AFLP analysis reveals a clonal population of *Phytophthora pinifolia* in Chile

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Abstract

Phytophthora pinifolia is the causal agent of the recently discovered needle disease of *Pinus radiata* in Chile, referred to as "Daño Foliar del Pino" (DFP). The genetic structure of the pathogen population is unknown, which hinders our understanding of its appearance and spread in Chile since 2004. In this study, a population of 88 cultures of *P. pinifolia* isolated from *P. radiata* at several localities in Chile was evaluated for genotypic diversity using amplified fragment length polymorphisms (AFLPs). Results of the AFLP analyses showed that the *P. pinifolia* population in Chile consists of two near identical genotypes but with no genetic differentiation based on geography, year of isolation or the part of the tree from which the isolates were obtained. Mating experiments did not lead to the production of gametangia suggesting that the organism is sterile. The fact that a single clonal genotype dominates the population of *P. pinifolia* in Chile supports the hypothesis that *P. pinifolia* was recently introduced into this country and that its impact is due to a new and susceptible host encounter.

Introduction

Phytophthora pinifolia causes a needle disease of *Pinus radiata* in plantations in Chile. The disease, locally known as Daño Foliar del Pino (DFP) affects *P. radiata* (Monterrey pine) of all ages. In young and adult trees, infection occurs in current year needles towards the end of the growing season, resulting in severe needle loss. In seedlings, with severe damage, the disease is characterized by the rapid death of young terminal shoots and death of the entire plant (Durán *et al*. 2008).

DFP was first observed in Raqui, on the Arauco coast of Chile in 2004 and the pathogen rapidly spread from the initial detection, confined to an area of 70 ha, to 60 000 ha in 2006 (Durán *et al*. 2009). Between 2007 and 2008 the affected area has reduced to less than 500 ha, and is confined to road borders and specific zones in the plantation, which are in most of the cases closest to the coast (Durán *et al*. 2009). The *Phytophthora* sp. causing DFP was new to science at the time of the initial detection of the disease, but is now known to reside in Clade 6 in the phylogeny of *Phytophthora* spp. presented by Cooke *et al*. (2000). The behavior of *P. pinifolia* is unusual because it is the only

species residing in Clade 6 without a known soil borne phase and it also lacks nested or extended sporangium proliferation. Furthermore, it is the only *Phytophthora* species known to cause a foliar disease of *Pinus* spp. in plantations (Durán *et al*. 2008, 2009).

Observations in the field suggest that *P. pinifolia* is specific to *P. radiata*. Other conifer species planted in the affected areas remain asymptomatic. For example, *Pinus pinaster* and *Pseudotsuga menziessii* do not show any signs of disease in Chile in areas where *P. radiata* is heavily infected with *P. pinifolia* (Durán *et al*. 2008).

At present, nothing is known regarding the population genetic structure of the *P. pinifolia* population in Chile. Management strategies such as breeding and selection programs focused to reduce the damage of DFP rely on information concerning the genetic structure of the pathogen population. Studies considering the genetic structure of populations of other *Phytophthora* species have provided evidence in support of an introduction hypothesis, e.g. for *Phytophthora cinnamomi* in Australia and South Africa (Linde *et al*. 1997, 1999) and *Phytophthora ramorum* in USA and UK ([Ivors *et al*. 2006; Prospero *et al*. 2007]). Knowledge of the genetic diversity of *P. pinifolia* might also provide clues to the possible origin of the pathogen.

The production of gametangia in culture represents the standard technique to determine the sexual status of *Phytophthora* spp. (Erwin & Ribeiro 1996). The production of oospores in single cultures is representative of a homothallic species, while the requirement of a culture of the opposite mating type (A1 or A2) is indicative of heterothallic species (Erwin and Ribeiro 1996; Judelson and Blanco 2005). Where no gametangia are produced under a wide range of conditions, *Phytophthora* spp. are considered sterile or silent (Brasier et al. 1993, 1999). Previous experiments on *P. pinifolia* failed to produce oospores despite repeated attempts to do so (Durán *et al*. 2008). Hence, additional studies are required to more reliably determine the nature of the sexuality in *P. pinifolia*.

