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Evidence of low levels of genetic diversity for the *Phytophthora austrocedrae* population in Patagonia, Argentina

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Phytophthora austrocedrae is a recently discovered pathogen that causes severe mortality of *Austrocedrus chilensis* in Patagonia. The high level of susceptibility of the host tree, together with the distribution pattern of the pathogen, have led to the hypothesis that *P. austrocedrae* was introduced into Argentina. The aim of this study was to assess the population structure of *P. austrocedrae* isolates from Argentina in order to gain an understanding of the origin and spread of the pathogen. Genetic diversity was determined based on amplified fragment length polymorphisms (AFLPs). In total, 48 isolates of *P. austrocedrae* were obtained from infected *A. chilensis* trees, representing the geographical range of the host. Four primer combinations were used for the AFLP analysis. Of the 332 scored bands, 12% were polymorphic. Gene diversity (*h*) ranged from 0·01 to 0·03; the Shannon index (*I*) ranged from 0·01 to 0·04. A high degree of genetic similarity was observed among the isolates (pairwise *S* values = 0·958–1; 0·993 ± 0·009, mean ± SD). A frequency histogram showed that most of the isolate pairs were identical. Principal coordinate analysis using three-dimensional plots did not group any of the isolates based on their geographical origin. The low genetic diversity (within and between sites) and absence of population structure linked to geographic origin, together with the aggressiveness of the pathogen and the disease progression pattern, suggest that *P. austrocedrae* might have been introduced into Argentina.

Keywords: AFLP analysis, *Austrocedrus chilensis*, *Austrocedrus* root disease, biological invasions, forest phytophthoras, 'mal del ciprés'

Introduction

Austrocedrus chilensis (ciprés de la cordillera, cypress) is an endemic tree in the Cupressaceae, found in southern Argentina and Chile. It is the most widely distributed species of the small number of conifers found in southern Argentina. *Austrocedrus chilensis* is found across 140 000 ha, in a wide variety of ecological niches, and in different soil types. It grows between 36° 30′ and 43° 35′S on the eastern, and between 32° 39′ and 44° S on the western, slopes of the Andes (Veblen *et al.*, 1995). In Argentina, the tree grows in a 60–80 km-wide strip along the Andean foothills, across a broad moisture gradient (170 cm rainfall year^{-1} in the west to 50 cm year^{-1} in the east). In the west, *A. chilensis* can be found either in mixed stands with *Nothofagus* spp. or in pure stands on drier sites. In the north, *A. chilensis* trees grow in forests mixed with *Araucaria araucana*. They also grow in open, xeric forests or in isolated clumps at the limit of the Andean forest and the Patagonian steppe, acting as a barrier against the advancing desert. *Austrocedrus chilensis* is important not only because of its ecological functions but because of the high quality of its wood and its aesthetic value.

High levels of *A. chilensis* mortality were first detected in 1948 on Victoria Island (Nahuel Huapi National Park) in Neuquén Province. The disease was later detected during 1953 in a stand located near a forest nursery *c.* 150 km from Victoria Island, in Chubut Province. Since then, mortality has extended to almost all of the range of *A. chilensis* in Argentina, where the disease is referred to as 'mal del ciprés' (Greslebin & Hansen, 2010).

Phytophthora austrocedrae (Pythiales, Peronosporomycetes, Straminipila) was recently described as a new species and is believed to be the primary cause of *A. chilensis* mortality in Patagonia (Greslebin & Hansen, 2010). The disease is generally referred to as Austrocedrus Root Disease (ARD). *Phytophthora austrocedrae* is considered to be the main component in the complex pathology that leads to 'mal del ciprés' (Greslebin *et al.*, 2007; Greslebin & Hansen, 2010). The pathogen resides in clade 8 of the *Phytophthora* phylogenetic tree of Cooke *et al.* (2000). Phylogenetic analysis of the nuclear and mitochondrial genes has shown that *Phytophthora*

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syringae and *Phytophthora obscura* are the closest relatives of *P. austrocedrae* (Greslebin *et al.*, 2007; Robideau *et al.*, 2011; Grünwald *et al.*, 2012). Among these species, *P. austrocedrae* is homothallic and is characterized by a combination of very slow growth, semipapillate, non-caducous and non-proliferating sporangia, oogonia with amphigynous antheridia, and low (17·5°C) optimal temperature for growth (Greslebin *et al.*, 2007).

Phytophthora austrocedrae affects *A. chilensis* trees of all ages and is present across the native growth range of the trees in Argentina (Greslebin & Hansen, 2010). Symptoms of ARD include chlorosis, a wilting of the foliage and root rot. Trees, and especially seedlings, can die rapidly, in which case the foliage changes from chlorotic to a red colour. Some infected trees die slowly, first becoming chlorotic followed by a progressive defoliation and eventual tree death after several years (Filip & Rosso, 1999). Studies have shown that the disease originates in the root systems, and is associated with poor drainage and high moisture levels in soils (Havrylenko *et al.*, 1989; Filip & Rosso, 1999; La Manna & Rajchenberg, 2004). Additional symptoms include necrotic lesions on the roots and stems that can affect the entire breadth of the phloem. The pathogen is able to colonize the xylem, which is evident by superficial discoloration of woody tissue and the presence of hyphae blocking rays and tracheids in xylem sections (Greslebin & Hansen, 2010; Vélez *et al.*, 2012).

