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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* spp. 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

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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 & Ribeiro 1996; Judelson & 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 & 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 H_2O and 10 % glycerol at room temperature in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

DNA extraction

Three 5 mm agar blocks were transferred from the edges of actively growing cultures to 65 mm 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 H_2O 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 µl 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/µl 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