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Phytophthora austrocedrae sp. nov., a new species associated with *Austrocedrus chilensis* mortality in Patagonia (Argentina)

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ABSTRACT

Phytophthora austrocedrae is a new species isolated from necrotic lesions of stem and roots of *Austrocedrus chilensis*. It is a homothallic species characterized by semipapillate sporangia, oogonia with amphigynous antheridia, and very slow growth (1–2 mm d^{-1} on V-8 agar at 17.5 °C optimum temperature). Phylogenetic analysis of ITS rDNA sequence indicates that its closest relative is *Phytophthora syringae*, another species frequently isolated from soil and streams in *A. chilensis* forests.

Introduction

The mortality of *Austrocedrus chilensis* in Patagonia—'Mal del Ciprés'—has been studied for many years but its causes remain unclear. Several studies of biotic and abiotic factors have tried to elucidate the origin and causes of this disease, but in spite of the amount of information gathered, a satisfactory aetiology has not emerged. For a detailed description of the disease and background see Greslebin *et al.* (2005).

Pythiaceous fungi (*Pythiaceae*, *Oomycetes*) have been suggested by several authors as a possible causal agent (Filip & Rosso 1999; Hansen 2000; Havrylenko *et al.* 1989; Hennon & Rajchenberg 2000; La Manna & Rajchenberg 2004; Rajchenberg *et al.* 1998). For this reason, a survey of phytophthoras of *Austrocedrus* forests was conducted. Five *Phytophthora* species were detected inhabiting soil of declining *A. chilensis* forests but none of them showed a clear relationship with the decline (Greslebin *et al.* 2005).

In further work, necrotic inner bark lesions at the root collar and lower stem, even though rarely reported in previous studies, were frequently detected on affected trees. These lesions originated in the roots and looked very similar to those caused by

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other *Phytophthora* taxa, such as *P. lateralis* on *Chamaecyparis lawsoniana* in the western United States (Hansen 2000). Initial isolation attempts from the *Austrocedrus* lesions were negative, but DNA extraction from the necrotic tissues and PCR with Phytophthora-specific primers showed the presence of a *Phytophthora* species (Sutton, unpublished data). After some changes in the isolation procedures, the new *Phytophthora* species was finally isolated from the lesions. This paper describes this new species.