A possible mechanism to explain the decline of *A. chilensis* caused by *P. austrocedrae* has been presented by Vélez *et al.* (2012). Inoculation studies on 2-year-old saplings with *P. austrocedrae* led to a progressive and significant reduction in net photosynthesis, stomatal conductance and stem-specific hydraulic conductivity. The substantial negative impact on plant physiology was mainly attributed to the extensive death of the bark and cambium tissue, resulting in a disruption of phloem transport. Additionally, blockage of xylem transport by hyphal colonization, presence of resinous plugs, and death of xylem ray parenchyma, leads to a loss of hydraulic conductivity. Involvement of effectors secreted by *P. austrocedrae*, or host-derived responses to the pathogen, have also been suggested (Vélez *et al.*, 2012).

The geographic origin of *P. austrocedrae* is unknown, although the impact of the pathogen suggests that it has been introduced into Argentina. Until recently, *P. austrocedrae* was not known from any region outside of Argentina, or on any other host than *A. chilensis*. However, in 2011, the pathogen was reported as the causal agent of root disease and mortality on *Chamaecyparis nootkatensis* in a public park in East Renfrewshire, UK (EPPO, 2011). This was followed by a report describing a damaging disease in rare native juniper bushes in the Upper Teesdale National Nature Reserve in the UK (Forestry Commission Great Britain, 2012). The source of *P. austrocedrae* could not be determined, but it was assumed that the pathogen had been introduced into these areas.

The presence of *P. austrocedrae* outside Argentina has raised concern regarding the origin of the pathogen. Because of its limited geographic range, and the high level of susceptibility of its primary host, it has been hypothesized that *P. austrocedrae* was introduced into Patagonia. However, the population genetic structure of the pathogen has not been established, precluding a more definitive view of this question. The aim of this study was, therefore, to assess the genetic diversity of *P. austrocedrae* in Argentina. The study was also undertaken to gain knowledge that would inform disease management strategies.

Materials and methods

Isolate selection and growth conditions

Isolates of *P. austrocedrae* were obtained from the culture collection of the Centro de Investigación y Extensión Forestal Andino Patagónico (CIEFAP). These isolates were collected during several surveys of declining *A. chilensis* stands in Patagonia, Argentina (Table 1). Sampling and isolation methods followed the protocols of Greslebin *et al.* (2007) and Greslebin & Hansen (2010). Forty-eight isolates from 19 stands were selected for analysis, reflecting the geographical range of *A. chilensis* mortality known at the time of the study, with a distance of *c.* 550 km between the two most distant locations (Fig. 1).

In order to verify the identity of the isolates collected, DNA sequence comparisons were made for a subset of the isolates using the internally transcribed spacer regions (ITS1 and ITS2) and the cytochrome oxidase I gene (*coxI*). These gene regions were amplified and sequenced for isolates 13-Phy-309, 15-Phy-271 and 16-Phy-270 (GenBank accession numbers ITS: JX121857, JX121856, JX121855; *coxI*: JX448319, JX448318, JX448317, respectively). DNA was extracted as outlined below. The ITS region was amplified using PCR with the primers ITS4 and ITS5 (White *et al.*, 1990); the *coxI* gene was amplified with primers FM 83 and FM 84 (Martin & Tooley, 2003). PCR reaction mixtures were the same for both regions. The mixture comprised dNTPs (0·25 mM each), $MgCl_2$ (2·5 mM), PCR buffer, 0·1 μM of each primer, DNA (50–100 ng) and *Taq* DNA polymerase produced at the Forestry and Agricultural Biotechnology Institute (2·5 U). The PCR conditions for both regions were identical and followed those described by Martin & Tooley (2003). PCR products were separated on a 1% agarose gel stained with GelRed and visualized under UV light. PCR products were purified using a MSB Spin PCRapace purification kit (Invitek). DNA sequencing reactions were done using a BigDye Terminator v. 3.1 cycle sequencing kit (ABI) and sequences were determined on an ABI 3100 DNA automated sequencer. The regions were sequenced in both directions with the primers used for PCR amplification. Contigs were assembled and edited with the STADEN software package (Staden, 1996). DNA sequences were compared to those available in GenBank using BLASTN.

Four isolates of *P. syringae* were included in this study for comparative purposes. These isolates were obtained from soil samples collected in forests located in different regions of Patagonia using soil baiting. The isolates were: syr-40, obtained in Corcovado (near site 15); syr-157, from Los Alerces National Park (near site 9); syr-259, from Lanin National Park (near site 3); and syr-319, from Futaleufú, Río Grande valley (near site 13; Fig. 1).

