Gayana Bot. 74(1): 176-188, 2017
Original Article
ISSN 0016-5301

Genetic diversity of *Chondrostereum purpureum* (Pers.) Pouzar causing silverleaf disease on blueberries in Chile

Diversidad genética de *Chondrostereum purpureum* (Pers.) Pouzar causante del plateado en arándano en Chile

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ABSTRACT

Blueberry (*Vaccinium corymbosum* L.) in Chile is being affected by silver disease whose causative agent is *Chondrostereum purpureum* (Pers.) Pouzar. This fungus causes a decrease in the yield of the fruit and the death of the plant. So far, this disease has not been reported in the literature attacking blueberry plants in Chile. The main objective of this research was to characterize genetically the populations of *Ch. purpureum* at molecular level using the ITS-RFLP markers. The results of the ITS-RFLP indicate a low level of diversity in the Chilean populations of *Ch. purpureum*. When analyzing the isolates collected in blueberry, two large groups were configured in the dendrogram. The first one grouped 49 isolates with the same molecular profile and in the second cluster there was a greater diversity among the isolates. On the other hand, when we analyzed *Ch. purpureum* isolates collected in other hosts, 12 isolates constituted a group with identical molecular profile with the 49 isolates collected from blueberry. Analysis of variance of the molecular data showed that there were no statistical differences between *Ch. purpureum* collected in different areas or in different hosts.

Keywords: *Chondrostereum purpureum*, ITS-RFLP diversity, molecular markers.

RESUMEN

El arándano en Chile está siendo afectado por la enfermedad del "plateado" cuyo agente causal es *Chondrostereum purpureum* (Pers.) Pouzar. Este hongo produce una disminución en el rendimiento de la fruta y la muerte de la planta. Hasta ahora, esta enfermedad no se ha reportado en la literatura atacando plantas de arándano en Chile. El objetivo principal de esta investigación fue caracterizar genéticamente las poblaciones de *Ch. purpureum* a nivel molecular utilizando los marcadores ITS-RFLP. Los resultados de los ITS-RFLP indican un bajo nivel de diversidad en las poblaciones Chilenas de *Ch. purpureum*. Al analizar los aislados recolectados en arándano se configuraron en el dendrograma dos grandes grupos. El primero de ellos agrupó 49 aislamientos con el mismo perfil molecular y en el segundo cluster hubo una mayor diversidad entre los aislados. Por otro lado, cuando se analizaron aislados de *Ch. purpureum* recolectados en otros huéspedes, 12 aislados constituyeron un grupo con idéntico perfil molecular con los 49 aislados recolectados de arándano. El análisis de varianza de los datos moleculares mostró que no había diferencias estadísticas entre *Ch. purpureum* recolectado en zonas diferentes o en diferentes huéspedes.

Palabras clave: *Chondrostereum purpureum*, diversidad de ITS-RFLP, marcadores moleculares.

INTRODUCTION

Blueberry (*Vaccinium corymbosum* L.) is the fastest growing fruit crop in Chile based on both area and production. Currently, blueberry has a planted area of approximately 14,506 hectares and a production of 81,000 t mainly for export (ODEPA 2014). Chilean blueberry production has a competitive advantage due to its off-season production that allows it to offer this product to the main fresh markets in the Northern Hemisphere (United States, Canada, and some

European countries) when there is no fresh fruit available. In this context, Chile is the second most important producer and main exporter of fresh blueberry fruit worldwide (PROCHILE 2011, ODEPA 2014).

Agroclimatic conditions, especially in the central and southern zone of the country, are favourable to establish and produce an excellent quality blueberry fruit for the international market and compete favourably with others countries and other fruit species in these regions. Currently, 89% of the total blueberry production is concentrated in the central-southern (8,646 has) and southern regions (4,221 has) of the country (ODEPA-CIREN 2014). The increasing interest for producing blueberry in the country has led to the exploration of new agroecological areas and the possibility of being attacked by new pathogens.

In Chile, blueberry is attacked by various diseases, such as *Agrobacterium tumefaciens* (Smith & Townsend 1907) Conn, 1942, *Armillaria mellea* (Vahl) P. Kumm., *Botrytis cinerea* Pers. ex Fr., *Diaporthe* spp. (Elfar *et al*. 2013), *Neofusicoccum* spp. (Espinoza *et al*. 2009, Pérez *et al*. 2014), *Phoma spp*. (Cisterna & France 2009), *Phomopsis vaccinii* Shear., and *Phytophthora cinnamomi* Rands. Since 2009, blueberry has been attacked by a new fungal disease that produces symptoms characteristics of the "silverleaf". These symptoms that were originated in the southern area, are detected right now, in the northern area of the country (France *et al*. 2009).

Currently, the causal agent of this disease has been identified as *Chondrostereum purpureum* (Pers.) Pouzar (Basidiomycota), using morphometric and molecular tools (France *et al*. 2009). Various studies indicate that *Ch. purpureum* causes damage in other fruit and forest genera such as *Prunus*, *Pyrus*, *Actidinia*, and *Rubus*, and *Salix* and *Populus*, respectively (Farr *et al*. 2008). In 2014, *Ch. purpureum* was detected for the first time in blueberry in Oregon, EE.UU. (Seth Elkington, pers. com., Simplot Advisor, U.S.A.).