The fact that *P. radiata* is not native to Chile, and that plantations in this country have been free of this diseases for more than a century suggests that the *P. pinifolia* pathogen population may have recently been introduced into the country. An introduced pathogen such as *P. pinifolia* in Chile, would be expected to show a low level of genetic diversity due to the founder effect which makes the population go through a significant bottleneck (Parker and Gilbert 2004). A good example of such a situation is *Phytophthora infestans*, where a single A1 mating type isolate escaped from its ancestral area in central Mexico in the 1840s, giving rise to the global distribution of only a single clone (Fry *et al*. 1993). Studies on populations from the centre of origin of *P. infestans* revealed high levels of gene and genotypic diversity (Grünwald & Flier 2005). A second global migration of this pathogen took place in the 1980s, including both mating types which gave rise to sexual reproduction, and thus far more variable pathogen populations (Fry *et al*. 1992; 1993; Drenth *et al*., 1994). The presence of high levels of gene and genotypic diversity in populations of *P. pinifolia* would also be indicative of sexual reproduction involving both mating types, and/or a high level of gene flow due to continuous introduction of the pathogen over time (McDonald & Linde 2002).

In order to gain insight into the structure, reproductive biology and origin of the *P. pinifolia* population in *P. radiata* plantations in Chile our first aim was to determine the genetic diversity of the population using AFLP analysis. The resulting data would allow us to test the hypothesis that the pathogen population originates from a recent introduction. Our second aim was to test a wider range of experimental conditions than those used in Durán *et al*. (2008) under which gametangial production may occur, to more rigorously determine the likelihood of sexual reproduction in this population. Determining the population genetic structure of the pathogen population will assist in the development of disease management strategies and the identification and deployment of potential resistance to this important plant pathogen.

Materials and Methods

Isolates

Isolates of *Phytophthora pinifolia* were obtained from several locations in Chile (Fig 1) representing the geographic range of the occurrence of DFP, with more than 600 km between the two most distant locations. Isolations were made from symptomatic *Pinus radiata* needles (PRNs) (Table 1) as described by Durán *et al*. (2008). Hyphal tip cultures were made from each isolate on V8 agar (Erwin & Ribeiro 1996). All the isolates are maintained in H2O and 10 % glycerol at room temperature in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

