Phytophthora pinifolia sp. nov. associated with a serious needle disease of *Pinus radiata* in Chile

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During the course of the past three years, a new disease of *Pinus radiata*, referred to as 'Daño Foliar del Pino' (DFP) has appeared in the Arauco province of Chile and subsequently spread to other areas. The disease is typified by needle infections, exudation of resin at the bases of the needle brachyblasts and, in younger trees, necrotic lesions in the cambium, which eventually girdle the branches. The disease causes the death of young seedlings and mature trees can also succumb after a few years of successive infection, probably hastened by opportunistic fungi such as *Diplodia pinea*. Isolations on selective medium for *Phytophthora* spp. led to the consistent isolation of a *Phytophthora* sp. from needle tissue. DNA sequence comparisons for the ITS rDNA and *cox* II gene regions, and morphological observation showed that this oomycete represents a previously undescribed species for which the name *Phytophthora pinifolia* sp. nov. is provided. This new species is characterized by unbranched sporangiophores, and non-papillate, sub-globose to ovoid sporangia that are occasionally free from the sporangiophore with medium length pedicels. Despite using a number of oospore inducing techniques, oogonia/antheridia were not observed in isolates of *P. pinifolia*. Pathogenicity trials with *P. pinifolia* showed that it is pathogenic to *P. radiata* and causes rapid death of the succulent apical parts of young plants. *Phytophthora pinifolia* is the first *Phytophthora* known to be associated with needles and shoots of a *Pinus* sp. and its aerial habit is well matched with the occurrence and symptoms of DFP in Chile.

Keywords: Monterey pine, oomycete phylogeny, plantation forestry

Introduction

Forestry plantations based on non-native trees, particularly in the tropics and Southern Hemisphere, represent substantial business enterprises of great international relevance (Boyle *et al.*, 1999). These forestry companies primarily plant species of *Pinus*, *Eucalyptus* and *Acacia*, and they produce solid–wood products and pulp for paper and rayon production. Growth of the trees, used to sustain wood and fiber farms, is in many cases exceptional and typically far beyond that associated with the trees in their natural environment (Richardson *et al.*, 2007). This is widely attributed to the separation of the trees from their natural enemies (Wingfield, 2003; Brockerhoff *et al.*, 2006). Thus, one of the greatest threats to plantation forestry based on non-native trees clearly lies in dangers surrounding the accidental introduction of pests and pathogens (Wingfield *et al.*, 2001, 2006; Brockerhoff

et al., 2006), or native pests and diseases that can affect these exotic hosts (Slippers *et al.*, 2005; Carroll, 2007).

Pinus radiata (Monterey pine), one of the most widely planted forest trees in the Southern Hemisphere, has its origin on the California coast (USA) and nearby islands (Rogers *et al.*, 2006; Richardson *et al.*, 2007). Within its native range, it is ecologically restricted to coastal Mediterranean climates, growing in only five locations; three along central coastal California and two on Mexican islands of Baja California (Rogers, 2004; Rogers *et al.*, 2006). Consistent with its native range, *P. radiata* has performed best in other areas of the world that have a Mediterranean climate, including parts of South Africa, Australia, Chile, New Zealand and Spain (Rogers, 2004). Chile has a substantial investment in plantations of *P. radiata*, which was introduced for ornamental purposes in 1885, and has grown exceptionally well over a relatively long period (Toro & Gessel, 1999). In surface area, Chilean *P. radiata* plantations represent more than one third of the total area planted to this species in the world, with approximately 1·5 million ha established and forming the basis of a major industry important to the economy of the country (Guerrero & Bustamante, 2007).

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Several important pests and diseases occur on *P. radiata* plantations in Chile. Needle blight caused by *Dothistroma septosporum* (Barnes *et al.*, 2004) and the pine shoot moth *Rhyacionia buoliana* (Lanfranco, 2000) have resulted in the most serious sanitary problems on *P. radiata* in Chilean plantations (Ahumada, 2003). *Fusarium circinatum*, the causal agent of pitch canker in *P. radiata* plantations in Spain and South Africa (Landeras *et al.*, 2005; Coutinho *et al.*, 2007), occurs in forestry nurseries in Chile, but has not been found in the field (Jacobs *et al.*, 2007).

In February 2004, an unusual tree mortality appeared in a 6-year-old *P. radiata* stand of about 70 ha on the Arauco coast of Chile. In October 2004 and in the same area, a serious needle blight disease was observed and associated with this mortality. The damage increased dramatically expanding to approximately 60 000 ha by the end of 2006. The *P. radiata* needle blight disease in Chile has been referred to as 'Daño Foliar del Pino' (DFP) and it is generally accepted to be the most serious problem yet to have affected pine forestry in the country.

The overall pattern of disease development, the species of tree affected and the symptoms provided a robust indication that DFP is caused by a biotic agent. Previous isolations from disease symptoms on routine growing media, such as streptomycin–amended 2% malt extract agar (MEA), yielded many fungi including *Strasseria geniculata*, *Allantophomopsis lycopodina*, *Phoma herbarum*, *Microsphaeropsis olivaceae*, *Diplodia pinea* and *Pestalotiopsis* spp. *Strasseria geniculata* and *A. lycopodina* were also commonly found sporulating at the bases of needles in the early stages of infection. However, inoculation of these fungi onto the needles and stems of young and old *P. radiata* plants with spore suspensions or with mycelial plugs, did not result in any symptoms (unpublished data). Thus none of these fungi appear to be responsible for the disease and they probably represent opportunistic endophytes or secondary inhabitants.

Isolations from diseased needles in July 2007 on *Phytophthora*–selective media consistently yielded a *Phytophthora* species (http://www.fabinet.up.ac.za/tpcp/pinifolia). There are more than 80 species in the genus *Phytophthora* (www.phytophthoradb.org) and most are destructive plant pathogens (Goodwin, 1997). Thus, the close association of a *Phytophthora* sp. with the needle and shoot disease in Chile suggested that this could be the causal agent. The aim of this study was to describe DFP and to characterize the *Phytophthora* species isolated from diseased tissue based on morphology and DNA sequence comparisons. In addition, the ability of the oomycete to cause disease was considered in pathogenicity tests using young *P. radiata* plants.

