelencia Internacional” program of the Universidad Politécnica de Valencia. Financial support for this research was provided by Ministerio de Agricultura, Alimentación y Medio Ambiente and INIA, and the United States Department of Agriculture–Agricultural Research Service CRIS 5358-22000-039-00. We thank M. Larsen and M. León for technical assistance. Isolates provided by the different research centers working with *Fusarium circinatum* are acknowledged (Table 1).

LITERATURE CITED

1. Agapow, P. M., and Burt, A. 2001. Indices of multilocus linkage disequilibrium. *Mol. Ecol. Notes* 1:101-102.
2. Alonso, R., and Bettucci, L. 2009. First report of the pitch canker fungus *Fusarium circinatum* affecting *Pinus taeda* seedlings in Uruguay. *Australas. Plant Dis. Notes* 4:91-92.
3. Arnaud-Haond, S., and Belkhir, K. 2007. GENCLONE: A computer program to analyse genotypic data, test for clonality and describe spatial clonal organization. *Mol. Ecol. Notes* 7:15-17.
4. Arnaud-Haond, S., Duarte, C. M., Alberto, F., and Serrao, E. A. 2007. Standardizing methods to address clonality in population studies. *Mol. Ecol.* 16:5115-5139.
5. Bragança, H., Diogo, E., Moniz, F., and Amaro, P. 2009. First report of pitch canker on pines caused by *Fusarium circinatum* in Portugal. *Plant Dis.* 93:1079-1079.

6. Breuillin, F., Dutech, C., and Robin, C. 2006. Genetic diversity of the Chestnut blight fungus *Cryphonectria parasitica* in four French populations assessed by microsatellite markers. *Mycol. Res.* 110:288-296.
7. Britz, H., Coutinho, T. A., Gordon, T. R., and Wingfield, M. J. 2001. Characterization of the pitch canker fungus, *Fusarium circinatum*, from Mexico. *S. Afr. J. Bot.* 67:609-614.
8. Britz, H., Coutinho, T. A., Wingfield, B. D., Marasas, W. F. O., and Wingfield, M. J. 2005. Diversity and differentiation in two populations of *Gibberella circinata* in South Africa. *Plant Pathol.* 54:46-52.
9. Britz, H., Wingfield, M. J., Coutinho, T. A., Marasas, W. F. O., and Leslie, J. F. 1998. Female fertility and mating type distribution in a South African population of *Fusarium subglutinans* f. sp. *pini*. *Appl. Environ. Microbiol.* 64:2094-2095.
10. Britz, H., Wingfield, B. D., Coutinho, T. A., and Wingfield, M. J. 2002. Sequence characterized amplified polymorphic markers for the pitch canker pathogen, *Fusarium circinatum*. *Mol. Ecol. Notes* 2:577-580.
11. Bruvo, R., Michiels, N. K., D'Souza, T. G., and Schulenburg, H. 2004. A simple method for the calculation of microsatellite genotype distances irrespective of ploidy level. *Mol. Ecol.* 13:2101-2106.
12. Carlucci, A., Colatruglio, L., and Frisullo, S. 2007. First report of pitch canker caused by *Fusarium circinatum* on *Pinus halepensis* and *P. pinea* in Apulia (Southern Italy). *Plant Dis.* 91:1683-1683.
13. Clark, L. V., and Jasieniuk, M. 2011. Polysat: an R package for polyploid microsatellite analysis. *Mol. Ecol. Resour.* 11:562-566.
14. Correll, J. C., Gordon, T. R., and McCain, A. H. 1992. Genetic diversity in California and Florida populations of the pitch canker fungus *Fusarium subglutinans* var. *pini*. *Phytopathology* 82:415-420.
15. Dlugosch, K. M., and Parker, I. M. 2008. Founding events in species invasions: genetic variation, adaptive evolution, and the role of multiple introductions. *Mol. Ecol.* 17:431-449.
16. Dwinell, L. D., Adams, D., Guerra-Santos, J. J., and Aguirre, J. R. M. 1998. Pitch canker disease of *Pinus radiata*. Paper 3.7.30 in: *Proc. 7th Int. Cong. Plant Pathol.* Edinburgh.