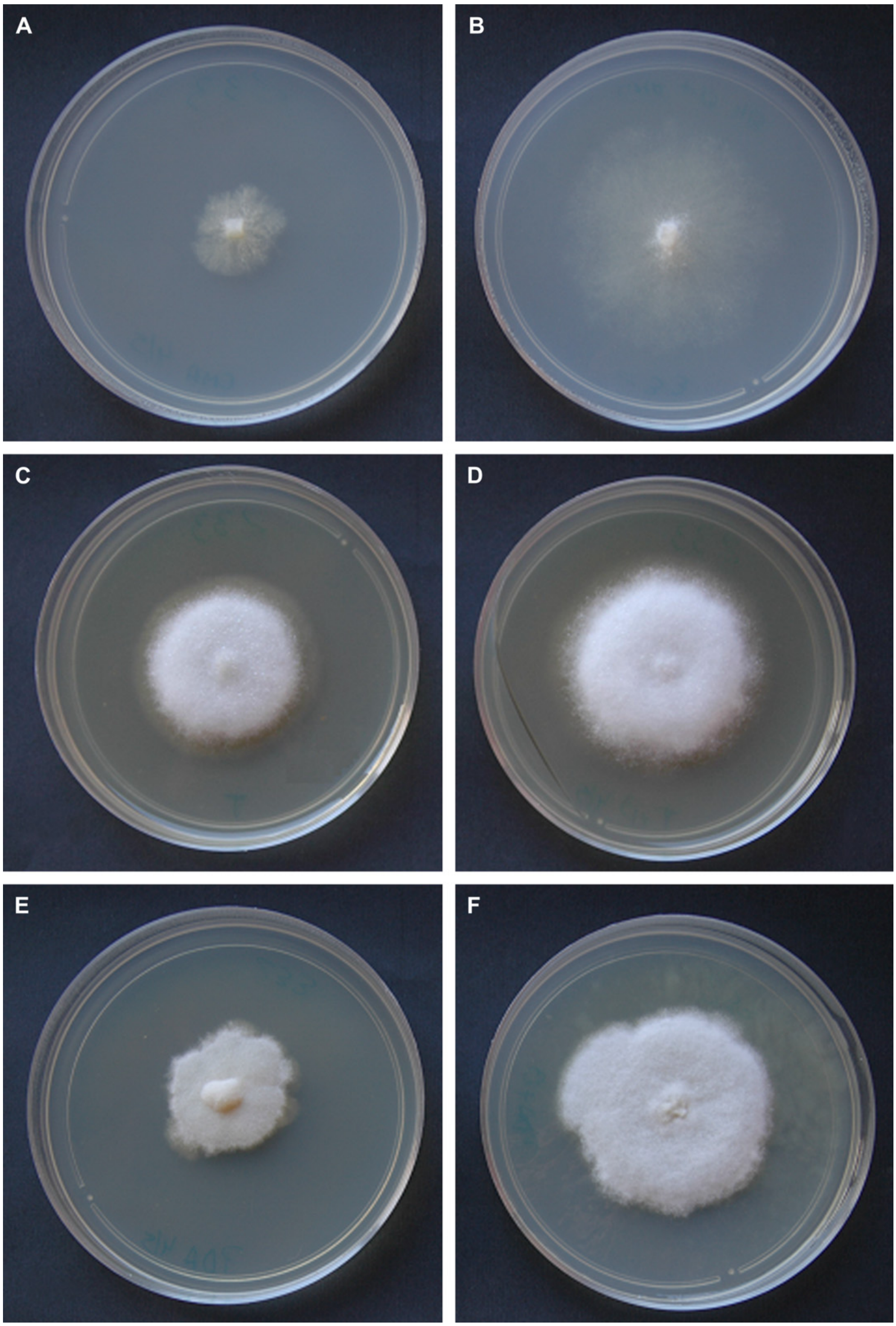

Fig 1 – Colony morphology of *Phytophthora austrocedrae* at 16 °C after four weeks on: (A) CMA. (B) CMAβ. (C) TA. (D) TAβ. (E) PDA. (F) PDAβ.

Materials and methods

Isolation methods

All isolates were gathered from the advancing necrotic zone of phloem lesions located in the lower stem, root collar, or the main roots of symptomatic trees. Isolations were made in the field by direct plating of necrotic tissues into selective media. Samples of necrotic tissues were also removed from the trees and brought to the laboratory where they were washed with running tap water for 24–48 h, and a second isolation was attempted. Five media were compared. Selective media used in both field and laboratory isolations included: corn meal agar (CMA, Sigma, St Louis, MO) amended with: (1) PARNBP (10 mg l^{-1} pimaricin, 200 mg l^{-1} ampicillin, 10 mg l^{-1} rifampicin, 50 mg l^{-1} nystatin, 15 mg l^{-1} benomyl, 50 mg l^{-1} PCNB); (2) PAR (10 mg l^{-1} pimaricin, 200 mg l^{-1} ampicillin, 10 mg l^{-1} rifampicin); (3) NAR (25 mg l^{-1} nystatin, 200 mg l^{-1} ampicillin, 10 mg l^{-1} rifampicin); or (4) BARP (10 mg l^{-1} benomyl, 200 mg l^{-1} ampicillin, 10 mg l^{-1} rifampicin, 50 mg l^{-1} PCNB). In laboratory isolations, unamended CMA was also used. Antibiotics were dissolved in ethanol (up to 0.5 %) and added to the liquid media after autoclaving. Isolation plates were incubated at 16 °C in the dark. The low incubation low temperature was chosen because of the negative results obtained when previous isolates were grown at 22 °C. Isolates were transferred to clarified V8-juice agar (V8A) (Erwin & Ribeiro 1996) or tomato juice agar (TA) and stored at 16 °C in the dark. Clarified TA was prepared as clarified V8A, but using tomato juice (La Campagnola) instead of V8 juice.

Morphology and physiology

To describe colony pattern and mycelium morphology, isolates were grown at 16 °C in the dark on: V8A, TA, TA amended with β-sitosterol (30 mg l^{-1}), Sigma CMA, Sigma CMA amended with β-sitosterol (30 mg l^{-1}), potato dextrose agar (PDA; made with fresh potatoes) and PDA amended with β-sitosterol (30 mg l^{-1}). Colony morphology was recorded after 28 d. Mycelial growth rates of eight isolates at temperatures from 10–25 °C were measured on clarified V8 agar. The colony radii were measured at 2-d intervals for 8 d and the average radial increment per day was calculated. The test was conducted in triplicate.

Sporangia, oogonia and antheridia were studied on TA and CMA amended with β-sitosterol (30 mg l^{-1}). Sporangia were obtained by transferring agar discs (5 mm diam) cut from the growing edge of 14 d-old cultures into tomato broth culture (100 ml clarified tomato juice La Campagnola in 400 ml distilled water) for 4 d. Blocks were then carefully rinsed ten times with distilled water and flooded with river water or soil extract water. Measurements in the Latin diagnosis are from the holotype.

Isolates studied

Measurements of sexual and asexual organs were obtained from ten strains isolated from necrotic lesions at root collar in two different geographical areas (Fig 8): (1) Argentina, Chubut, Río Grande Valley (isolates 190, 191, 193, 194 and 195); and (2) Argentina, Chubut, Los Alerces National Park (isolates 203, 206, 213, 215, 223 and 225) (Table 2).

DNA extraction

A 5 mm plug with mycelium was removed from colonies growing on CMA and genomic DNA was extracted as described in Winton & Hansen (2001). PCR was performed in 50 μl reactions [1 × Buffer, 200 nM dNTP, 0.4 μM DC6 (Cooke et al. 2000) and ITS4 primers (White et al. 1990), 0.05 U μl^{-1} RedTaq DNA polymerase (Sigma), 0.5 μl 5 % blocking powder (Schleicher & Schuell, Keene, NH, 03431) and 2 μl template DNA]. Reaction conditions were: 60 s at 94 °C, 34 cycles of 60 s at 94 °C, 60 s at 55 °C and 60 s at 72 °C, and a final incubation for 7 min at 72 °C. After amplification, PCR products were separated on a 1.5 % agarose gel to evaluate concentration and quality.