Materials and methods

Disease occurrence and symptoms

Symptoms of DFP were first observed on the Arauco coast at Paillacahue (37°17′40″S; 73°36′44″W) on 18-year-old
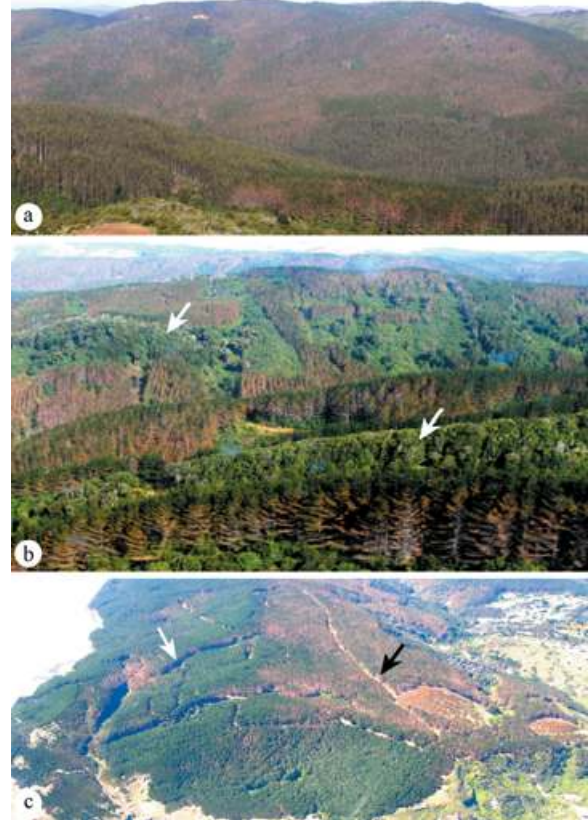

Figure 1 Aerial views of *Pinus radiata* plantations affected by 'Daño Foliar del Pino' (DFP) on the Arauco coast of Chile. (a) Extensive damage due to needle blight. (b) Unaffected native vegetation (white arrow) alongside affected pine stands. (c) Severe infection on the eastern slopes (black arrow) of a plantation with little damage on western the slopes (white arrow).

P. radiata trees in October 2004 and they have subsequently been monitored regularly. During this period, the disease expanded from an area of 70 ha to approximately 60 000 ha at the end of 2006 with varying levels of damage depending on specific sites (Fig. 1a–b). Symptoms were thus studied over a period of four years and their description reflects observations over a relatively long period of time.

Isolations from infected tissue

Isolations were made from the bases of newly infected needles, the resinous bands on needles, as well from infected phloem tissue below infected needles on the branches and stems. For isolations, a sharp scalpel was used to cut small (1 mm) pieces of infected tissue that were transferred to plates of modified selective CMA–NARP (17 g L^{-1} corn meal agar [Difco], amended with 1 mg L^{-1} nystatin, 100 mg L^{-1} ampicillin, 100 mg L^{-1} pentachlornitrobenzene (PCNB) and 10 mg L^{-1} rifampicin) (Shearer

& Dillon, 1995). Plates were incubated at 20°C for 10 days. Resultant colonies were transferred to carrot agar (CA) and maintained at 20°C (Erwin & Ribeiro, 1996). All cultures used in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria and representative isolates have been deposited in the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands, and the World Phytophthora Genetic Resource Collection (WPC), University of California, Riverside, CA, USA.

DNA sequence comparisons

Five isolates of the *Phytophthora* sp. (CMW 26667–26671), tentatively identified based on mycelial characteristics and the presence of sporangia, were selected for DNA extractions. DNA was extracted from mycelium scraped from the surface of agar plates using the PrepMan Ultra sample preparation reagent (Applied Biosystems) following the manufacturer's instructions. The ITS region of the rDNA was amplified using the primers ITS6 and ITS4 (White *et al.*, 1990). Primer ITS6 is similar to ITS5 (White *et al.*, 1990), but was modified according to the *P. megasperma* 18S rDNA sequence to allow more efficient amplification in *Phytophthora* spp. (Cooke & Duncan, 1997; Cooke *et al.*, 2000). The *cox* II region was amplified using the primers FM35 and FM58 (Martin, 2000). Amplifications were performed in an iCycler thermocycler (Bio–Rad). The thermocycle sequence for ITS and *cox* II regions was as follows: an initial denaturation step of 2 min at 95°C was followed by 30 cycles of 20 s denaturation at 95°C, 25 s of annealing at 55°C and 50 s extension at 72°C, and a final extension step of 72°C for 10 min (Cooke *et al.*, 2000). The PCR reaction mixture (25 μL) consisted of 10–20 ng of template DNA, 200 μM dNTPs, 1U *Taq* DNA polymerase, 10X buffer (Roche Molecular Biochemicals), 1·5 mM MgCl$_2$, and 25 ng of each primer. Successful amplification was confirmed by gel electrophoresis (1% agarose gel stained with ethidium bromide and visualized under UV light).

PCR products were purified on Sephadex columns (Multiscreen HV, Millipore) following the manufacturer's recommendations, to remove excess primers and nucleotides. These PCR products were sequenced with the forward and reverse primers used in the amplification reactions. Reactions were performed using an ABI PRISM™ Big Dye terminator sequencing reaction kit following the manufacturer's instructions (Perkin–Elmer, Applied BioSystems). Sequencing was done using an ABI 3100™ automated DNA sequencer.

The forward and reverse sequences were assembled using the software Vector NTI Advance™ v. 10·3 (Invitrogen Corporation), and aligned using the software MEGA v. 3·0 (Kumar *et al.*, 2004) followed by manual improvement of the alignment. The sequences were subjected to an NCBI BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/) prior to phylogenetic analyses to identify the closest related sequences.

The obtained sequences for the *Phytophthora* spp. were compiled into DNA sequence data matrixes. The ITS sequences were incorporated in a DNA sequence dataset that represents the diversity of the genus *Phytophthora*, produced by Cooke *et al.* (2000) and obtained from TreeBASE (www.treebase.org; Matrix #M751). Additional DNA sequences for *Phytophthora ramorum* (Martin & Tooley, 2003), *P. inundata* (Brasier *et al.*, 2003a), and *P. austrocedrae* (Greslebin *et al.*, 2007) were added. Similarly, the *cox* II sequences were incorporated in a DNA sequence dataset representative of the genus *Phytophthora* published by Martin & Tooley (2003) (Treebase matrix # M1228), and DNA sequences of *P. ramorum* (Ivors *et al.*, 2004) were added. *Cox* II sequences corresponding to the region used here were not available for *P. inundata*, *P. humicola* and *P. austrocedrae*.

Maximum parsimonious trees were obtained with PAUP (Phylogenetic Analysis Using Parsimony) version 4·0b10 (Swofford, 2002), with heuristic searches of only informative characters and tree bisection and reconstruction (TBR) as branch–swapping algorithm (random stepwise addition). Gaps were treated as missing, and all characters were unordered and of equal weight. Maxtrees were unlimited, branches of zero length were collapsed, and all multiple, equally parsimonious trees were saved. Estimated levels of homoplasy and phylogenetic signal (retention and consistency indices and g1–value) were determined (Hillis & Huelsenbeck, 1992). Branch supports were determined using 1000 bootstrap replicates (Felsenstein, 1985). Phylogenetic species hypotheses were also tested using distance analyses with the neighbour-joining algorithm (implemented in MEGA v. 3·0 [Kumar *et al.*, 2004]), and compared to maximum parsimony analysis. Individual sequences generated in this study have been deposited in GenBank.