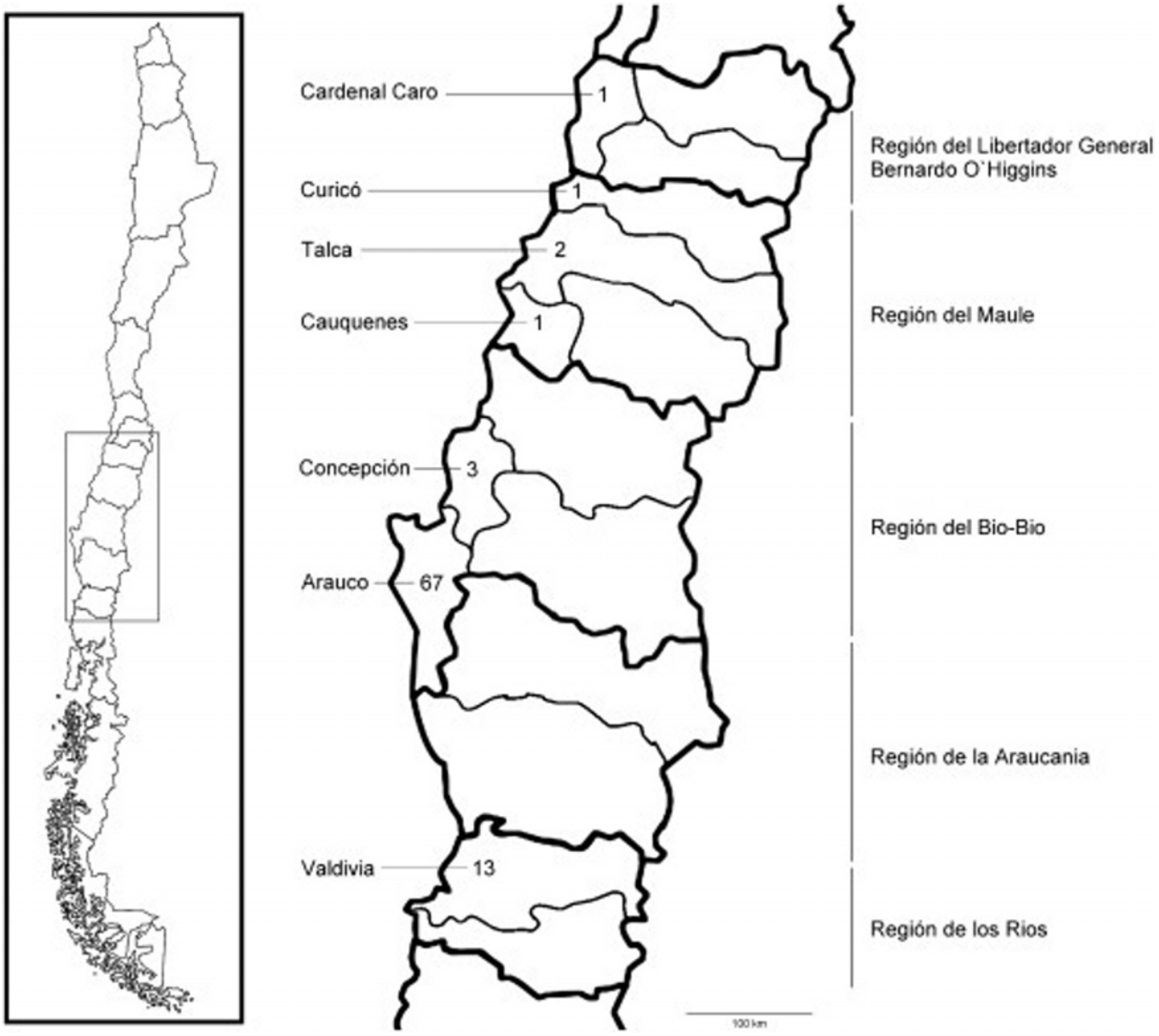

Fig 1. Map of Chile and total number of *Phytophthora pinifolia* isolates obtained in each province.

Table 1. Origin and isolation dates of *Phytophthora pinifolia* cultures used in this study.

Province	Compartment	2007			2008						2009	
		Jun.	Aug.	Oct.	May	Jun.	Jul.	Aug.	Sep.	Oct.	Apr.	May
Cardenal Caro	Sta Consuelo							1				
Curicó	El Alamo							1				
Talca	Quivolgo						1					
	Risco Afuera							1				
Cauquenes	Bellavista								1			
Concepción	La Posada							1				
	Las Palmas								1			
	Malal Aparte							1				
Arauco	Camaron	1		17		5	1					
	Cordillera							1				
	La Colcha							1				
	Llico		5									
	Llico Pira 03			14				1		1		
	Llico Pira 94					5		1				1
	Molino del Sol							1				
	Nalcahue						1					
	Paillacahue				1			1			1	
	Quidico						1				1	
	Quilachanquín							1				
	Rumena						1					
	Trana 3						1			1		
	Trehuaco						1					
	Tropen Efa											1
Valdivia	El Duero								1			
	Naguilan								1			
	Rochela 4								1			
	San Guillermo								1			
	Sta Clara								1			
	Tres Chiflones			8								

DNA extraction

Three 5 mm agar blocks were transferred from the edges of actively growing cultures to 65mm plates with 20 ml of V8 broth and these were incubated for 2 weeks at 25 °C. The mycelium was harvested from these cultures, excluding the agar blocks, under sterile conditions, air dried for 5 min in the plate lid and transferred to 2 ml Eppendorf tubes. The harvested mycelium was washed by filling the tubes with 1.5 ml double autoclaved distilled H2O and vortexed for 30 s. The tubes were then centrifuged for 5 min at 5000 rpm and the water was removed with a pipette, taking care to eliminate the solid pieces generated from the V8 broth at the bases of the tubes. This washing step was repeated twice. Cleaned mycelium was freeze dried (24 h) and ground to a fine powder using the Retsch MM301 mixer mill (Haan, Germany)for 5 min (1/30 mHz).