DNA extraction

Isolates were maintained on clarified tomato juice agar (TA) at 16°C in the dark (Greslebin *et al.*, 2007). To produce mycelium

Table 1 Origin of *Phytophthora austrocedrae* cultures used in this study

Site	Isolate	Province	Location	Description	Date of isolation
1	Phy-255	Neuquén	40°9′45·79″S	Lanin National Park, Cte. Díaz hill, San Martín de los Andes	May 2008
	Phy-263		71°20′44·88″ W		
2	Phy-308	Neuquén	40°29′58·4″ S	Lanin National Park, Filo-Huaum lake	May 2009
			71°21′6·39″W		
3	Phy-256	Neuquén	40°40′34·95″S	Lanin National Park, road from Confluencia to Traful	May 2008
	Phy-257		71°18′41·62″W		
	Phy-258				
4	Phy-298	Neuquén	40°39′48·024″S	Lanin National Park, various stands of *A. chilensis* in the surroundings of Traful	May 2009
	Phy-304		71°22′20·71″W		
	Phy-314				
	Phy-299		40°40′9·66″S		
			71°21′6·39″W		
	Phy-300		40°40′0·007″S		
	Phy-305		71°21′36·12″W		
	Phy-312				
5	Phy-290	Neuquén	40°58′37·03″S	Nahuel Huapi National Park, Puerto Totora and Bella Vista Hill, Victoria Island	Jan 2009
			71°30′59·02″W		
	Phy-292		40°57′41·26″S		
			71°31′52·25″W		
6	Phy-286	Río Negro	41°13′44·32″S	Nahuel Huapi National Park, Gutierrez Lake	Oct 2008
			71°25′11·12″W		
7	Phy-276	Chubut	42°0′26·98″S	Epuyén, Golondrinas, Reserva Forestal del INTA	Sep 2008
	Phy-278		71°32′11·27″W		May 2009
	Phy-279				
	Phy-281				
	Phy-318				
8	Phy-203	Chubut	42°46′29·97″S	Los Alerces National Park, Braese stream	Oct 2005
	Phy-205		71°32′11·27″W		Jan 2010
	Phy-209				
	Phy-211				
	Phy-213				
	Phy-215				
	Phy-338				
9	Phy-219	Chubut	42°48′26·8″S	Los Alerces National Park, Quebrada del León stream	Oct 2005
	Phy-221		71°38′58·9″W		
	Phy-223				
	Phy-225				
	Phy-232				
10	Phy-243	Chubut	42°53′5·21″S	Los Alerces National Park, Las Rocas camping area	Apr 2006
	Phy-244		71°35′52·58″W		
11	Phy-294	Chubut	43°9′55·8″S	Los Alerces National Park, seccional Río Grande	Nov 2008
			71°42′18·4″W		
12	Phy-234	Chubut	43°7′34·36″S	Trevelin, Aldea Escolar, Estación Experimental INTA	Jan 2006
	Phy-237		71°33′46·10″W		
13	Phy-191	Chubut	43°12′55·5″S	Futaleufú, Trevelin, Rio Grande Valley, 'La 106' property	Sep 2005
	Phy-195		71°32′50·9″W		Jun 2009
	Phy-201				
	Phy-309				
14	Phy-238	Chubut	43°11′39·7″S	Futaleufú, Trevelin, Río Grande valley, Nant y Fall falls	Jan 2006
	Phy-239		71°28′23·2″W		
15	Phy-271	Chubut	43°34′31·11″S	Corcovado, Momberg property	Aug 2008
	Phy-273		71°41′10·64″W		
16	Phy-267	Chubut	43°33′51·97″S	Corcovado, Underwood property	Aug 2008
	Phy-270		71°38′54·83″W		

for DNA extraction, tomato juice broth was inoculated with agar plugs cut from the actively growing margins of fresh cultures. After 30 days' growth at 16°C, the mycelium was harvested under sterile conditions, washed with sterile DNase/RNase-free water and centrifuged to discard the supernatant. Washed mycelium was transferred to Eppendorf tubes and DNA was extracted using the procedure described by Möller *et al.* (1992) with minor modifications. Mycelium was ground in