Morphology

To induce the production of fruiting structures, isolates were grown on either CA or V8 agar (354 mL V8 juice, buffered with 5 g of CaCO$_3$, 15 g of agar, 1 litre with distilled water) (Erwin & Ribeiro, 1996). For sporangia, blocks (5 × 5 mm) were cut from the edges of actively growing 7-day-old CA and V8 cultures, and immersed directly into non-sterile soil water (Erwin & Ribeiro, 1996), with modifications (60 g soil in 1 litre of water, allowing the soil to settle for 8 h and filtering the supernatant through four layers of cheese cloth). Blocks of agar bearing mycelium in the soil-water mixture were incubated at room temperature (20–22°C) for 12 hrs and then transferred to a 4°C incubator for 4 hrs. Cultures were then returned to room temperature and inspected every 30 min for the presence of fruiting structures. In an attempt to produce oogonia, pure cultures growing in CA and V8 were incubated in the dark at 20°C and 25°C and checked at 14 and 30 d. Isolates were also paired with each other in all combinations on the same media and under the same incubation conditions, and observed at 14 and 30 days.

The morphology of the *Phytophthora* sp. was studied using conventional microscopy techniques. Structures were mounted in 85% lactic acid. Twenty measurements of all structures were made, but fifty measurements were taken for the isolate representing the ex-type. Measurements and photography were done with a HRc Axiocam digital camera system with Axiovision 4·6 software (Carl Zeiss Ltd.).

To test for the optimum growth temperature, five isolates (CMW 26667–26671) were transferred to two types of growing media (V8 and CA) with five replicates for each isolate. Circular agar blocks (3 mm diam) were placed at the centres of 90 mm diameter Petri dishes and these were incubated at temperatures between 10 and 35°C at five degree intervals, in the dark. Colony growth was assessed after 10 days by taking two diameter measurements at right angles to each other for each replicate plate. The averages were computed and the data statistically compared with a one–way ANOVA test using Statistica v. 6 (StatSoft Inc., 2004).

Pathogenicity tests

A pathogenicity test was conducted using the same *Phytophthora* isolates (CMW 26667–26671) used in the DNA sequence comparisons. The isolates were grown on CA at 20°C for two weeks prior to inoculation. Inoculations were conducted in Chile where *P. radiata* seedlings, approximately 12-month-old, were used. A total of 70 plants, approximately 400 mm high and 6 mm collar diameter, were acclimatized into the screening facilities with artificial day light at approximately 20–25°C for two weeks prior to inoculation. Small (3 mm diam) discs of bark were removed from the apical parts of the stems where the tissue was green and plugs of mycelium of similar size, taken from the edges of actively growing cultures, were placed into the wounds. Discs of clean CA were used as negative controls. Ten plants were inoculated for each of the cultures. Inoculation wounds were covered with Parafilm (Pechiney Plastic) to reduce desiccation and contamination.

Two weeks after inoculation, the outer bark was removed from the inoculation points with a sterile scalpel, and the inner bark lesion lengths (mm) were measured. Small pieces of wood from the leading edges of the lesions were plated onto NARP medium to ensure that the inoculated organism was associated with the lesions. The means of the measured lesions were compared with a one-way ANOVA test using Statistica v. 6 (StatSoft Inc., 2004).

Results

Disease occurrence and symptoms

The symptoms of DFP on mature trees, up to 18-years-old, begin with a reddening of the past year's needles in early winter (Fig. 2a–b). The first needles to display symptoms are those on the lower sides of the branches (Fig. 2d). Needles die and assume a distinctly grey colour and begin to fall. Initially, dead and dying needles are retained on the branches giving the trees the appearance of having been severely scorched. Needles then fall from the trees, which can be almost entirely defoliated. New needle growth is not affected and the trees appear to recover until infection re-occurs in the following season. After two or three years of defoliation, trees occasionally die and this appears to be hastened due to infection by *D. pinea*, which is a well-known opportunistic pathogen (Swart & Wingfield, 1991).

One of the earliest symptoms on affected needles is the emergence of dark resinous bands on the green needles, which appear transparent when viewed with backlighting (Fig. 2c). These bands are found at various positions, either close to the bases or higher up on the needles. Drops of resin are often found at the base of the needles (Fig. 2f) and the tissue within the papery brachyblasts is commonly collapsed and has a light grey colour. Needles often collapse from their bases just above the branches and hang at right angles from the branches. When the bark is removed, a distinct brown or reddish brown discolouration can be seen in the phloem and cambium, particularly where it is associated with dying needles (Fig. 2g–i).

Symptoms on young trees between one and 4-year-old appear different to those on mature trees. Damage to young trees is most common and most severe where stands occur alongside larger trees affected by DFP. One of the first and most obvious symptoms to appear on young trees is where young growing terminal shoots wilt and die rapidly (Fig. 2e–j). Lesions are typically found on these shoots or on the needles associated with them, depending on the time of the year. The damage resulting in the wilted growing shoots is typically found on parts of branches or shoots a short distance below the obviously affected tissue. Close inspection reveals needles with symptoms similar to those on older trees with infected bases leading to girdling of the stems. Dark resinous bands can be found on the needles in the early stages of symptom development and infection appears to be concentrated within the papery brachyblasts at the base of the needle fascicles.

On younger trees, necrosis of the cambium is more pronounced than that on mature trees and the impact of infection is commonly more severe. Resin can be found exuding profusely from the brachyblasts or the bases of the needles (Fig. 2h). Branches can have large numbers of needles hanging at right angles, apparently from their bases and infections begin on the lower sides of branches. Removal of the outer bark reveals distinct lesions below the needle bases and in many cases, due to multiple infections, they coalesce to form cankers in the phloem and outer cambium. These cankers result in girdling of the stems or branches and wilting of the needles and shoots proximal to them. Naturally regenerated plants and newly planted seedlings are equally affected by DFP, and appear to wilt and die rapidly due to their small size.