17. Dwinell, L. D., Barrow-Broadus, J., and Kuhlman, E. G. 1985. Pitch canker: a disease complex of southern pines. *Plant Dis.* 69:270-276.
18. EPPO. 2006. EPPO Reporting Service 2006/104.
19. EPPO. 2009. EPPO Reporting Service. 2009/093.
20. Estoup, A., and Guillemaud, T. 2010. Reconstructing routes of invasion using genetic data: why, how and so what? *Mol. Ecol.* 19:4113-4130.
21. Excoffier, L., and Schneider, S. 2005. Arlequin ver. 3.0: an integrated software package for population genetics data analysis. *Evol. Bioinf.* 1:47-50.
22. Excoffier, L., and Smouse, P. E. 1994. Using allele frequencies and geographic subdivision to reconstruct gene trees within a species: molecular variance parsimony. *Genetics* 136:343-359.
23. Excoffier, L., Smouse, P. E., and Quattro, J. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction data. *Genetics* 131:479-491.
24. Fincham, J. R. S., and Day, P. R. 1963. *Fungal Genetics*. Blackwell Scientific Publication, Oxford.
25. Ganley, R. J., Watt, M. S., Manning, L., and Iturriza, E. 2009. A global climatic risk assessment of pitch canker disease. *Can. J. For. Res.* 39:2246-2256.
26. Garbelotto, M. 2008. Molecular analysis to study invasions by forest pathogens: Examples from Mediterranean ecosystems. *Phytopathol. Mediterr.* 47:183-203.
27. Gordon, T., Storer, A. J., and Wood, D. L. 2001. The pitch canker epidemic in California. *Plant Dis.* 85:1128-1139.
28. Gordon, T. R. 2006. Pitch canker disease of pines. *Phytopathology* 96:657-659.
29. Gordon, T. R., Kirkpatrick, S. C., Aegerter, B. J., Wood, D. L., and Storer, A. J. 2006. Susceptibility of Douglas fir (*Pseudotsuga menziesii*) to pitch canker, caused by *Gibberella circinata* (anamorph = *Fusarium circinatum*). *Plant Pathol.* 55:231-237.
30. Goss, E. M., Larsen, M., Chastagner, G. A., Givens, D. R., and Grünwald, N. J. 2009. Population genetic analysis infers migration pathways of *Phytophthora ramorum* in U.S. nurseries. *PLoS Pathog.* 5:e1000583.
31. Goss, E. M., Larsen, M., Vercouteren, A., Werres, S., Heungens, K., and Grünwald, N. J. 2011. *Phytophthora ramorum* in Canada: Evidence for migration within North America and from Europe. *Phytopathology* 101:166-171.
32. Grünwald, N. J., Garbelotto, M., Goss, E. M., Heungens, K., and Prospero, S. 2012. Emergence of the sudden oak death pathogen *Phytophthora ramorum*. *Trends Microbiol.* 20:131-138.
33. Grünwald, N. J., and Goss, E. M. 2011. Evolutionary and population genetics of exotic and re-emerging pathogens: Traditional and novel tools and approaches. *Annu. Rev. Phytopathol.* 49:249-267.
34. Grünwald, N. J., and Hoheisel, G. A. 2006. Hierarchical analysis of diversity, selfing and genetic differentiation in populations of the oomycete *Aphanomyces euteiches*. *Phytopathology* 96:1134-1141.
35. Hepting, G. H., and Roth, E. R. 1946. Pitch canker, a new disease of some southern pines. *J. For.* 44:724-744.
36. Hepting, G. H., and Roth, E. R. 1953. Host relations and spread of the pine pitch canker disease. (Abstr.) *Phytopathology* 43:475.
37. Hodge, G. R., and Dvorak, W. S. 2000. Differential responses of Central American and Mexican pine species and *Pinus radiata* to infection by the pitch canker fungus. *New For.* 19:241-258.
38. Iturriza, E., Ganley, R. J., Wright, J., Heppe, E., Steenkamp, E. T., Gordon, T. R., and Wingfield, M. J. 2011. A genetically homogenous population of *Fusarium circinatum* causes pitch canker of *Pinus radiata* in the Basque Country, Spain. *Fungal Biol.* 115:288-295.