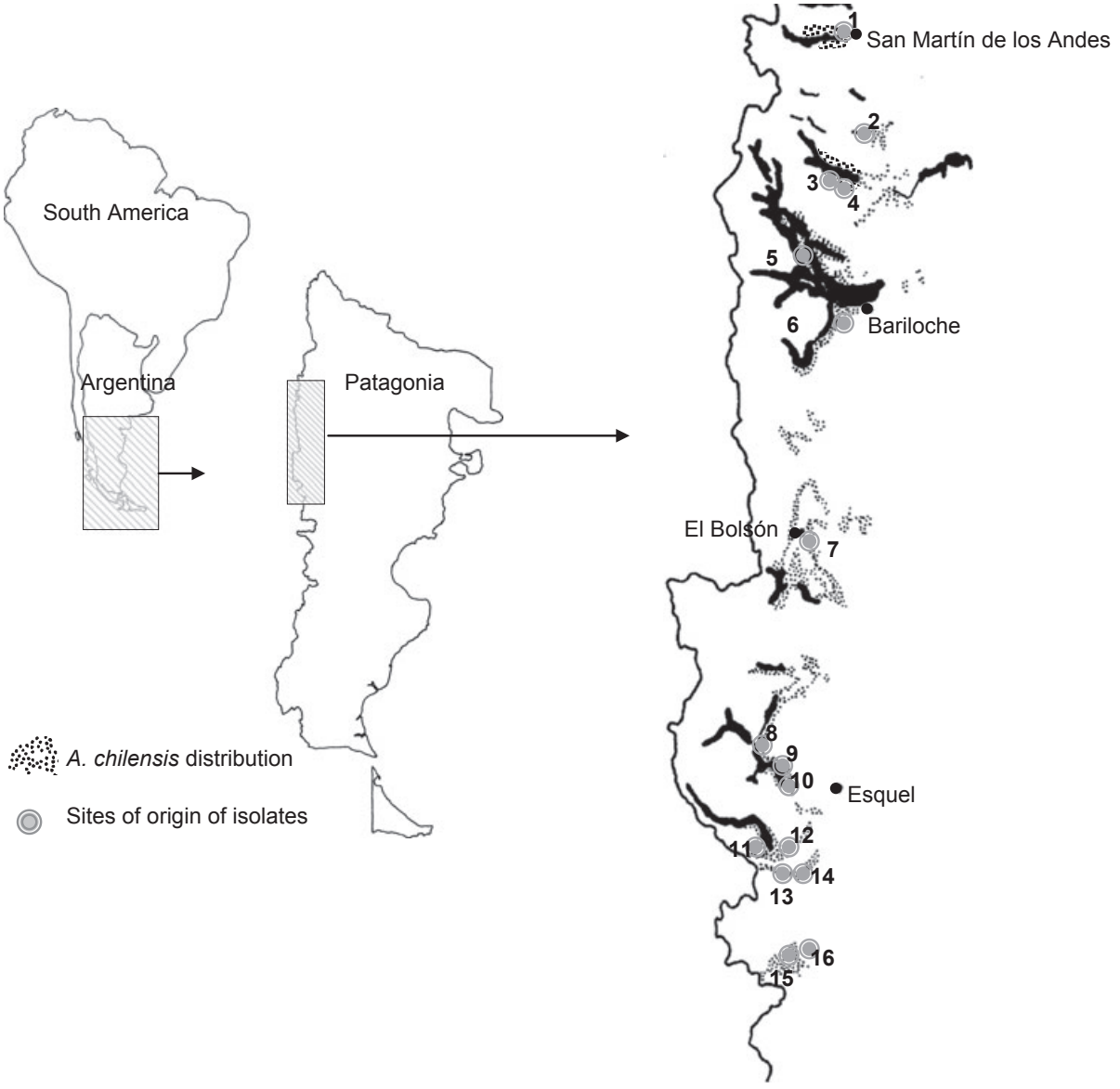

Figure 1 Map showing the origin of *Phytophthora austrocedrae* isolates from *Austrocedrus chilensis* in forests of Patagonia. For location details, see Table 1.

liquid nitrogen, then 600 μL extraction buffer (200 mM Tris–HCl pH 8, 150 mM NaCl, 25 mM EDTA, 50 μg proteinase K, 100 μg RNase A) was added. This mixture was homogenized at 4 m s^{-1} for 20 s using a Fastprep FP120 (QBIOgene) homogenizer. The mixture was frozen in liquid nitrogen and then incubated for 60 min at 60°C. After incubation, 140 μL 5 M NaCl and 65 μL 10% CTAB were added, vortexed and incubated at 60°C for 10 min. One volume of chloroform/isoamyl alcohol (24:1) was added to the mixture, mixed gently and incubated at 4°C for 30 min. Samples were centrifuged at 11 700 ***g*** for 20 min at 4°C, the supernatant was transferred to new Eppendorf tubes and 0·55 volumes of isopropanol were added, followed by centrifugation at 11 700 ***g*** at room temperature. The supernatant was discarded and the pellet washed with 70% cold ethanol, at 6200 ***g*** for 5 min at 4°C. This step was repeated, the pellet dried and dissolved in 50 μL DNase/RNase-free water. Successful DNA extraction was confirmed by electrophoresis (1% agarose gel stained with GelRed and visualized under UV light). The DNA quality and concentration was determined using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies Inc.). The final concentration was adjusted to 20 ng μL^{-1}.

Amplified fragment length polymorphism (AFLP) analysis

Of the 48 *P. austrocedrae* isolates, five were duplicated in the AFLP analysis to serve as internal controls and to ensure reproducibility. AFLP analysis was performed following the protocol of Vos *et al.* (1995) with minor modifications. Restriction digestion of genomic DNA (100 ng) was done

using *Eco*RI and *Mse*I. The resulting restriction fragments were ligated to the corresponding enzyme-specific oligonucleotide adapters (Vos *et al.*, 1995). Preselective amplifications were performed with zero-base-addition *Eco*RI and *Mse*I adapter-specific primers, using the PCR conditions described in De Vos *et al.* (2007). Successful restriction, ligation and preamplification were confirmed by gel electrophoresis (1% agarose gel stained with GelRed and visualized under UV light). The product of the preamplification step was diluted 1:10 in TE buffer (10 mM Tris–HCl pH 8, 0·1 mM EDTA) and used as the template for subsequent amplifications. Final selective amplifications were made using *Eco*RI and *Mse*I primers with two-base-additions. Before the final amplification with all the samples, a screening test with five isolates (13-Phy-191, 8-Phy-205, 1-Phy-255, 16-Phy-267 and 5-Phy-290) and 16 primer combinations (E-AC, E-AT, E-AA and E-TT, in combination with each of M-AC M-CC, M-AG and M-TG) was done. The *Eco*RI primers were labelled with the infrared dyes, IRDye 700 or IRDye 800 (LI-COR). PCR conditions were: 13 cycles of 10 s at 94°C, 30 s at 65°C with a decrease of 0·7°C per cycle, 1 min at 72°C; followed by 23 cycles of 10 s at 94°C, 30 s at 56°C, 1 min at 72°C with an increase of 1 s per cycle; and a final elongation step of 1 min at 72°C.