The pine needle blight in Chile has a distinctly seasonal pattern of occurrence. Trees begin to show symptoms in early winter from about July onwards when the
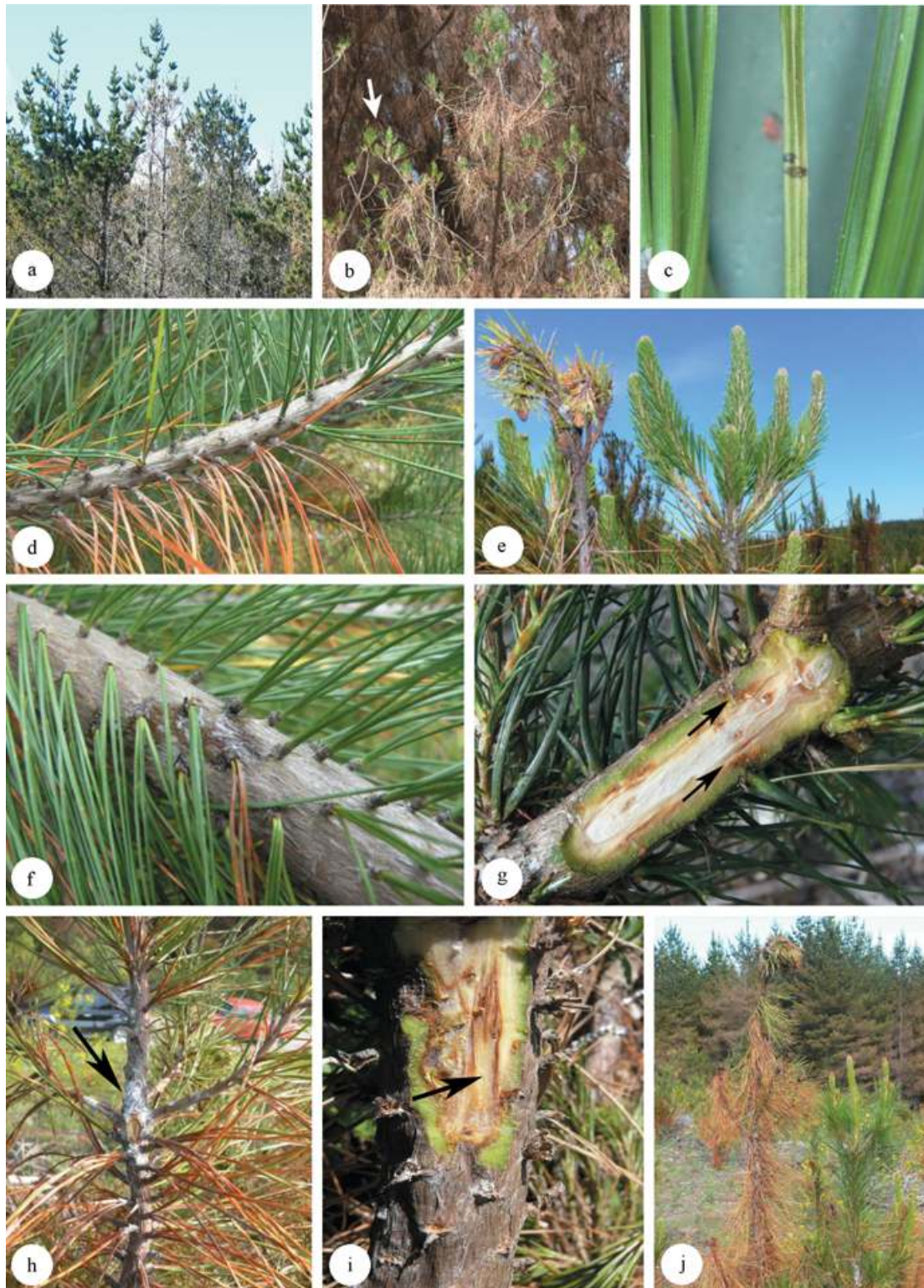
Figure 2 Symptoms on *Pinus radiata* associated with infection by *Phytophthora pinifolia*. (a) Dying trees with scorched appearance. (b) New growth at branch tips (arrow) after heavy infection of the previous season's needles. (c) Black band on a needle representing one of earliest symptoms of infection. (d) Dead needles on the lower and less affected needles on the upper side of a branch. (e) Wilted shoots on a young tree, resulting from cankers lower down the stem and associated with infection of needles. (f) Resin flowing from the bases of infected needles. (g) Lesions in cambium at the bases of infected needles. (h) Copious resin exudation on a stem associated with infected needles. (i) Cankers developing from the coalescence of lesions at the needle bases. (j) Naturally regenerated trees showing needle blight and tip death.
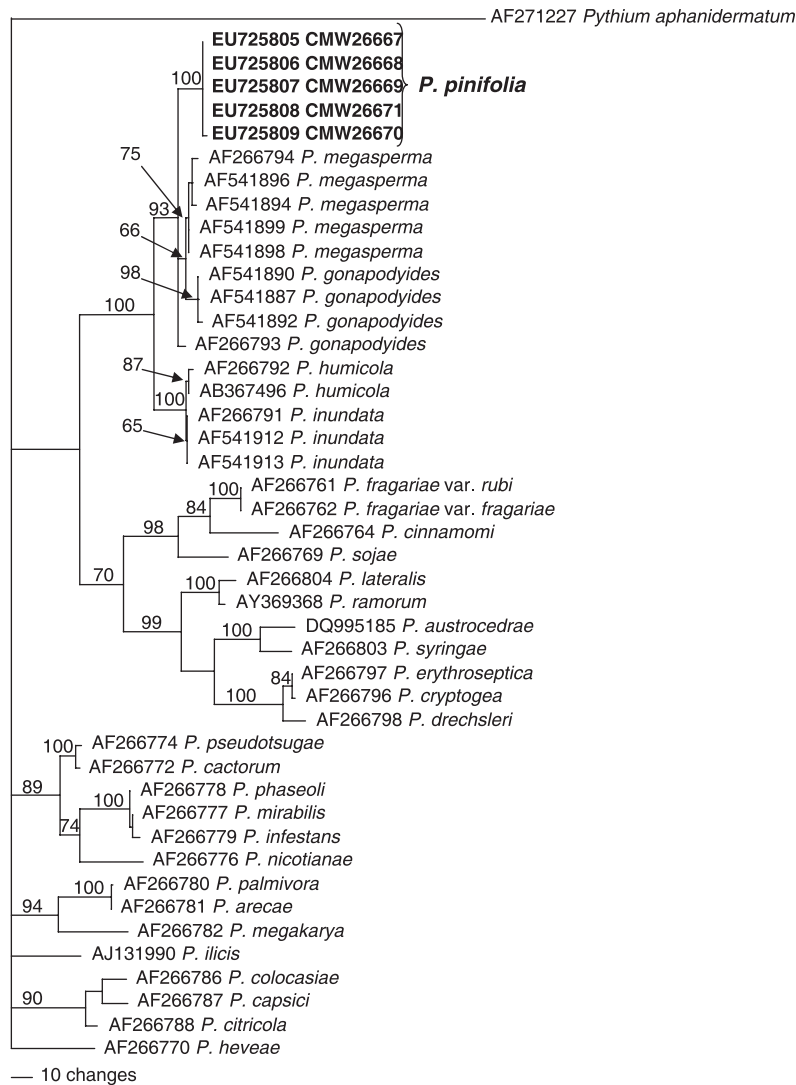
Figure 3 One of 10 most parsimonious trees having the same overall topology obtained through analysis of ITS rDNA sequence data, reflecting hypotheses of phylogenetic relationships for *Phytophthora pinifolia* and other *Phytophthora* species. The tree is rooted to *Pythium aphanidermatum*. Bootstrap support values (1000 replicates) are given at the branch nodes.

temperatures begin to drop to between 6 and 12°C. This is also the start of the rainy season and there appears to be a very close association of the disease with rainfall. A spatial analysis based on data from 2006 (data not shown), has shown that the southern slopes (higher humidity, most free water and low sun radiation) are most severely affected (Fig. 1c).

DNA sequence analyses

GenBank BLAST searches using ITS and *cox* II sequences for the *Phytophthora* isolates from *P. radiata* in Chile all indicated that the organism is unequivocally a species of *Phytophthora*, with the highest similarity to sequences obtained for isolates of *P. megasperma* and *P. gonapodyides*. These searches included sequences of undescribed lineages in Clade 6 defined by Brasier *et al.* (2003b). The ITS dataset contained a total of 935 characters after alignment,

492 of which were constant, 187 variable and parsimony uninformative, and 256 were variable and parsimony informative. Heuristic searches using the TBR algorithm found 10 most parsimonious trees (g1 = −0·406866, tree length = 996 steps; consistency index (CI) = 0·603; retention index (RI) = 0·806; rescaled consistency index (RC) = 0·486). These trees differed only in re-arrangements within the major clades and not between them, and consequently one was chosen for presentation (Fig. 3). The clades identified using these analyses were identical to those revealed by neighbour-joining analysis (data not shown).