DNA was extracted using the protocol described by Goodwin *et al*. (1992) and resuspended in 50 ml of nuclease free water. Successful DNA extraction was confirmed by gel electrophoresis (1 % agarose gel stained with ethidium bromide and visualized under UV light). The DNA concentration was determined using a Nanodrop ND 1000 spectrophotometer and NanoDrop 3.2.1 Software (NanoDrop Technologies Inc., Rockland, DE) and adjusted to 30 ng/ml with nuclease free water. To confirm the suitability of the DNA to perform PCR and to confirm the identity of each isolate, the extracted DNA was used as template in amplification with the species specific primers Pfoli1F and Pfoli1R developed by Durán *et al*. (2009), and following the same protocols described therein. Amplified fragment length polymorphism (AFLP) analysis

Eighty-eight isolates were selected for the analysis, with eight isolates duplicated as internal controls to ensure reproducibility of the banding patterns (Table 1). AFLP profiles were generated from the extracted DNA using the method described by Vos *et al*. (1995) with minor modifications. These included the initial DNA concentration and the restriction and ligation incubation times. From the diluted genomic DNA, 5 µl (150 ng) was digested with two units of the restriction

endonuclease EcoRI (Fermentas, Vilnius, Lithuania) and MseI (New England Biolabs, Beverly, MA) for 3 h at 37 °C. The digested fragments were ligated to the adapters EcoRI (5′ CTC GTA GAC TGC GTA CC/CAT CTG ACG CAT GGT TAA 5′) and MseI (5′ GAC GAT GAG TCC TGA G/TAC TCA GGA CTC AT 5′) (Vos *et al*. 1995) using T4 DNA ligase (Fermentas, Vilnius, Lithuania) for 3 h at 22 °C.

The DNA fragments were subjected to an initial preamplification step with the reaction mixture including 0.3 µM EcoRI + A (5′ GAC TGC GTA CCA ATT CA 3′) primer, 0.3 µM MseI + C (5′ GAT GAG TCC TGA GTA AC 3′) primer (Vos *et al*. 1995), 0.2 mM dNTPs, PCR buffer containing 1.5 mM $MgCl_2$, 0.6 U Taq Polymerase (Roche Diagnostics, Mannheim, Germany) and 5 µl of the restriction–ligation mix. The preamplification PCR was carried out using the following conditions: 30 s at 72 °C followed by 25 cycles of 30 s at 94 °C; 30 s at 56 °C; 1 min + 1 s per cycle at 72 °C, and a final cycle of 2 min at 72 °C. Successful restriction, ligation and amplification were confirmed by gel electrophoresis (1 % agarose gel stained with ethidium bromide and visualized under UV light).

The product of the preamplification step was diluted 1/10 with nuclease free water, and used as template for the subsequent final amplification step. The reaction mixture included 5 µl of the diluted preamplification product, PCR buffer, 0.5 mM MgCl2, 0.2 mM dNTPs, 0.04 µM Infrared dye (IRD) labeled (Li-COR, Lincoln, NE, USA) EcoRI + 2 primer (E), 0.25 µM MseI + 2 primer (M) and 0.6 U Taq Polymerase (Roche Diagnostics). Reaction conditions for the final amplification step were 13 cycles for 10 s at 94 °C; 30 s at 65 °C with temperature decreasing by 0.7 °C per cycle during subsequent cycles; 1 min at 72 °C; followed by 23 cycles of 10 s at 94 °C; 30 s at 56 °C; 1 min + 1 s per cycle at 72 °C, and a final cycle of 1 min at 72 °C. The enzyme restriction, ligation, preamplification and final amplification were carried out in 200 µl Eppendorf tubes in an Eppendorf Mastercycler ep (Eppendorf, Hamburg, Germany).

AFLP fragment analysis was performed on a model 4200 LI-COR automated DNA sequencer with the electrophoresis parameters similar to those described by Wingfield *et al*. (2009). Before the final amplification with all the samples, a screening test was done using four isolates (CMW 26667, CMW 26668, CMW 26669, and CMW 26670) and 16 primer combinations (M + AA, M + AG, M + AC, and M + CC in combination of each E + AA, E + AC, E + TC, and E + CC). Based on the clarity of the resulting fingerprinting profiles (allowing unambiguous scoring) and reproducibility, four primer combinations (M + AA–E + TC, M + AG–E + AA, M + AG–E + AC, and M + AA–E + AA) were selected and used to evaluate the larger group of isolates. From the digital image, bands were visually evaluated with JelMarker Software V1.3 Demo (Softgenetics, State College, PA, USA).