39. Ivors, K., Garbelotto, M., Vries, I. D. E., Ruyter-Spira, C., Hekkert, B. T. E., Rosenzweig, N., and Bonants, P. 2006. Microsatellite markers identify three lineages of *Phytophthora ramorum* in U.S. nurseries, yet single lineages in U.S. forest and European nursery populations. *Mol. Ecol.* 15:1493-1505.
40. Jombart, T. 2008. ADEGENET: a R package for the multivariate analysis of genetic markers. *Bioinformatics* 24:1403-1405.
41. Jombart, T., Devillard, S., and Balloux, F. 2010. Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. *BCM Genet.* 11:94.
42. Kobayashi, T., and Muramoto, M. 1989. Pitch canker of *Pinus luchuensis*, a new disease of Japanese forests. *For. Pest* 40:169-173.
43. Komada, H. 1975. Development of a selective medium for quantitative isolation of *Fusarium oxysporum* from natural soil. *Rev. Plant Prot. Res.* 8:114-125.
44. Landeras, E., García, P., Fernández, Y., Braña, M., Fernández-Alonso, O., Méndez-Lodos, S., Pérez-Sierra, A., León, M., Abad-Campos, P., Berbegal, M., Beltrán, R., García-Jiménez, J., and Armengol, J. 2005. Outbreak of pitch canker caused by *Fusarium circinatum* on *Pinus* spp. in Northern Spain. *Plant Dis.* 89:1015.
45. Lee, J. K., Lee, S. H., Yang, S. I., and Lee, Y. W. 2000. First report of pitch canker disease on *Pinus rigida* in Korea. *Plant Pathol. J.* 16:52-54.
46. Leslie, J. F. 1995. *Gibberella fujikuroi*: Available populations and variable traits. *Can. J. Bot.* 73:282-295.
47. Leslie, J. F., and Klein, K. K. 1996. Female fertility and mating type effects on effective population size and evolution in filamentous fungi. *Genetics* 144:557-567.
48. Linde, C. 2010. Population genetic analyses of plant pathogens: new challenges and opportunities. *Australas. Plant Pathol.* 39:23-28.
49. MAPA. 1996. Informe de la reunión del grupo de trabajo de laboratorio de diagnóstico y prospecciones fitosanitarias. MAPA, Madrid.
50. McCain, A. H., Koehler, C. S., and Tjosvold, S. A. 1987. Pitch canker threatens California pines. *Calif. Agric.* 41:22-23.
51. McDonald, B. A., and Linde, C. 2002. Pathogen population genetics, evolutionary potential, and durable resistance. *Annu. Rev. Phytopathol.* 40:349-379.
52. Milgroom, M. G. 1996. Recombination and the multilocus structure of fungal populations. *Annu. Rev. Phytopathol.* 34:457-477.
53. Milgroom, M. G., and Fry, W. E. 1997. Contributions of population genetics to plant disease epidemiology and management. *Adv. Bot. Res.* 24:1-30.
54. Milgroom, M. G., Lipari, S. E., and Powell, W. A. 1992. DNA fingerprinting and analysis of population structure in the chestnut blight fungus, *Cryphonectria parasitica*. *Genetics* 131:297-306.
55. Peever, T. L., Salimath, S. S., Su, G., Kaiser, S. W., and Muehlbauer, F. J. 2004. Historical and contemporary multilocus population structure of *Ascochyta rabiei* (teleomorph: *Didymella rabiei*) in the Pacific Northwest of the United States. *Mol. Ecol.* 13:291-309.
56. Pérez, G., Slippers, B., Wingfield, B. D., Hunter, G. C., and Wingfield, M. J. 2010. Micro- and macroscale analyses illustrates mixed mating strategies and extensive geneflow in populations of an invasive haploid pathogen. *Mol. Ecol.* 19:1801-1813.
57. Pérez-Sierra, A., Landeras, E., León, M., Berbegal, M., García-Jiménez, J., and Armengol, J. 2007. Characterization of *Fusarium circinatum* from *Pinus* spp. in northern Spain. *Fungal Biol.* 111:832-839.