The *cox* II dataset had a total of 568 characters after alignment, of which 403 were constant, 46 were variable and parsimony uninformative, and 119 were variable and parsimony informative. Parsimony analysis resulted in 4 most parsimonious trees (Fig. 4) with 494 steps (g1 = −1·149137; CI = 0·496, RI = 0·677, RC = 0·336).
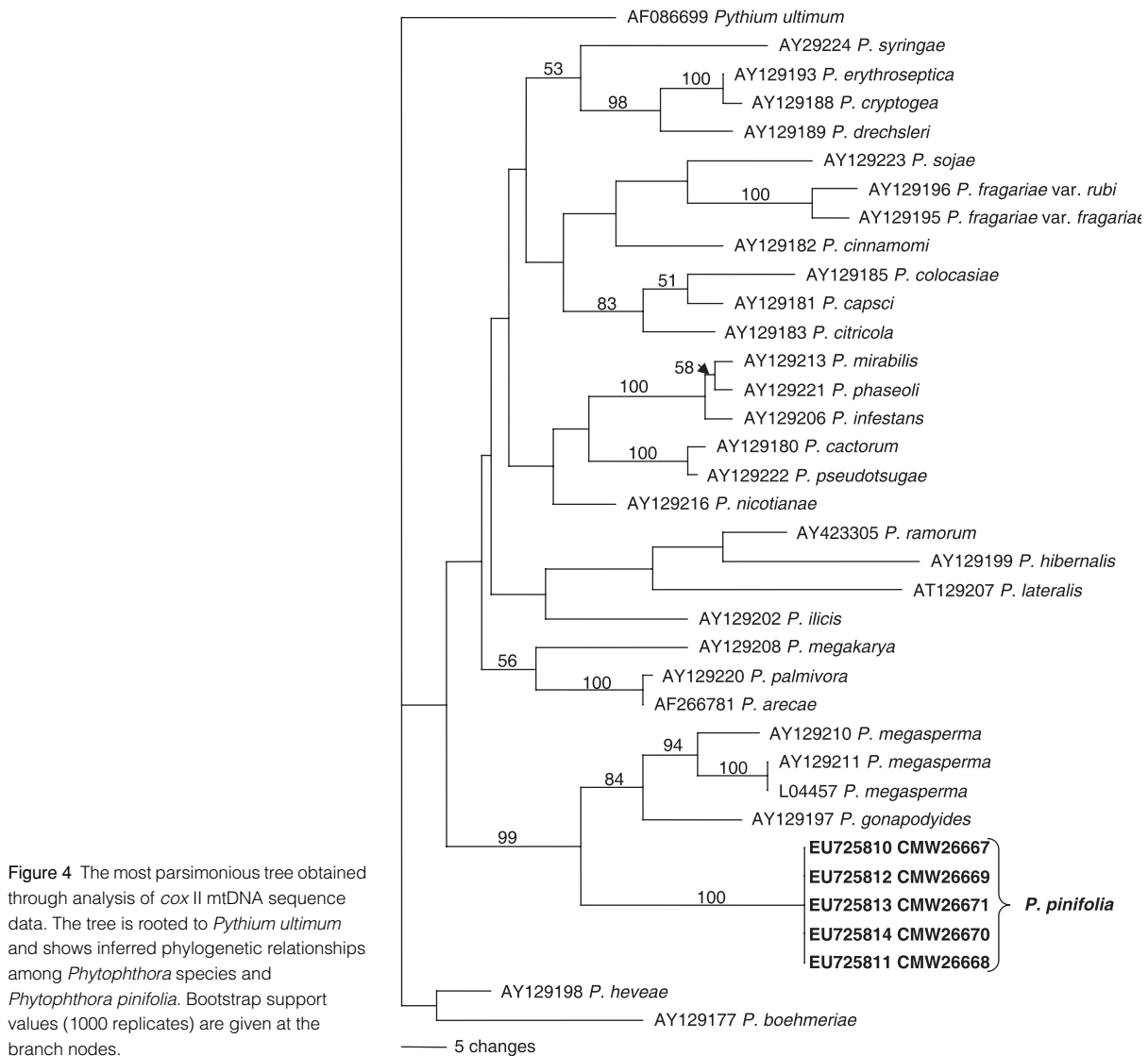
Figure 4 The most parsimonious tree obtained through analysis of *cox* II mtDNA sequence data. The tree is rooted to *Pythium ultimum* and shows inferred phylogenetic relationships among *Phytophthora* species and *Phytophthora pinifolia*. Bootstrap support values (1000 replicates) are given at the branch nodes.

The clades identified by parsimony analysis were identical to those revealed by neighbour-joining analysis (data not shown).

Both the analyses of the ITS and *cox* II datasets revealed that the isolates from *P. radiata* in Chile resided in a well-resolved clade (100% bootstrap support) distinct from all other *Phytophthora* spp. (Figs 3–4). In both trees, the Chilean isolates grouped in Clade 6 of the *Phytophthora* phylogeny described by Cooke *et al.* (2000), with *P. megasperma* and *P. gonapodyides* its closest relatives. In the ITS tree, the isolates from *P. radiata* in Chile grouped in a sub-clade with *P. megasperma* and *P. gonapodyides* (93% bootstrap support) separate from *P. humicola* and *P. inundata* (100% bootstrap support). In the *cox* II tree, the isolates from *P. radiata* in Chile form a distinct clade (99% bootstrap support) with *P. megasperma* and *P. gonapodyides*, separate from all other *Phytophthora* spp. currently available in GenBank.