Alternatively, DNA was extracted from mycelial homogenates on FTA cards (Whatman International, Florham Park, NJ). A 5 mm plug with mycelium was taken from colonies growing on CMA, placed in 100 μl 1 × phosphate-buffered saline (PBS) and homogenized. Twenty-five microlitres of homogenate were applied to a Whatman FTA card and stored at room temperature until processing. A 2 mm disk was taken from the dried card, placed in a 0.65 ml PCR tube, rinsed three times with FTA purification reagent (Whatman) then rinsed twice with TE buffer (5 ml 0.5 M Tris–HCI pH8, 5 ml 0.5 M EDTA pH8, 495 ml dd water). PCR was performed in 25 μl reactions (1 × buffer, 100 nM dNTP, 0.4 μM DC6 (Cooke et al. 2000) and ITS4 primers (White et al. 1990), 0.05 U μl^{-1} RedTaq DNA polymerase (Sigma, St Louis, MO), 0.2 μl 5 % blocking powder (Schleicher & Schuell) and FTA disk). PCR reaction conditions were as above.

ITS sequence analysis

PCR products were prepared for DNA sequencing by addition of 1 μl EXOSAP-IT (USB, Cleveland OH) and incubation overnight at 17 °C followed by 15 min at 80 °C. Direct sequencing of PCR products (BigDye Terminator version 3.1 Cycle Sequencing Kit, Applied Biosystems, Foster City, CA) was performed with primers DC6 (Cooke et al.. 2000) and ITS2, ITS3 and ITS4 (White et al. 1990) and run on an ABI 3730 capillary

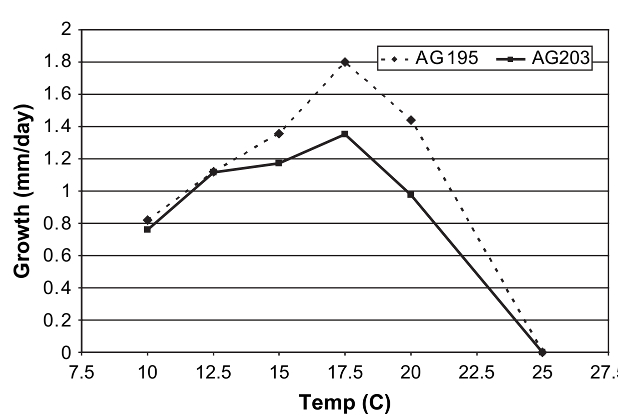

Fig 2 – Radial growth rate of two isolates of *P. austrocedrae* on V8A at different temperatures. Mean of three trials.