Amplified fragment length polymorphism (AFLP) fragment analysis was performed on a model 4200 LI-COR automated DNA sequencer. Parameters for electrophoresis were: 1500 V, 35 mA, 35 W, 45°C, motor speed 3 and signal filter 3. Electrophoresis pre-run time was set to 30 min and run time to 4 h. Digital gel images obtained from the LI-COR system were analysed and scored manually. Based on the clarity of the resulting fingerprinting profiles (allowing unambiguous scoring), reproducibility and maximal variability obtained between the isolates, four primer combinations (E-AC/M-AC, E-AA/M-TG, E-AC/M-CC, E-CC/M-CC) were selected and used to evaluate the larger group of isolates. The AFLP procedure was replicated from the initial restriction digest step for all study isolates.

Each clearly resolved AFLP band was assigned a number based on its migration distance. For each isolate, the presence or absence for all monomorphic and polymorphic bands was scored visually. The presence (1) or absence (0) matrices of each primer combination and a matrix that included all data were analysed using POPGENE v. 1.31 (Yeh *et al.*, 1997) to assess the genetic diversity based on Nei's gene diversity index (*h*; Nei, 1973) and Shannon's information index (*I*; Lewontin, 1972). Values of these indices for each set of primers were compared using one-way ANOVA. In order to analyse the degree of genetic similarity between isolates, a pairwise distance matrix for each species was generated using the Jaccard, Dice and simple matching coefficients of similarity as calculated in INFOSTAT v. 2011 (Di Rienzo *et al.*, 2011). Because isolate grouping was shown to be highly similar among the different coefficients, only results from Dice's index (S_j) are presented. Dice's coefficient measures the proportion of shared AFLP markers between each pair of isolates, while correcting for the dominance of the AFLP data by disregarding shared absence of bands. Distance dendrograms were then constructed using the INFOSTAT program. *Phytophthora syringae* was used as the out-group to root the dendrogram. The binary matrix was also analysed in PAUP, v. 4.0b10 (Swofford, 2002) to obtain bootstrap values at the nodes for a tree generated from Nei and Li genetic distances (1000 bootstrap replicates). To test possible grouping of the isolates by provenance and according to variables (band profiles), the binary matrix was also analysed in a principal coordinate analysis using INFOSTAT.

Results

In total, after analysis in duplicate, 332 clearly resolved AFLP bands were visually evaluated from the digital images generated using four different primer combinations (Table 2). The banding patterns for each of the five duplicated isolates, which served as internal controls, were the same, indicating intra-assay AFLP reproducibility. Of the 332 bands that were scored, only 40 (12%) were polymorphic. The total number of bands scored per primer combination ranged from 65 (E-CC/M-CC) to 97 (E-AC/M-AC). The lowest percentage of polymorphic loci (8·2%) was generated by the E-AC/M-AC primer combination, whilst the E-AC/M-CC primer combination yielded the highest percentage (16·2%) of polymorphic loci (Table 2). Twenty-four rare alleles (i.e. present in five or fewer isolates) were identified and none of these could be associated with the region or stands from which the isolates were collected.

Genetic analysis of the bands yielded gene diversities (*h*) that ranged from 0·01 to 0·03. The Shannon index (*I*) ranged from 0·01 to 0·04 (Table 2). Comparison of the values obtained from the four different primers sets using ANOVA showed that they were not statistically different (*h*, $P = 0{\cdot}065$; *I*, $P = 0{\cdot}092$).

A high degree of genetic similarity was found for the *P. austrocedrae* isolates. The pairwise similarity (S_j) values were close to 1, ranging from 0·958 to 1 ($0{\cdot}993 \pm 0{\cdot}009$; mean ± SD) for the majority of comparisons (Fig. 2). The largest distances were obtained between isolates 8-Phy-203, 16-Phy-270 and 15-Phy-271 (0·04), all originating from Chubut Province (8-Phy-203 from Los Alerces National Park, and 16-Phy-270 and 15-Phy-271 from two sites geographically close to each other in Corcovado; Fig. 1). Isolates obtained from the most distant locations (Corcovado and San Martín de los Andes) yielded high similarity values (0·969–0·998). Isolates originating from the same stand, in general, yielded similarity values (S_j values: $0{\cdot}993 \pm 0{\cdot}006$) that were comparable with those from different stands (S_j values: $0{\cdot}993 \pm 0{\cdot}004$).