Morphology

Cultures on CMA-NARP (Fig. 5a–b) were submerged and had coenocytic hyphae typical of *Phytophthora* spp. with a coralloid morphology (Fig. 6a–b), while CA and V8 cultures were fluffy with a regular margin to rosaceous or petallate margin (Fig. 5c–f). Sporangia formed abundantly in soil water, but more rarely in sporangium-inducing solutions, such as Petri Solution, and they were generally absent in pure culture. Sporangia were borne on predominantly unbranched sporangiophores and were non-papillate, sub-globose to ovoid (Fig. 6c–d), releasing the zoospores (Fig. 6i–j) or occasionally germinating directly with apical elongations (Fig. 6e–f). Free sporangia with pedicels were occasionally observed in the medium immersed in soil water and were usually released after the liquid medium was stirred (Fig. 6g–h). Despite regular observation of plates, including those where isolates of the
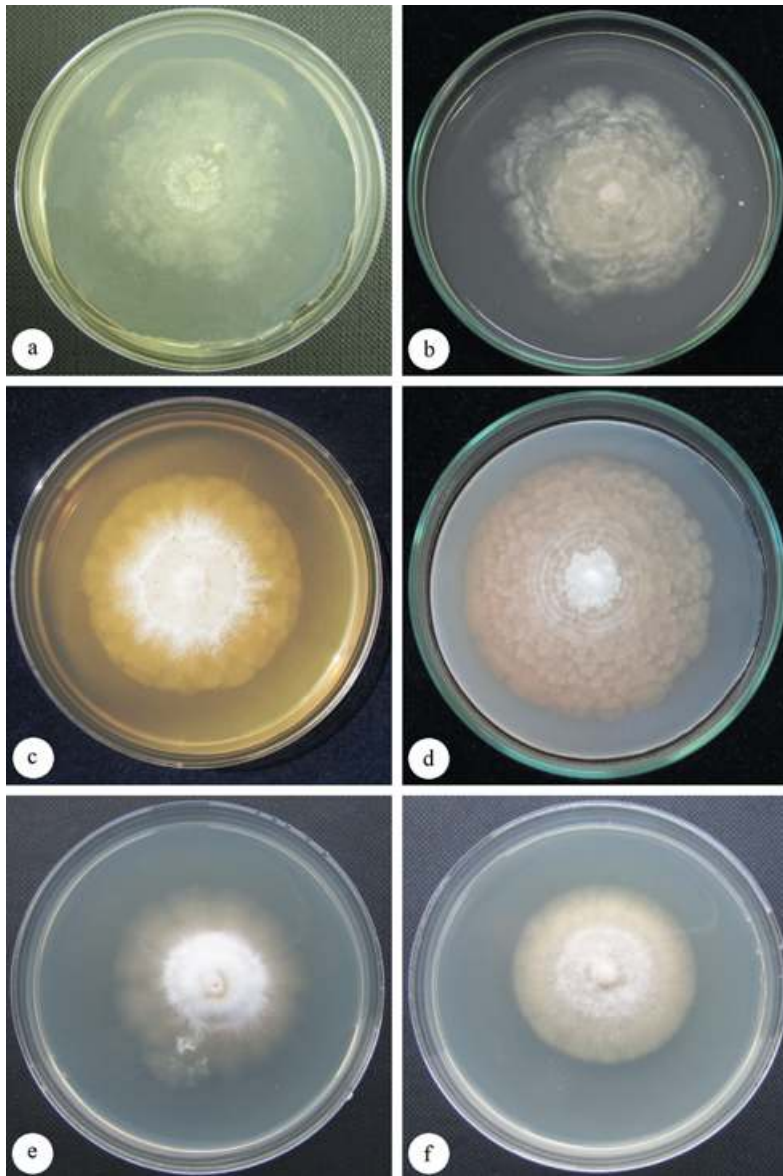
Figure 5 Colony morphology of *Phytophthora pinifolia* at 20°C on CMA-NARP (a–b) carrot agar (c–d) and V8 Juice agar (e–f) after six (left column) and three (right column) weeks of growth.

Phytophthora sp. were paired with each other in all possible combinations, sexual structures of the oomycete where not observed.

Taxonomy

The *Phytophthora* sp. isolated from the bases of newly infected needles, the resinous bands on needles and infected phloem tissue below infected needles of *P. radiata* in Chile, resides in Group 6 of the phylogeny-based classification of Cooke *et al.* (2000). Other species in this group include *P. gonapodyides*, *P. megasperma*, *P. humicola* and *P. inundata*. These species are all ecologically and morphologically different to the *Phytophthora* sp. from Chile (Table 1) despite their phylogenetic affinity. For

example, they are all soil fungi whereas the Chilean *Phytophthora* sp. has an aerial habit, with occasionally caducous sporangia that do not proliferate internally or externally unlike those found in *P. gonapodyides*, *P. megasperma*, *P. inundata* and *P. humicola*. DNA sequence comparisons, the ecology and the morphology of the *Phytophthora* sp. from diseased *P. radiata* needles in Chile thus provide convincing evidence that it represents a new taxon. The following description is provided for it.

Phytophthora pinifolia A. Durán, Gryzenh. & M.J. Wingf., sp. nov., MB 511870, Figs 5–6. Etymology: 'pinifolia' refers to the occurrence of the organism on the needles of *Pinus radiata*.

Coloniae in CA et V8 albae mycelio laxo, margine regulari, rosoide vel petalloide, appressae vel in medio
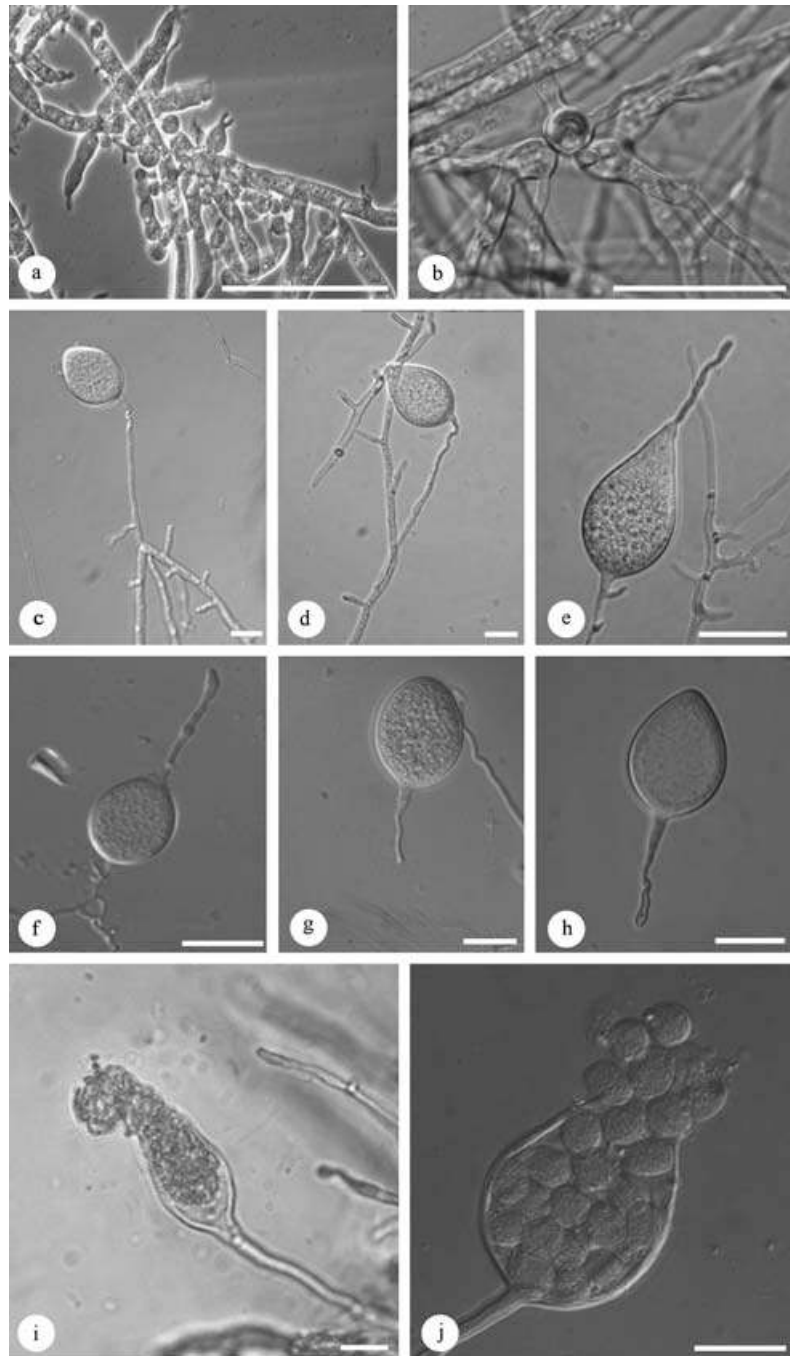
Figure 6 Morphology of *Phytophthora pinifolia*. (a) Coenocytic hyphae. (b) Spherical hyphal swelling with radiating hyphae. (c–h) Different shapes of non-papillate sporangia, (c–d) unbranched sporangiophores, sporangia with direct germination (e–f) and non-papillate free sporangia with pedicles (g–h). (i–j) Sporangia releasing zoospores. Scale bars = 20 μm.