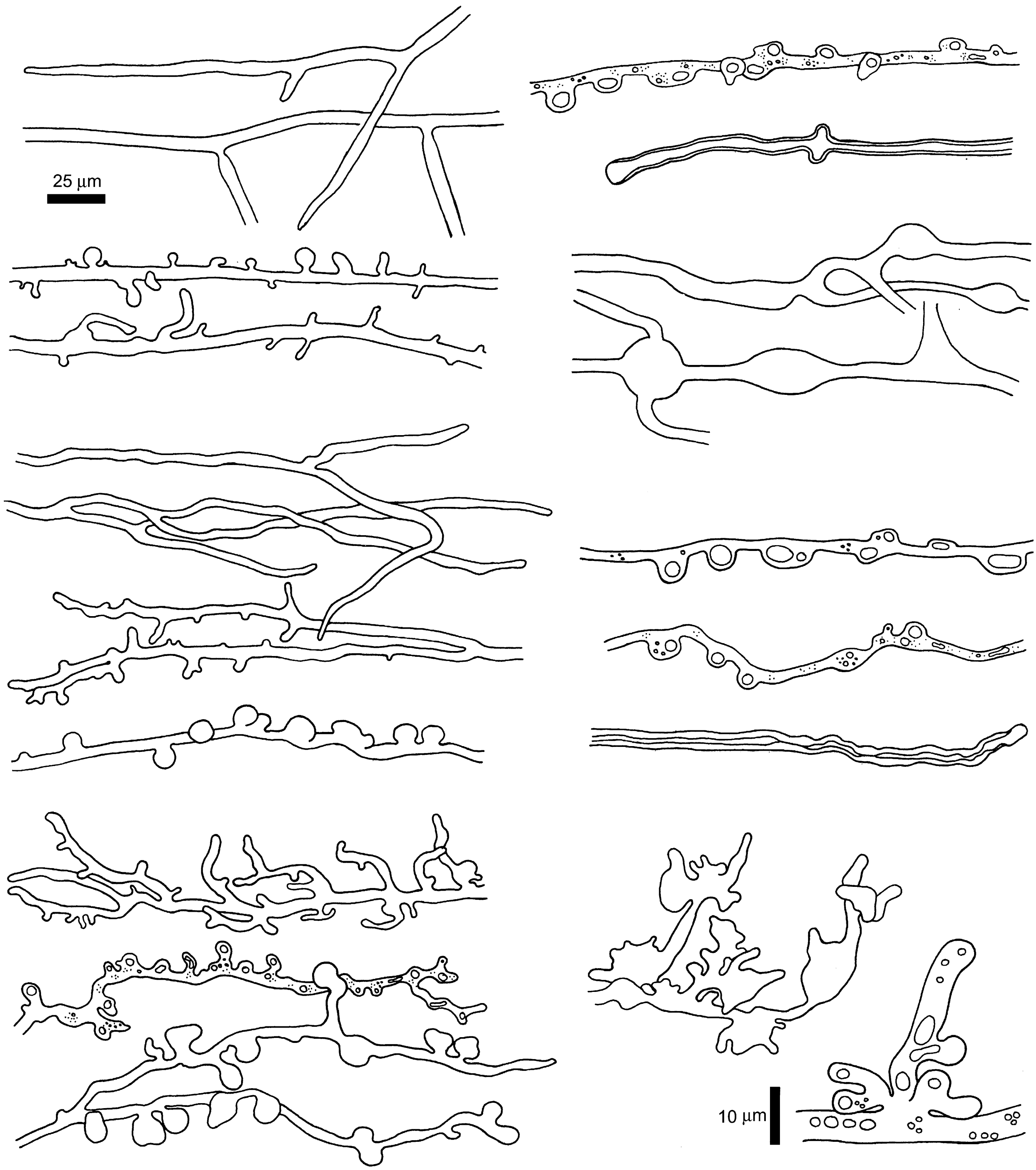

Fig 3 – Morphology of hyphae of *Phytophthora austrocedrae*. (A–E) In TAβ: (A–B) Hyphae from the margins of the colony. (C–E) Hyphae from older areas of the colony. (F–J) In PDAβ. (F–H) Hyphae from the margins of the colony. (I–J) Hyphae from older areas of the colony. (K–N) In CMA. (K–L) Hyphae from the margins of the colony. (M) Hyphae from older areas of the colony. Bar.: 25 μm (N) Detail of branches originated from the same point on the main hyphae. Bar.: 10 μm.

sequence machine (Applied Biosystems). Contigs were assembled and edited with the Staden software package (1996).

Edited sequences (Table 2) were compared with *Phytophthora* sequences available in GenBank with the BLASTN search utility (Altschul *et al.* 1997). Sequences were aligned to the data set of Cooke *et al.* (2000) excluding clades 9 and 10 with the addition of sequences from more recently described species. The multiple alignment program ClustalX (Thompson *et al.* 1997) was employed. Distance-based phylogenetic analysis was performed in ClustalX with NJ tree-building options. Support for tree stability was obtained from 1K BS replicates. The phylogram was drawn using TreeView (Win32)1.6.6 (Page 1996).

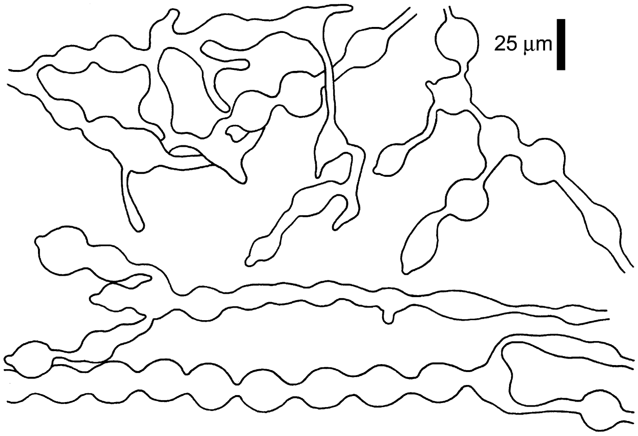

Fig 4 – *Phytophthora austrocedrae*. Hyphal swellings on liquid media. Bar.: 25 μm.

Taxonomy

Phytophthora austrocedrae Gresl. & E. M. Hansen, **sp. nov.** (Figs 1, 3–6)

MycoBank no.: MB 510267

Etym.: austrocedrae refers to the host *Austrocedrus chilensis*.

Coloniae lente crescentes, incrementum radiatum optime a 17.5 °C in agaris "V8 juice (V8A)" 1–1.8 mm d^{-1}. Nulla incrementum a 25 °C. Coloniae uniformia, sine ordinatione proprio. Hyphae hyalinae, non septatae, maturitate septatae, (3–)4–8 μm diametros. Inflationis hypharum globosus, subglobosus vel asymetricus, catenatus, paucae in agaris solidis, sed abundantes in cultura liquida. Sporangiophora simplicia. Sporangia terminalia, persistentia, semipapillata ellipsoidea, ovoidea vel obpyriformia, in medio 58 × 39 μm (34–75 × 28–51 μm), ratio longitudinis ad altitudinem in medio in medio 1.5 (1.2–1.8), prolificatio nulla. Oogonia praesens in cultura singularis, in medio 39 diametros (24–51 μm); tunica leve, tenuis vel leviter incrassata, hyalina vel infuscatus. Oosporae globosae, in medio 32 μm. diametros (17–44 μm), pleroticae vel apleroticae; tunica leve, incrassata 1–2 μm, hyalina. Antheridia amphigyna, in medio 15 × 14 μm (12–27 × 8–20 μm).

Typus: **Argentina**: *Chubut*: Parque Nacional Los Alerces, ad radice et caule *Austrocedrus chilensis*, Oct. 2005, *A. Greslebin* (CIEFAP 203—holotypus; ATCC MYA-4074).

Colony in V8A, TA and TAβ was uniform, without growth pattern, cottony, dome-shaped in the centre and appressed or mostly submerged at the margins (Fig 1). In CMA and CMAβ the colony was appressed, with little or no aerial mycelium; the submerged mycelia showed an arachnoid pattern. In PDA the colony was uniform, without growth pattern, densely felty to woolly, with abundant and dense aerial mycelium and defined margins. *Growth*: Growth was very slow and favoured by cool temperatures. Optimum temperature was 17.5 °C, with no growth at 25 °C (Fig 2). Maximum radial growth rate of eight isolates on V-8 agar ranged from 1–1.8 mm d^{-1}. Growth rate on CMA and PDA was affected by β-sitosterol, being faster when β-sitosterol was added to the media. *Hyphae* were (3–)4–8 μm in diam. Morphology of hyphae varied according to the growth medium. This variation seemed to be related to the amount of sitosterol in the media. In V8A, TA, TAβ, PDA and PDAβ hyphae of the margin of the colony were mostly straight (or nearly so), with sparse, long branches