Gametangial induction

The ability of *Phytophthora pinifolia* to produce gametangia was evaluated on various growth media. Three isolates of *P. pinifolia*, including the extype isolate (CMW 26668) and two paratypes (CMW 26667, CMW 26669), were grown for 2 weeks in V8A (Erwin & Ribeiro 1996) at 25 °C in the dark. A single block (5 × 5 mm) of agar bearing mycelium was transferred to the centre of 65 mm Petri dishes containing either CA, CMA, OMA (Erwin and Ribeiro 1996), V8A, WA (15 g agar l^{-1}) growth media including PRNs and *Pinus radiata* needle extract (PRNE). To prepare the PRN, five pieces of 5 mm long autoclaved PRNs, were scattered randomly on the surface of V8A, CMA and CA growing media before the agar solidified. The PRNE was made by mixing V8A and CA, respectively, with PRN broth that was prepared by autoclaving 50 g of fresh, cut PRNs in 800 ml of distilled water and then filtering the broth through a double layer of cheese cloth. For the V8A–PRNE, 150 ml of centrifuged V8 and 15 g of agar were added to 800 ml of PRN extract, and made up to 1 l before autoclaving. The CA–PRNE was made by adding 300 ml of cheese, cloth filtered

carrot juice (200 g carrot, crushed in a blender and boiled for 20 min) and 15 g of agar. Cultures were maintained at 25 °C in the dark and inspected every 3 weeks for the presence of gametangia.

Phytophthora pinifolia isolates were tested for their ability to produce gametangia in crosses with tester strains of other *Phytophthora* spp. of opposite mating types (Erwin & Ribeiro 1996). Three *P. pinifolia* isolates (CMW 26667, CMW 26668, and CMW 26669) were paired with known A1 and A2 tester isolates of *Phytophthora cambivora*, *Phytophthora cinnamomi*, *Phytophthora cryptogea*, *Phytophthora drechsleri*, and *Phytophthora palmivora* (Table 2). Agar plates (CA, CMA, OMA, V8A, or WA) were inoculated with a block (5 × 5 mm) of *P. pinifolia* culture placed 15 mm from the edge of a 65 mm Petri dish and maintained at 25 °C in the dark for 1 week prior to the start of these tests. The tester isolates were transferred to the opposite sides of the plate, 15 mm from the edge, and incubated in the dark at 25 °C and inspected for the presence of gametangia every 3 weeks.

Table 2. Isolates of *Phytophthora* species used for the mating tests.

Phytophthora spp.	Isolate number[a]		Mating type	Host or source	Location
P. cryptogea	P29	CMW 33002	A2	*Begonia* sp.	Germany
P. cryptogea	P30	CMW 33003	A1	*Lewisia* sp.	Germany
P. cambivora	P31	CMW 33004	A2	Soil (*Quercus robur*)	Germany
P. cambivora	P32	CMW 33005	A1	*Chamaecyparis lawsoniana*	Germany
P. cinnamomi	P33	CMW 33006	A1	*Camellia* sp.	Germany
P. cinnamomi	P34	CMW 33007	A2	*Rhododendron simsii*	Germany
P. drechsleri	P35	CMW 33008	A2	*Beta vulgaris*	Iran
P. drechsleri	P36	CMW 33009	A1	*Hedera* sp.	Germany
P. palmivora	UQ 3689	CMW 29599	A1	*Howea forsteriana*	Australia
P. palmivora	UQ 3694	CMW 29601	A1	*Arecastrum romanzoffianum*	Australia
P. palmivora	UQ 3716	CMW 29602	A1	Palm	Australia
P. palmivora	UQ 3717	CMW 29603	A2	Soil	Australia
P. palmivora	UQ 3718	CMW 29604	A2	Soil	Australia
P. cinnamomi	UQ 3652	CMW 29598	A2	*Ananas comosus*	Australia
P. cinnamomi	UQ 3651	CMW 29597	A2	*Ananas comosus*	Australia
P. cinnamomi	UQ 3648	CMW 29596	A2	*Ananas comosus*	Australia
P. cinnamomi	UQ 789	CMW 29606	A1	*Eucalyptus gummifera*	Australia
P. cinnamomi	UQ 795	CMW 29607	A1	Soil	Australia
P. drechsleri	WPC 1899	CMW 29592	A1	*Beta vulgaris*	USA
P. drechsleri	WPC 3808	CMW 29594	A1	*Vigna unguiculata*	USA