Dendrograms based on similarity indices from each individual AFLP primer combination were concordant and differed only slightly (data not shown). Data from the four primer combinations were, therefore, concatenated to generate a single dendrogram (Fig. 3). The isolates resided in one large group that was supported by a 100% bootstrap value (Fig. 3). Within this group, 45 isolates grouped in a single cluster (92% bootstrap support), while three isolates (16-Phy-270, 15-Phy-271 and 13-Phy-309) grouped separately but with no bootstrap support (54%; Fig. 3). DNA sequence comparisons for these three isolates with sequences on GenBank showed high similarity (maximal identity from 99 to 100%, *E*-values $<6e^{-177}$) to *P. austrocedrae* and therefore were assumed to be *P. austrocedrae*. These three isolates derived from stands relatively close to each other in terms of the geographic distribution of the host. Stands

Table 2 Total number of amplified fragment length polymorphism (AFLP) bands, proportion of monomorphic and polymorphic bands, gene diversity (*h*) and Shannon index (*I*) obtained with each primer pair combination

AFLP primer combination	Total no. of bands	Monomorphic bands	Polymorphic bands	Polymorphic bands (%)	h^a	*I*
E-AC/M-AC	97	89	8	8·2	0·01	0·02
E-AA/M-TG	96	86	10	10·4	0·01	0·01
E-AC/M-CC	74	62	12	16·2	0·01	0·03
E-CC/M-CC	65	55	10	15·4	0·03	0·04
Total	332	292	40	12·0	0·02	0·03

[a]Gene diversity according to Nei (1973).

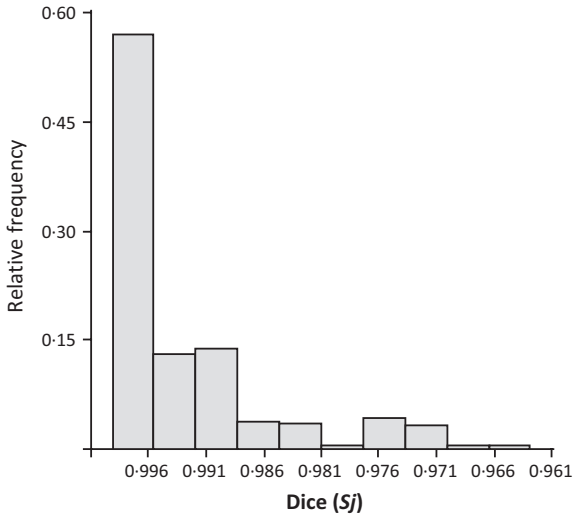

Figure 2 Distribution of Dice's coefficient of similarity (S_j) for *Phytophthora austrocedrae*. Frequency histogram showing the distribution of all pairwise S_j for the 48 isolates (n = 1128). Frequency indicates percentage of comparisons with a given S_j value; S_j values of 1 indicate pairs with 100% similarity.

in Corcovado (sites 15 and 16) were very close to each other, whilst 'La 106' property in Rio Grande Valley (site 13) is about 60 km from those in Corcovado. Although there seemed to be a geographic pattern in this case, the bootstrap did not support this partition; in addition to this, other isolates obtained in the same stands grouped with isolates collected in other distant stands. This is the case for isolates 13-Phy-191, 13-Phy-195 and 13-Phy-201 from 'La 106' property, and 15-Phy-273 and 16-Phy-267 from the two stands in Corcovado (Fig. 3). Among the 45 isolates that grouped together, a subgroup of five isolates from two different stands located *c*. 90 km from each other could be distinguished (89% bootstrap support; Fig. 3). This subgroup was, in turn, subdivided into two according to isolate provenance (90% bootstrap support). The subgroup of five isolates was obtained from plants that were relatively close to each other (stands with small areas); therefore an effect caused by sampling could have occurred. However, as stated above, there does not seem to be geographic partitioning because other isolates from the same sites (13-Phy-309 and 8-Phy-209, 8-Phy-211, 8-Phy-213, 8-Phy-215, 8-Phy-338) grouped apart and mixed with isolates obtained in other stands and sites (Fig. 3). Thus, there was no overall clear partitioning of genetic diversity that corresponded to geographic origin. In the principal coordinate analyses, three-dimensional plots did not resolve any clear grouping of isolates on the basis of geographical origin (Fig. 4).

Discussion

This is the first study to consider the genetic structure and diversity of *P. austrocedrae* in Argentina. Results revealed high levels of within-species genetic similarity and no evidence of partitioning of genetic diversity among the collection sites. This suggests that the pathogen represents a single population with low heterogeneity in Patagonia and a possible explanation is that *P. austrocedrae* might have been introduced into Argentina. The fact that isolates in this study were collected from across the broad distribution of *A. chilensis*, during different times of the year and in different years, adds credence to this view.

The fact that the only known host of *P. austrocedrae* in Argentina exhibits high levels of susceptibility is consistent with the behaviour of an introduced pathogen, and the results obtained in this study indicate that there is a possibility that *P. austrocedrae* might be exotic to the region. Generally, exotic species, especially near-obligate or obligate pathogens such as *Phytophthora* spp., undergo population bottlenecks when they are introduced into new areas (Goodwin, 1997). Therefore, lower genetic variation is expected for these populations in comparison to those where they occur naturally. Moreover, endemic species exhibit population genetic structures that reflect the presence of geographic or environmental barriers, or a combination of both, to gene flow (Goodwin, 1997). In this study, a low genetic variation and absence of geographical differentiation was observed for isolates of *P. austrocedrae*, supporting the notion that it might be introduced into Argentina.