immersae; crescunt optime in 25°C (minime 10°C, maxime 30°C), diametro maxime 45–55 mm post 4 hebdomades; in CMA–NARP mycelio immerso pariete valde irregulari. *Hyphae* coralloideae tumoribus paucis singulis 4–8 μm diametro, interdum cup hyphis radiatis. *Sporangiophorae* 28–44 μm, mediocriter 36 μm longae, simplices. *Sporangia* terminalia, semiglobosa vel ovoidea, non papillata, 39–61 × 27–45 μm, mediocriter 48 × 35 μm, aliquando proxime elongatione apicali poro exitus

15 μm lato germinantia, aliquando liber pedicellis 14–32 μm, mediocriter 23 μm longis.

Colonies on CA and V8 white with fluffy aerial mycelium, with a regular border or a rosaceous to petallate pattern, appressed or submerged in the medium, optimal temperature for growth 25°C (min 10°C; max 30°C), plates never completely covered but reaching maximum diam (45–55 mm) on CA in 4 weeks. On CMA-NARP, mycelium submerged with a very irregular border. *Hyphae*

Table 1 Comparison of ecological and morphological characteristics of *Phytophthora pinifolia* and other *Phytophthora* species in Clade 6

Characteristics	*Phytophthora pinifolia*	*Phytophthora gonapodyides*[a]	*Phytophthora humicola*[a]	*Phytophthora inundata*[a]	*Phytophthora megasperma*[a]
Habitat	Aerial parts of *Pinus radiata*	Soil, root	Soil	Soil, root, river water or from pond debris	Soil, root
Occurring on *Pinus*	Yes	No	No	No	No
Sporangial proliferation	None	Internal or internal nested or external proliferation	Mostly internal proliferation	Internal proliferation	Internal proliferation
Sporangium	Non-papillate	Non-papillate	Non-papillate	Non-papillate	Non-papillate
Hyphal swellings	Yes	No	Yes	No	Yes
Hyphal swelling morphology	Spherical, radiating hyphae	n/a[b]	Spherical, radiating hyphae	n/a	Rounded or angular, in chains or clusters
Sexuality	unknown	Heterothallic	Homothallic	Partially heterothallic	Mostly homothallic

[a]From Erwin & Ribeiro (1996), Brasier *et al.* (2003a).
[b]Not assessed.

coralloid with unusual single spherical swellings, sometimes with radiating hyphae, swellings 4–8 μm diam. *Sporangiophores* 28–44 μm long (avg. 36 μm), simple. *Sporangia* produced abundantly in soil extract water and rare in culture; in soil extract water mostly occurring within agar plugs; terminal, semi-globose to ovoid, non-papillate, 39–61 × 27–45 μm (avg. 48 × 35 μm), occasionally germinating directly with apical elongation or releasing zoospores directly, exit pore 15 μm wide, occasionally free with pedicels 14–32 μm (avg. 23 μm) long.

Specimens examined: CHILE. Arauco province, Arauco, Llico plantation, *Pinus radiata*, July 2007, M.J. Wingfield (**holotype** PREM 59887, ex–holotype isolate CMW 26668/CBS 122924/WPC P16100; paratypes PREM 59888, PREM 58889; living cultures CMW 26667/CBS 122923/WPC P16101, CMW 26669/CBS 122922/WPC P16102).

Distribution – Arauco province, Chile.

Pathogenicity

Distinct lesions developed on *P. radiata* shoots 15 days after inoculation with *P. pinifolia* isolates. In contrast, no lesions developed on shoots inoculated as negative controls. The average lengths of the lesions for the treated shoots ranged from 15 mm (CMW 26669) to 35 mm (CMW 26671) (Fig. 7). Inoculated shoots wilted and the needles turned brown, in a manner very similar to symptoms on infected shoots observed in nature (Fig. 8a–b). *Phytophthora pinifolia* was consistently re-isolated from the lesions at various positions across the necrotic area, and the identity of the isolates was confirmed using DNA sequence comparisons.

Discussion

This study considered a serious new needle blight disease of *P. radiata* in Chile and led to the discovery of a *Phytophthora* sp. consistently isolated from affected needles, needle bases and stems of young trees. Furthermore, DNA
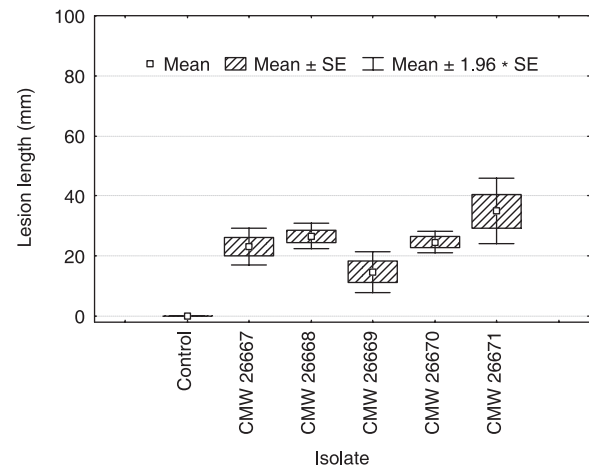
Figure 7 Histogram showing average lesion lengths for *Pinus radiata* plants 15 days after inoculation with *Phytophthora pinifolia* or a sterile agar control.

sequence comparisons and morphological observations provided convincing evidence to show that the oomycete represents an undescribed species of *Phytophthora*, which was provided with the name *P. pinifolia*. Pathogenicity tests with a suite of isolates also showed that *P. pinifolia* is able to rapidly kill inoculated shoots, leading to symptoms similar to those seen on naturally infected trees in Chile.