a Isolates with P numbers were provided by Prof. Everett M. Hansen, Oregon State University, Corvallis, OR, USA; those with UQ notations are from the *Phytophthora* culture collection, University of Queensland, Brisbane, Queensland, Australia, those with WPC numbers from the world *Phytophthora* culture collection, University of California, Riverside, CA, USA and the CMW numbers represent the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Results

AFLP analysis

A total of 200 clearly resolved bands were visually evaluated from the digital images emerging from the four different primer combinations. The profiles for the isolates with the primers M + AA–E + TC, M + AG–E + AA, M + AG–E + AC, and M + AA–E + AA, which were generated during the primer screening phase, were the same as those generated for the same isolates when they were evaluated together with the rest of the population. The eight isolates duplicated as internal controls, had the same profiles in both samples for the four primer combinations evaluated.

Other than two isolates, all the remaining 86 isolates included in this study had identical profiles for all of the primer combinations used. For isolates CMW 33987 and CMW 34012, one band was not detected for the primer combination (M + AA–E + TC). This difference was considered to be a polymorphic site. Other than this polymorphism, these isolates were identical to all other isolates in this study for the other three primer combinations used; M + AG–E + AA, M + AG–E + AC, M + AA–E + AA.

Gametangial induction

After 12 weeks of growth on a wide variety of media, including those amended with pine needle tissue, no sign of gametangia was found in *Phytophthora pinifolia* cultures. Likewise, where *P. pinifolia* isolates were crossed with A1 and A2 mating type testers for *Phytophthora cambivora*, *Phytophthora cinnamomi*, *Phytophthora cryptogea*, *Phytophthora drechsleri*, or *Phytophthora palmivora*, there were no signs of gametangia in the *P. pinifolia* cultures or in the tester strains. All tester strains produced oospores in all cases when mated with testers of the opposite mating type of the same species.

Discussion

This is the first study to consider the genetic structure of the population of the new and important pine pathogen *Phytophthora pinifolia*. Results of this study based on a broadly collected population of *P. pinifolia* isolates from its currently known geographic distribution in Chile, showed that the pathogen population is represented by two near identical genotypes, one of which constitutes more than 97 % of the isolates sampled. Isolates included in this study were broadly sampled from across the area in which *P. pinifolia* occurs and they were also collected at different times of the year as well as in different years. The clonal nature of the pathogen population provides compelling evidence that the pathogen has spread rapidly from a single entry point, with the first detection in Raqui in 2004 (Durán *et al*. 2008). The population structure of *P. pinifolia* observed in this paper is indicative of an introduced pathogen population (Goodwin *et al*. 1994; Loo, 2009).

Two isolates (CMW 33987 and CMW 34012) included in this study had a single missing band for primer (M + AA–E + TC). This difference is equivalent to 0.5 % of the scored bands and is a much lower level of diversity than that found in other *Phytophthora* spp. such as *P. infestans* (Goodwin *et al*. 1994), *Phytophthora cinnamomi* (Linde *et al*. 1997; Linde *et al*. 1999) and *P. ramorum* (Ivors *et al*. 2004; Ivors *et al*., 2006) that have been defined as clonal. The missing band in isolates CMW 33987 and CMW 34012 could be a consequence of a single mutation in one of the restriction enzyme sites in these two *P. pinifolia* strains.