Another plausible explanation for the finding of the low genetic diversity in the pathogen population is that *P. austrocedrae* could have recently evolved from another species. With regard to this, *P. syringae* and

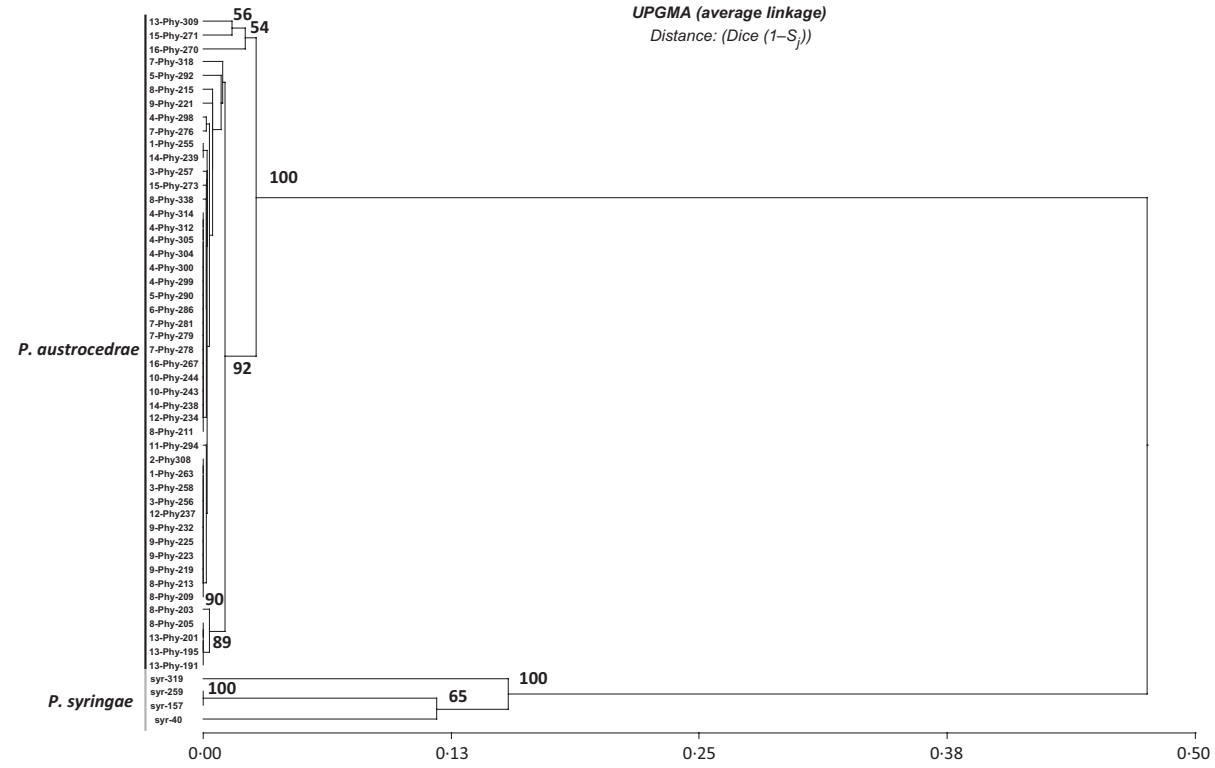

Figure 3 Dendrogram generated from AFLP distance matrix (1–S_j) for 48 *Phytophthora austrocedrae* isolates obtained along the geographical range of *Austrocedrus chilensis*. The first number of each isolate name indicates its site of origin (Table 1). Groups of isolates with zero branch length comprise samples with identical AFLP genotypes. Values at the branches are bootstrap values. *Phytophthora syringae* was used as the outgroup.

P. obscura are the closest relatives of *P. austrocedrae*, by evaluation of nuclear and mitochondrial genes (Greslebin *et al.*, 2007; Robideau *et al.*, 2011; Grünwald *et al.*, 2012). Only *P. syringae* has been previously isolated in Patagonia (Greslebin *et al.*, 2005). More studies are needed to investigate this possibility.

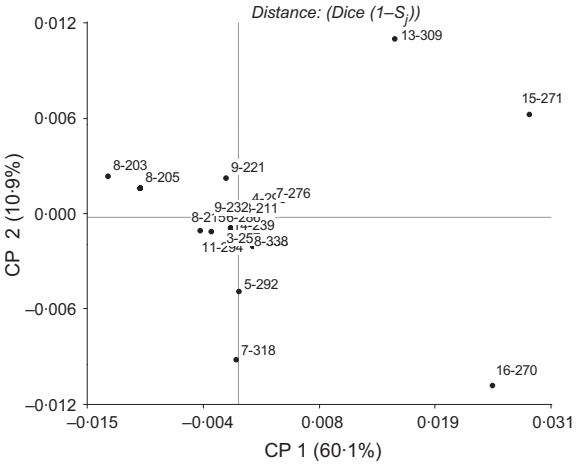

Figure 4 Principal coordinate analysis generated in INFOSTAT from the binary matrix obtained from AFLP patterns of 48 *Phytophthora austrocedrae* isolates obtained along the geographical range of *Austrocedrus chilensis*. The first number of each isolate name indicates its site of origin (Table 1).