Table 1 – Morphological characteristics of *Phytophthora austrocedrae* isolates

Strain	n	Sporangia			n	Oogonia		Oospore		Antheridia	
		Mean	Range	L:B ratio		Mean	Range	Mean	Range	Mean	Range
190	20	40.5 ± 8.1 × 30.6 ± 5	27–51 × 22–39	1.3 ± 0.17	20	42 ± 8	22–56	345 ± 7	17–49	17.5 ± 2.5 × 13.5 ± 1.5	12–22 × 12–17
191	20	45.3 ± 7 × 31.8 ± 5.4	37–54 × 22–41	1.4 ± 0.13	20	36.8 ± 8.4	22–56	26 ± 6	20–46	18 ± 3.5 × 15 ± 2.5	12–24 × 12–22
193	20	37.7 ± 9.3 × 29.6 ± 7.6	22–51 × 17–41	1.3 ± 0.15	20	32.8 ± 8.7	24–51	26 ± 7.5	17–44	17 ± 2.5 × 13.5 ± 1.5	12–20 × 10–17
194	20	48.3 ± 9.9 × 36.8 ± 6.9	32–61 × 27–46	1.3 ± 0.1	22	42.5 ± 7	27–54	33.5 ± 6.5	22–46	18.5 ± 2 × 14.5 ± 1.5	15–22 × 12–17
195	42	59.9 ± 11 × 42.6 ± 6	30–83 × 21–58	1.4 ± 0.14	77	37.5 ± 7.8	24–50	32 ± 6	22–45	20.5 ± 4.5 × 15 ± 2.5	10–30 × 10–20
203	53	58.5 ± 9.7 × 39 ± 5	34–75 × 28–51	1.5 ± 0.2	65	38 ± 6	24–51	32 ± 5.5	17–44	15.5 ± 3.5 × 14.5 ± 2.5	12–27 × 8–20
206	–	–	–	–	21	41.2 ± 9.3	22–56	32.5 ± 7.5	19–44	17.5 ± 2.5 × 14 ± 1.5	12–23 × 12–17
213	20	45.1 ± 9.9 × 32.9 ± 5.6	27–66 × 20–39	1.4 ± 0.13	–	–	–	–	–	–	–
215	20	44.9 ± 8.3 × 34 ± 7.3	27–61 × 17–49	1.3 ± 0.11	20	39 ± 6.5	27–54	32 ± 4.5	22–39	15.5 ± 3 × 13.5 ± 2	10–20 × 10–17
223	21	49.5 ± 9.1 × 36.2 ± 4.8	41–78 × 32–51	1.3 ± 0.11	22	36.5 ± 4.5	32–46	28.5 ± 3	24–32	17.5 ± 3 × 13.5 ± 1.5	15–22 × 12–17
225	20	46 ± 10.1 × 35.9 ± 6.3	34–68 × 27–46	1.3 ± 0.16	20	38.5 ± 5.4	29–49	38.5 ± 5.5	24–39	15.5 ± 1.5 × 13.5 ± 2	12–20 × 10–17

at nearly right angles (Fig 3A, F). Behind the margins, hyphae usually produced short, digitiform branches (Fig 3B, G). Hyphae of the central area of the colony were mostly irregular in diam. with lateral swellings (Fig 3C, H–I); these swellings were also observed, though less frequently, in hyphae at the colony margin. In unamended CMA, hyphae of the advancing zone looked tortuous, sometimes with lateral swellings (Fig 3K–L). They were ramified at more or less right angles, with each long branch with many very short, digitiform branches. In some cases branches originated from the same point on the main hyphae and were radiate (Fig 3N). The tortuous aspect of the hyphae and the short, digitiform branches were not present in hyphae from the advancing zone on CMA media when it was amended with β-sitosterol (30 μg l^{-1}). Hyphae with thickened walls were observed, though infrequently, on T, TAβ and PDAβ after four weeks (Fig 3D, J). *Hyphal swellings* usually formed in solid (Fig 3E) and liquid (Fig 4) media, but were more abundant in the former. Swellings were globose to subglobose and catennulated, sometimes with distorted shapes. *Sporangiophores* were mostly simple, 3–11 μm diam, frequently with hyphal swellings. *Sporangia* were borne terminally on mostly unbranched sporangiophores. They were ovoid, obpiriform, limoniform or ellipsoid; semi-papillate, papilla 1–3(–5) μm thick, non-papillate sporangia were infrequently observed. They measured in average 50 ± 12 × 36 ± 7 μm (range 22–83 × 15–58 μm) length:breadth ratio average 1.4 ± 0.2 (range 1.1–2) and infrequently had distorted shapes (Table 1). Sporangia with hyphal projections and lateral attachment of the sporangiophore were frequently observed in all isolates (Fig 5). The abundance of sporangia in water culture (soil extract or river water) was variable. Sporangia were not observed in solid media. *Oogonia* formed in single-strain culture in PAR, NAR, V8A, TA, CMA, CMAβ, PDAβ (Fig 6). Oogonia were usually formed in selective media after about 20 d. They usually formed more quickly and were more abundant on selective media than on media without antibiotics. Oogonia were globose or nearly so, on average 38.5 ± 7 × 39 ± 6 μm diam (range 22–56 μm), with hyaline to