Phytophthora pinifolia in Chile represents a clonal population that has spread rapidly over some area of *Pinus radiata* plantation in Chile. It is typical for introduced pathogens to have very limited genetic diversity. For example, *P. infestans* spread around the world as a single genotype for more than 100 y (Goodwin *et al*., 1994; Goodwin and Drenth 1997). *Phytophthora ramorum* in the USA and Europe and *P. cinnamomi* in Australia and South Africa are also introduced pathogens showing low levels of genetic diversity (Linde *et al*. 1997; Ivors *et al*. 2006; Prospero *et al*. 2007). Genetic bottlenecks giving rise to reduced levels of genetic diversity may have some long term evolutionary disadvantages as is often hypothesized by ecologists (Kolar & Lodge 2001). Most viral, bacterial and fungal epidemics in the plant and animal world are caused by pathogen populations showing low levels of genetic diversity in the initial expansionary phase of the epidemic. Once a specific genotype of a potential invasive pathogen finds a suitable agricultural or forest environment in which to become established, it can spread rapidly and cause significant damage, because the host and environment are often highly uniform (Drenth 2004; Parker and Gilbert 2004). The almost complete lack of diversity of *P. pinifolia* could be a consequence of low mutation pressure and absence of sexual recombination.

Efforts to induce sexual structures in *P. pinifolia* on different media in this study were not successful. Likewise, crossing of isolates with defined mating tester strains of several species of *Phytophthora* spp., including Phytophthora drechsleri and Phytophthora cambivora that have been shown to induce sexual structures in other Phytophthora species (Brasier *et al*. 2003a; 2003b; Brasier and Kirk 2004), did not induce the production of gametangia. *Phytophthora pinifolia* resides

in Clade 6 of *Phytophthora* phylogeny (Cooke *et al*. 2000; Durán *et al*. 2008) and there was a possibility that sexual structures might have been induced, as has been the case for *Phytophthora inundata* and *Phytophthora gonapodyides* (Brasier *et al*. 2003a) that also reside in this clade. This absence of sexual structures suggests that *P. pinifolia* is sterile, similar to the recently described species *Phytophthora gallica* (Jung & Nechwatal 2008). Sterility is found in approximately 4 % of *Phytophthora* spp. (Cooke *et al*. 2000), but is much more common among species in Clade 6 where *P. pinifolia* resides. In this clade, over 50 % of the taxa examined by Brasier *et al*. (2003a) are fully sterile, or sterile but acting as silent A1 mating type strains. The sterility in *Phytophthora* spp. is not fully understood and it could be an adaptation for adverse environments (Brasier *et al*. 2003b), although whether this is the case for *P. pinifolia* remains to be determined.

The absence of any sign of the pine disease caused by *P. pinifolia* prior to 2004, and the development of the disease subsequent to its first appearance in Chile, is typical of an introduced pathogen that is exposed to a highly susceptible host grown over vast areas in monoculture (Drenth 2004). The presence of a single dominant genotype in the *P. pinifolia* population in Chile further supports the hypothesis that this is a newly introduced pathogen. An alternative hypothesis is that *P. pinifolia* is native to Chile and that it has undergone a host shift (Slippers *et al*. 2005) from a native plant. Such host shifts might also result in a clonal population of a pathogen on a new host. However, a review of literature on *Phytophthora* epidemics (Hansen *et al*. 2000; Ivors *et al*. 2004; Ivors *et al*. 2006; Brasier *et al*. 2005; Denman *et al*. 2006; Prospero *et al*. 2007), as well as emerging plant diseases in general (Anderson *et al*. 2004), indicates that introduction is by far the most common explanation for such patterns. Hence, our data support the hypothesis that *P. pinifolia* is an introduced pathogen in Chile. The origin of *P. pinifolia* is unknown, but we hypothesize that it has most likely originated in an area where other *Pinus* spp. are native, and where cool moist conditions might favor infection.

Phytophthora pinifolia is an important pathogen of *P. radiata* and this study provides compelling evidence that it has been introduced into Chile. *Pinus radiata* appears susceptible to infection which occurs on seedlings, young as well as mature trees (Durán *et al*. 2008). The pathogen represents a threat to *P. radiata*, which is one of the most widely planted *Pinus* spp. in plantations in the southern hemisphere. The fact that the pathogen might have been accidentally introduced into Chile suggests that it has the capacity to be introduced into other areas of the world where *P. radiata* is grown in plantations.

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