58. Perry, J. P., Graham, A., and Richardson, D. M. 1998. Pine ecology and biogeography—an introduction. Pages 137-149 in: *Ecology and Biogeography of Pinus*. D. M. Richardson, ed. Cambridge University Press, Cambridge.
59. Pielou, E. C. 1969. *An Introduction to Mathematical Ecology*. John Wiley, New York.
60. Sakai, A. K., Allendorf, J. S., Holt, Lodge, D. M., Molofsky, J., With, K. A., Baughman, S., Cabin, R. J., Cohen, J. E., Ellstrand, N. C., McCauley, D. E., O'Neil, P., Parker, I. M., Thompson, J. N., and Weller, S. G. 2001. The population biology of invasive species. *Annu. Rev. Ecol. S.* 32:305-332.
61. Santana, Q. C., Coetzee, M. P. A., Steenkamp, E. T., Mlonyeni, O. X., Hammond, G. N. A., Wingfield, M. J., and Wingfield, B. D. 2009. Micro-

- satellite discovery by deep sequencing of enriched genomic libraries. *Biotechniques* 46:217-223.
62. Schuelke, M. 2000. An economic method for the fluorescent labeling of PCR fragments. *Nat. Biotechnol.* 18:233-234.
 63. Schweigkofler, W., O'Donnell, K., and Garbelotto, M. 2004. Detection and quantification of airborne conidia of *Fusarium circinatum*, the causal agent of pine pitch canker, from two California sites by using a real-time PCR approach combined with a simple spore trapping method. *Appl. Environ. Microbiol.* 70:3512-3520.
 64. Selkoe, K. A., and Toonen, R. J. 2006. Microsatellites for ecologists: A practical guide to using and evaluating microsatellite markers. *Ecol. Lett.* 9:615-629.
 65. Teacher, A. G. F., and Griffiths, D. J. 2011. HapStar: Automated haplotype network layout and visualization. *Mol. Ecol. Resour.* 11:151-153.
 66. Tibayrenc, M., Kjellberg, F., Arnaud, J., Oury, B., Brenipre, S. F., Dardi, M.-L., and Ayala, F. J. 1991. Are eukaryotic microorganisms clonal or sexual? A population genetics advantage. *Proc. Natl. Acad. Sci. USA* 88:5129-5133.
 67. Viljoen, A., Wingfield, M. J., Gordon, T. R., and Marasas, W. F. O. 1997. Genotypic diversity in a South African population of the pitch canker fungus *Fusarium subglutinans* f. sp. *pini*. *Plant Pathol.* 46:590-593.
 68. Viljoen, A., Wingfield, M. J., and Marasas, W. F. O. 1994. First report of *Fusarium subglutinans* f. sp. *pini* on seedlings in South Africa. *Plant Dis.* 78, 309-312.
 69. Vogler, D. R., Gordon T. R., Aegerter, B. J., Kirkpatrick, S. C., Stover, P., and Violett, P. 2004. First report of the pitch canker fungus (*Fusarium circinatum*) in the Sierra Nevada of California. *Plant Dis.* 88:772-772.
 70. Wallace, M. M., and Covert, S. F. 2000. Molecular mating type assay for *Fusarium circinatum*. *Appl. Environ. Microbiol.* 66:5506-5508.
 71. Wikler, K., and Gordon, T. R. 2000. An initial assessment of genetic relationships among populations of *Fusarium circinatum* in different parts of the world. *Can. J. Bot.* 78:709-717.
 72. Wingfield, M. J., Hammerbacher, A., Ganley, R. J., Steenkamp, E. T., Gordon, T. R., Wingfield, B. D., and Coutinho, T. A. 2008. Pitch canker caused by *Fusarium circinatum*—a growing threat to pine plantations and forests worldwide. *Australas. Plant Pathol.* 37:319-334.
 73. Wingfield, M. J., Jacobs, A., Coutinho, T. A., Ahumada, R., and Wingfield, B. D. 2002. First report of the pitch canker fungus, *Fusarium circinatum*, on pines in Chile. *Plant Pathol.* 51:397-397.
 74. Wostemeyer, J., and Kreibich, A. 2002. Repetitive DNA elements in fungi (Mycota): Impact on genomic architecture and evolution. *Curr. Genet.* 41:189-198.