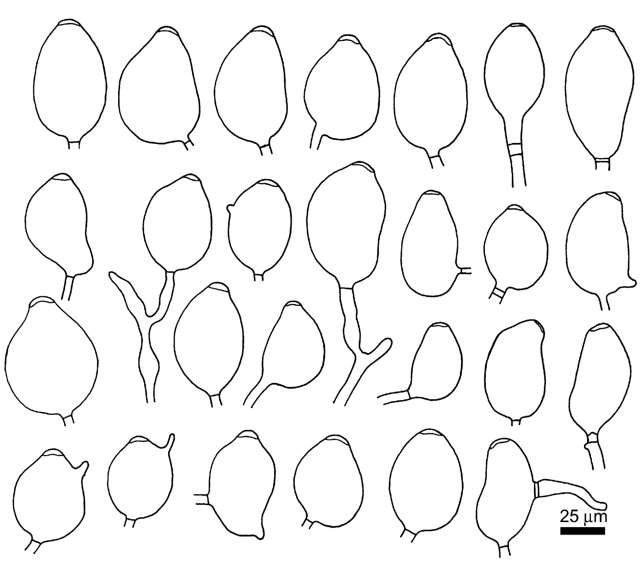

Fig 5 – *Phytophthora austrocedrae*. Morphology of sporangia. Bar.: 25 μm.

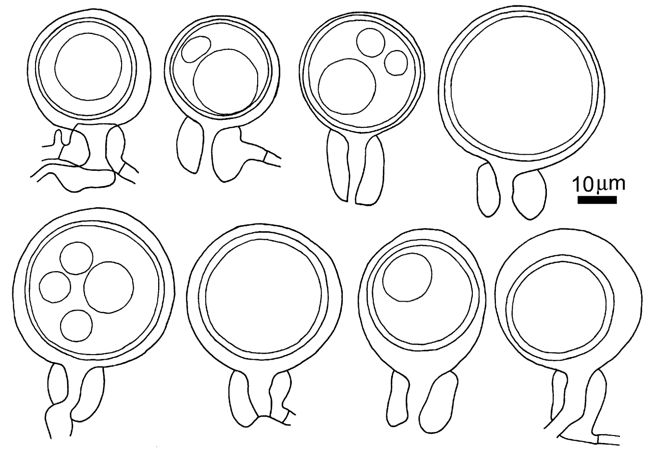

Fig 6 – *Phytophthora austrocedrae*. Morphology of oogonia, oospores and antheridia. Bar.: 10 μm.

light brown, smooth walls. *Oospores* were globose, in average 31 ± 6 μm diam (range 17–48 μm), hyaline, with smooth walls 1–2(–3) μm thick. *Antheridia* were amphigynous, hyaline, one-celled, in average 18 ± 3.5 × 14 ± 2 μm (range 10–30 × 8–20 μm; Table 1).

Phylogeny: Primers DC6 and ITS 4 amplified 1194 base pairs of ITS DNA. The ITS sequence of the two isolates examined was identical to sequences of *Phytophthora* DNA extracted from bark of five diseased trees. A double peak (A and G) was present at bp 1077 in all sequences. *P. austrocedrae* is in clade 8 of the Cooke *et al.* (2000) molecular phylogeny of the genus (Fig 7). Phylogenetic analysis of the ITS rDNA sequences identified *P. syringae* (GenBank AF266803 and AY787034) as the closest relative. *P. austrocedrae* differed from *P. syringae* at 32 of the 816 (4 %) *P. syringae* bases available for alignment.

Remarks: This new species is characterized by the combination of a very slow growth rate, semipapillate, non-caducous and non-proliferating sporangia, oogonia with amphigynous antheridia formed in single culture, and low (17.5 °C) optimal temperature for growth. In addition, the morphology of mycelia is often characteristic and may be useful for the identification of the species in combination with the other characters.

It can be distinguished from its closest relative *Phytophthora syringae* by colony pattern and antheridia. *P. syringae* has a petaloid colony pattern in V8A, TA and PDA, and oogonia with paragynous antheridia that, in isolates from Patagonia, are usually formed only in media amended with oil and at temperatures below 12 °C. Sporangia and hyphal swellings are very similar in both species, but although direct germination of sporangia is common in *P. syringae,* it was not observed in *P. austrocedrae*. Cardinal and optimal temperatures of growth of *P. syringae* are similar to those of. *P. austrocedrae*. Frezzi (1950) reported an optimal temperature of 15–18 °C and a maximum of 27–28 °C for Argentinean isolates of *P. syringae* and Erwin & Ribeiro (1996) reported a minimum <5 °C, optimum 15–20 °C, maximum 23–25 °C. Nonetheless, growth rate of *P. syringae* is more than twice that of *P. austrocedrae*.