The habitat of *P. pinifolia* is unusual in that it is the only species of this genus known to infect green shoots and needles of a *Pinus* sp. Results of this study, based on a consistent association with infected needles, as well as pathogenicity tests, have provided convincing evidence that it is the primary cause of the needle disease and ultimately death of young trees. All evidence, including consistent isolation from the needles with symptoms, suggests that *P. pinifolia* is also the cause of the needle cast on older *P. radiata* trees. It has, however, not been possible
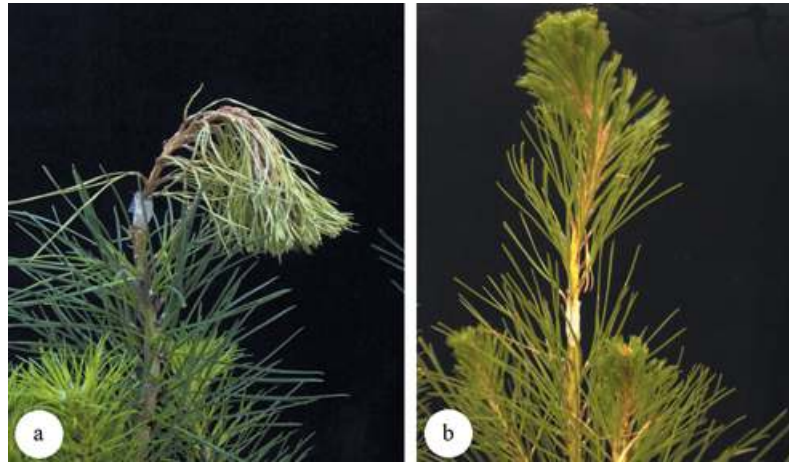
Figure 8 Symptoms on *Pinus radiata* plants 15 days after inoculation (a) with *Phytophthora pinifolia* or control (b).

to induce disease symptoms without wounding and a technique to test pathogenicity under natural conditions must still be developed.

While various *Phytophthora* spp. have been isolated from *Pinus* spp. (Erwin & Ribeiro, 1996; Sánchez *et al.*, 2002; Chavarriaga *et al.*, 2007), only *P. cinammomi* (Ali *et al.*, 1999), *P. citricola* (Sandlin *et al.*, 1992), *P. cryptogea* (Bumbieris, 1976) and *P. drechsleri* (Heather & Pratt, 1975) have been reported to be associated with *P. radiata*. All are soil–borne pathogens and they do not infect pine shoots and needles. The disease of *P. radiata* in Chile is clearly a new pine disease and it should not easily be confused with other *Phytophthora* diseases or indeed, any other pine needle disease.

The discovery of an aerial *Phytophthora* sp. causing a serious disease of *P. radiata* in Chile, adds to a number of new and serious tree-infecting aerial *Phytophthora* spp. that have recently been discovered. The most dramatic of these has been *P. ramorum* which was described from Germany and the Netherlands in 2001 (Werres *et al.*, 2001). This pathogen was discovered in the USA in 2001 (Rizzo *et al.*, 2002) where it has caused death of woody plants and particularity of tanoak (*Lithocarpus densiflorus*) and coast live oak (*Quercus agrifolia*) (Rizzo *et al.*, 2005). Another example is the more recently discovered *P. kernoviae*, which infects *Fagus sylvatica* and *Rhododendron ponticum* in England (Brasier *et al.*, 2005; Brown & Brasier, 2007). These newly discovered aerial *Phytophthora* spp. are apparently emerging as seriously threatening tree pathogens in various parts of the world (Denman *et al.*, 2006) and the discovery of *P. pinifolia* contributes to this view.

The closest relatives of *P. pinifolia* based on DNA sequence data are *P. megasperma*, *P. gonapodyides*, *P. humicola* and *P. inundata*. These species all reside in Clade 6 in the phylogenetic classification of *Phytophthora* spp. (Cooke *et al.*, 2000). Members of this clade mostly occur in forest or riparian ecosystems and cultures are mostly sexually sterile or inbreeding (Brasier *et al.*, 2003b; Kroon *et al.*, 2004). These species are unlikely to be confused with *P. pinifolia* because they are all soil–borne

pathogens (Ko & Ann, 1985; Hansen & Maxwell, 1991; Brasier *et al.*, 1993; Brasier *et al.*, 2003b) and all have nested or extended sporangium proliferation, in this regard very different to *P. pinifolia* (Table 1). It is consequently easy to discriminate between *P. pinifolia* and other species of *Phytophthora* even in the absence of oogonia, simply on the basis of habit and morphology of sporangia.

The discovery of a *Phythophthora* species consistently associated with symptoms of DFP in Chile, fits the aetiology of the disease. For example, DFP appears in close association with low temperatures and wet conditions. A particularly obvious symptom is that needles on the lower sides of branches die first and this is an area where free water typically accumulates. The disease is also more obvious on the southern hill slopes than on northern slopes, and thus in areas that are cool and moist. These conditions are all conducive to the formation of sporangia and the spread and development of the disease (Erwin & Ribeiro, 1996).

While the symptoms of DFP in Chile are consistent with the biology of an aerial *Phytophthora* sp., there are many questions regarding the biology of the pathogen that remain to be answered. While it is assumed that the sporangia are the infective propagules, this has yet to be shown experimentally. These structures were not abundant in culture and more natural conditions under which to produce them will need to be developed. Likewise, infection studies with zoospores and the infection biology and life cycle of the pathogen remain to be understood.

The association of a new species of *Phytophthora* with a serious pine needle blight disease in Chile raises the important question as to where it might have originated. One hypothesis regarding the origin of *P. pinifolia* would be that it is native and reproducing sexually on other hosts in Chile. In this regard, it would have undergone a 'host-jump' to *P. radiata*, a phenomenon quite common in tree pathogens (Slippers *et al.*, 2005). The absence of sexual structures in cultures of *P. pinifolia* could then be explained by the absence of conditions necessary for sexual reproduction in the laboratory. An alternative

hypothesis is that the oomycete is represented by a single mating type in Chile, and it is thus not able to undergo sexual recombination. This could imply that *P. pinifolia* has been introduced into Chile, as was the case with *P. infestans* that was accidentally introduced into Europe as a single mating type (Goodwin, 1997).

The origin of *P. pinifolia* on a native plant in Chile seems unlikely because there are no native pines in Chile and a host jump to *P. radiata* would imply a pathogen with a broad host range. The fact that only *P. radiata* is infected and that *P. pinaster* and *Pseudotsuga menziessii*, growing in close proximity, show no symptoms suggests that it has a limited host range and is, restricted to one or only a small number of pine species. Studies are continuing to consider the sexuality in *P. pinifolia*, to investigate its population biology and diversity, and to search for potential native hosts and origin of *P. pinifolia* in Chile.

The pine needle and shoot blight in Chile known as DFP has emerged and spread rapidly to cover an area of approximately 60 000 ha in only three years. It is currently the most important problem affecting *P. radiata* plantations in Chile and it seriously threatens the local forestry industry and consequently the economy of the country. Opportunities exist to select other *Pinus* spp. for plantation development and screening of species suited to the area is currently underway. There is early evidence to suggest that different genotypes of *P. radiata* differ in susceptibility to infection and this offers opportunities to reduce the impact for DFP. Alternatively, there are also possibilities for chemical control of the disease, for example through the application of fungicides.

Acknowledgements

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