Previous studies on *Phytophthora* spp. using AFLP analysis, and where pathogens have been suggested to be introduced into new areas, have reported similar levels of genetic diversity. The present study found that the percentage of polymorphic loci was slightly lower for *P. austrocedrae* than was reported for *Phytophthora nemorosa* in forests in the USA (Linzer *et al.*, 2009) or for *Phytophthora pinifolia* in Chile (Durán *et al.*, 2010), but was slightly higher than obtained for *Phytophthora quercina* in Europe (Cooke *et al.*, 2005) or *Phytophthora pseudosyringae* (Linzer *et al.*, 2009) in the USA and Europe. These species are homothallic, with the exception of *P. pinifolia* that is sterile. The very low levels of genetic diversity and the lack of geographic structuring that is unexpected for endemic organisms, including those that are able to reproduce homothallically, allowed the authors to hypothesize that they had been introduced. Although the AFLP primers used in these studies are not the same as those used in this present work, the relative number of polymorphic loci can be compared, because it is a parameter to indicate the proportion of variable loci. Therefore, a similar conclusion that *P. austrocedrae* has been introduced into Patagonia could be justified.

The first detection of mortality of *A. chilensis* trees occurred during 1948 in Piedras Blancas Bay on Victoria Island in Neuquén Province. The subsequent development of the disease is characteristic of an introduced pathogen that has encountered a highly susceptible host grown over an extended area. Victoria Island is known for the introduction of many exotic woody plants from different continents, especially during the 1920s and 1930s (Simberloff *et al.*, 2002). This, together with the fact that the first appearance of *A. chilensis* mortality occurred in this area, leads the authors to believe that *P. austrocedrae* was introduced to the island on infected plants. The results of this study support this view.

The recent discovery that *P. austrocedrae* has caused mortality of *C. nootkatensis* and *J. communis* in the UK (EPPO, 2011; Forestry Commission Great Britain, 2012) is relevant to the present study. These tree species belong to the same family (Cupressaceae) as *A. chilensis*. *Chamaecyparis nootkatensis* is endemic to the USA and *J. communis* occurs naturally in a wide distribution from the south Arctic to around 30° N latitude in USA, Europe and Asia, in the northern hemisphere. The source of *P. austrocedrae* in the UK has not been determined, but it has been suggested that the pathogen was also introduced (EPPO, 2011). Clearly, it is impossible to determine the origin of *P. austrocedrae* without having isolates from a hypothetical natural host or area of origin. The same situation is true for many *Phytophthora* spp. such as *P. ramorum, P. pinifolia, P. lateralis*, among others, that have unexpectedly appeared in new areas but for which the likely areas of origin are unknown (Hansen *et al.*, 2000; Ivors *et al.*, 2004; Durán *et al.*, 2010). In this regard, global surveys for *Phytophthora* spp. must continue and, in the case of *P. austrocedrae* that appears to be a conifer-specific pathogen, such surveys should include areas where related conifers occur naturally. For example, the recent discovery of *P. lateralis*, a serious root pathogen of *Chamaecyparis lawsoniana* in western North America, infecting *Chamaecyparis obtusa* var. *formosana*, a native species in Taiwan (Webber *et al.*, 2012), represents a good example of how the area of origin of a *Phytophthora* sp. might be found.

The possibility that *P. austrocedrae* could be native to Patagonia cannot be entirely excluded. The pathogen is a homothallic organism, therefore successful gene flow across the geographic range of the pathogen, together with self-fertile reproduction, could also lead to a population with low genetic diversity. However, this hypothesis is not consistent with the high levels of susceptibility of *A. chilensis* and the disease progression pattern that shows a progression from affected sites to healthy sites (La Manna & Matteucci, 2012).

The high level of genetic similarity found for *P. austrocedrae* isolates in this study could be accounted for if the pathogen had undergone a host shift from another native plant. However, a host jump following anthropogenic introduction (Anderson *et al.*, 2004; Woolhouse *et al.*, 2005) would be more plausible when one considers the ecology and epidemiology of other *Phytophthora* spp. (Hansen *et al.*, 2000; Ivors *et al.*, 2004; Brasier *et al.*, 2005; Prospero *et al.*, 2007; Linzer *et al.*, 2009; Durán *et al.*, 2010) and characteristics of emerging infectious diseases of plants in general (Anderson *et al.*, 2004). It has been shown that host shifts are more likely when the original host and the new host are phylogenetically closely related (Gilbert & Webb, 2007). In this regard, at least nine conifer species, phylogenetically related to *A. chilensis*, have been introduced into Victoria Island where the disease was first reported and *P. austrocedrae* is found. The areas of origin of these trees would be good targets for surveys aimed at discovering the origin of *P. austrocedrae*.

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