With semi-papillate sporangia and amphigynous antheridia this species belongs to group IV of the morphological classification system (Waterhouse 1963; Stamps *et al.* 1990; Erwin

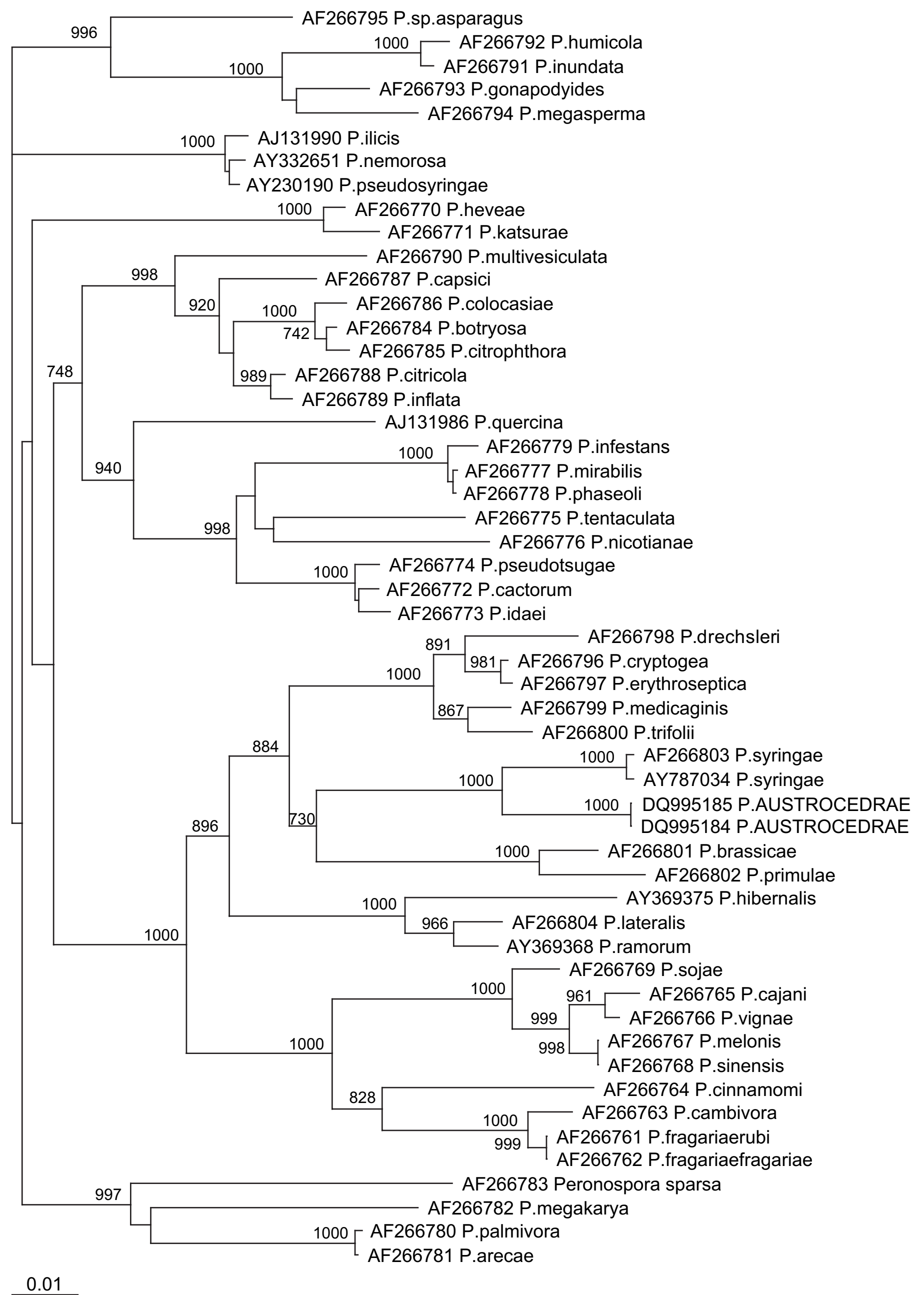

Fig 7 – Phylogeny of *Phytophthora austrocedrae* based on ITS rDNA sequence analysis, including clades 1–8 from Cooke *et al.* (2000). BS values for nodes with greater than 70 % support (1K iterations) are shown.

& Ribeiro 1996). Although most species in group IV have caducous sporangia, caducity has not been observed in this species. It is homothallic, readily distinguishing it from the heterothallic species in group IV (*P. colocasiae*, *P. infestans*, and *P. mirabilis*) and from *P. macrochlamydospora*. Non-caducous sporangia, hyphal swellings readily formed in water and solid culture and slow growth rate at optimal temperature differentiate this species from homothallic group IV species like *P. hibernalis*, *P. ilicis*, *P. phaseoli* and *P. psychrophila*. While there are similarities between *P. austrocedrae* and *P. psychrophila* (i.e. colony pattern, sporangia, sex organs, cardinal and optimal temperatures for growth) there are clear differences in their ITS rDNA sequences. *P. psychrophila* belongs to clade 3, whereas *P. austrocedrae* belongs to clade 8 of the Cooke *et al.* (2000) molecular phylogeny of the genus. In culture they can by differentiated by growth rate (*P. psychrophila* 4.2 mm d^{-1} in V8A) (Jung *et al.* 2002) and by lack of hyphal swellings in *P. psychrophila*.

Isolation from diseased trees was successful when plates were incubated at 17 °C, and unsuccessful at temperatures of 20 °C or above. *P. austrocedrae* isolation frequency was higher on PAR and NAR than BARP and PARNBP. CMA was as successful as PAR and NAR for laboratory cultures. No differences were detected between isolations made in the field by direct plating of necrotic tissues into selective media and those made in the laboratory after washing of tissues with

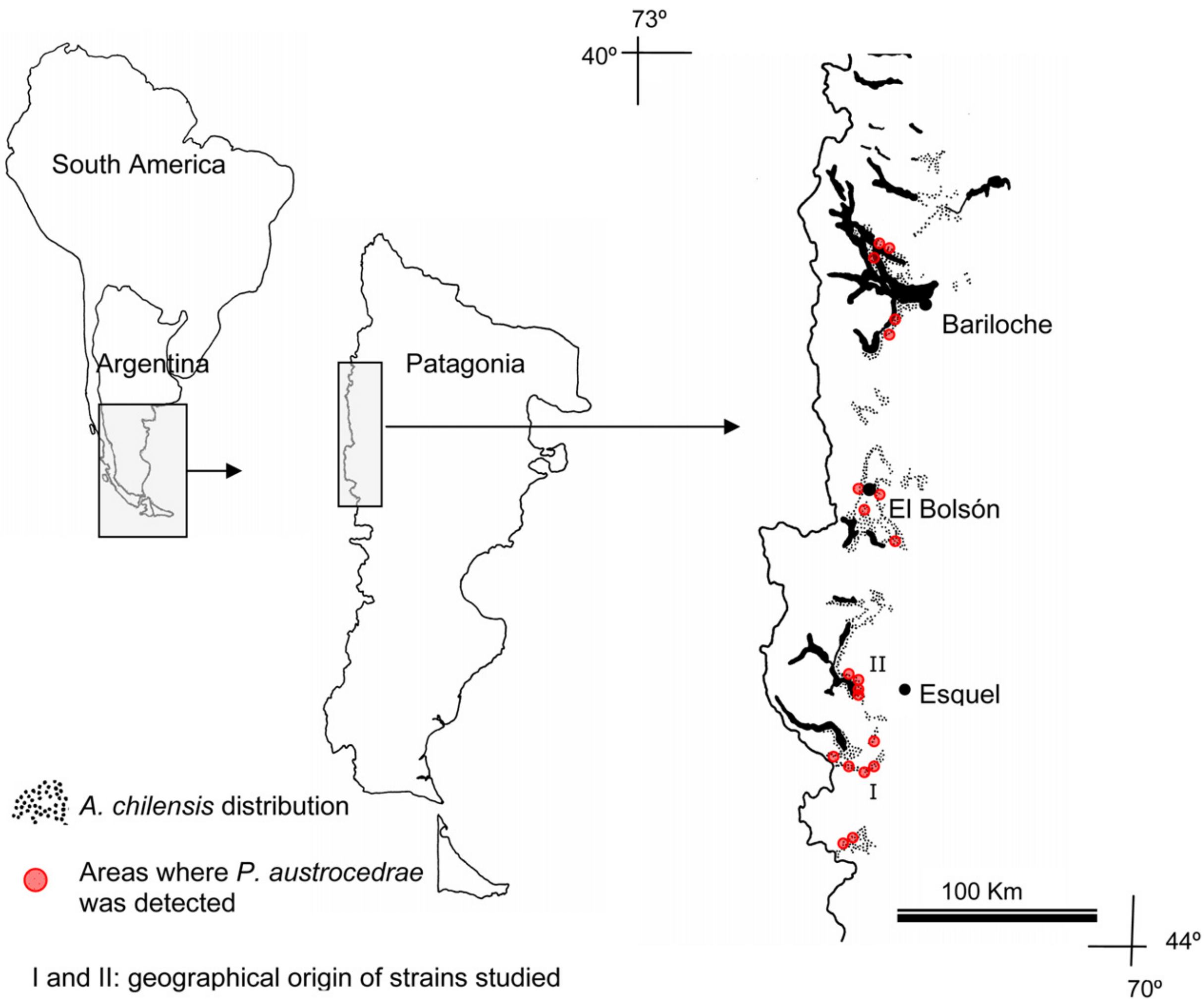

Fig 8 – Areas where *Pythophthora austrocedrae* has been detected to date and geographical origin of isolates studied.

running tap water for 24–48 h showing that phenolyc and other water-soluble compounds did not inhibit mycelial growth. In all media, first growth from bark pieces was not visible until 10 d or more after plating.

Discussion

Phytophthora austrocedrae is associated with the mortality of *Austrocedrus chilensis* known as 'mal del ciprés'. Evidence gathered to date suggests that it is the primary cause of the disease. *P. austrocedrae* was detected in the advancing zone of necrotic lesions in the inner bark of roots, root collar and stems of symptomatic trees in Isla Victoria (Nahuel Huapi National Park) the place where ''mal del ciprés'' was first reported, and in most of the areas that have been reported as affected by ''mal del ciprés'' (Fig 8). Pathogenicity tests to fulfill Koch's postulates are underway and will be reported separately.

There were two sites where *P. austrocedrae* was not detected in dying *A. chilensis,* but in these places symptomatology was

Table 2 – Isolates sequenced

Species	Deposited at[a]	Geographical location, year of isolation	Isolated from	GenBank accession number
Phytophthora austrocedrae strain 203 (holotype)	ATCC N° MYA-4074	Argentina, Chubut, Los Alerces National Park, 2005	Necrotic tissues	DQ995184
P. austrocedrae strain 195	ATCC N° MYA-4073	Argentina, Chubut, Río Grande Valley, 2005	Necrotic tissues	DQ995184

a Duplicates at Department of Botany and Plant Pathology, OSU and Area Protección Forestal, CIEFAP.

different (i.e. dead or dying crowns with healthy tissues at root collar) from the symptomatology described for 'mal del ciprés' (i.e. disease originated in the root system and death of roots and collar tissues preceding defoliation of the crown). A geographical review of the areas affected by 'mal del ciprés' is necessary to discriminate the different situations of mortality and better characterize the associated symptomatology.

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