The symptoms of this disease present in the Chilean blueberry are similar to those described for *Chondrostereum purpureum* attacking larger fruit trees; where plants exhibit less vigour in the branches and a gradual change in their color from green to greyish or silver causing a xylem necrosis. These symptoms initially affect few branches and finally cover to the whole plant, when the presence of the disease is severe. The xylem necrosis is the result of a colonization of the xylem by the fungus that induces to the plant to break easily over time (France *et al*. 2009). These morphological damages are caused by the effects of an endopoligalacturonase (endoPG) toxin produced by the pathogen, which migrates through the xylem to the leaves; producing the detachment of the foliar lamina from the palisade parenchyma, producing an air chamber, which gives it a silver appearance due to the optical effect of light passing through this chamber (Torres *et al*. 2006).

Based on the information presented above, it is necessary to broad the morphological information of this fungus available, in order to know the level of the genetic diversity of this pathogen and to determine the genetic relationships among isolates, their geographic distribution; the cultivar susceptibility to this disease, and to develop a strategy of control to face this problem.

Considering this situation, PCR based on *in vitro* amplification of specific DNA sequences, such as the nuclear ribosomal DNA, could contribute to determine the genetic diversity of this pathogen. Ribosomal DNA (rDNA) has a high copy number, repeated in tandem, and that includes three genes, the 18S, 5.8S, and 28S. These gene sequences are separated by two internal transcribed spacers (ITS), ITS1 and ITS2, with an estimated size of between 200 to 400 bases. These regions accumulate nucleotide mutations; when amplified by universal and/or species-specific primers can be used to determine the genetic diversity among various organisms, including fungal populations (Gomes *et al*. 2002, O'Brien *et al*. 2005, Becerra *et al*. 2007 a, b, Taylor & McCormick 2008).

The use of differential rates in nucleotide changes in nuclear ribosomal sequences has enabled the detection and comparison of fungus isolates at inter-and intraspecific levels attacking blueberries (Espinoza *et al*. 2008, Elfar *et al*. 2013, Pérez *et al.* 2014) and other species (Iturralde 2005, Toju *et al.* 2012). For example, the combined analysis of ITS and EF1- alfa gene sequences were able to identified five species of *Diaphortein* (Elfar *et al*. 2013) the ITS1-5.8S and ITS2-28S to detect *Neofusicoccum nonquaesitum* (Pérez *et al*. 2014) and *Pestalotiopsis* ssp. and *Truncatella* sp. attacking blueberries in Chile (Espinoza *et al*. 2008). Using specific ITS primers for basidiomycetes (ITS1F-ITS4) Prewitt *et al*. (2008) was able to determine a monophyletic group in the *Xylophagous* fungal species. In another study, the rDNA amplification and *Dra* I enzyme DNA restriction was used for early detection, identification and differentiation of basidiomycete and ascomycete fungal species in wood chips (Adair *et al.* 2002). Up to now, there are no studies on the genetic diversity of *Ch. purpureum* in Chile.

The objectives of this work were to determine the genetic diversity of the *Ch. purpureum* populations, by ITS-RFLP.

MATERIALS AND METHODS

Genetic material and geographic distribution

A total of 119 isolates of *Ch. purpureum* were collected from 10 hosts, 84% of the isolates were obtained from blueberry and 16% from other species such as *Populus nigra* L. (poplar), *Acacia dealbata* Link (acacia), *Prunus avium* L. (cherry), *Prunus armeniaca* L. (apricot), *Prunus persica* (L.) Batsch (peach), *Eucalyptus globulus* Labill. (eucalyptus), *Malus x domestica* Borkh. (apple), *Pistacia*

vera L. (pistachio) and *Rosa chinensis* Jacq. (rose) (Annex 1). The isolate Cho-391 was used as a reference line, since this isolate was identified as a *Ch. purpureum* in CABI, UK (France *et al*. 2009).

Ch. purpureum isolates were collected from plants that were displaying visible signs of silverleaf in the field orchards as described by France *et al*. (2009), then the isolates were morphometrically characterized at the Plant Pathology Laboratory at INIA Quilamapu, Chillán, to certify the presence of *Ch. purpureum*.

The isolates were collected from different locations during 2014: six (6) from the central zone (32°49'20''S; 71°03'48''W), 30 from the central-southern area and 83 from southern Chile (40°7'73''S; 73°30'12''W). Isolates were deposited at INIA Microbial Germplasm Bank (Annex 1) and the genetic study was conducted at the Biotechnology Laboratory, at INIA Quilamapu, Chillán.

DNA extraction

DNA was extracted from fungal mycelium of pure isolates culture grown on a solid nutrient malt agar (MA) medium (malt extract 20 g, agar 20 g, yeast extract 2 g, distilled water to complete 1 L) in Petri dishes incubated at 27 ºC. Mycelium maceration was done with liquid nitrogen and samples were incubated for 45 min with 700 µL extraction buffer (100 mM Trizma (Invitrogen); 1.4 M NaCl (Sigma); 20 mM EDTA (Sigma); 1% Polivinilpirrolidone (Sigma); 2% CTAB (Sigma); 1% β-mercaptoetanol (Sigma); pH 8.0) at 65 ºC, an aliquot of 10 µL proteinase K (Sigma) (50µg/ml) was added to each sample. Samples were cooled at room temperature and mixed twice with 2/3 v/v chloroform: isoamyl alcohol (Sigma) (24:1) and centrifuged at 5000 rpm for 15 min (Eppendorf centrifuge 5804, Hamburg, Germany). The aqueous supernatant was transferred to a clean tube and DNA was precipitated with isopropanol (Sigma) and then incubated at -20 ºC over night. The DNA pellet was washed with 70% and 90% ethanol (Sigma), dried at room temperature, and suspended in TE solution (10 mM Tris; 1 mM EDTA; pH 8). Finally, DNA was treated with RNAse (Sigma) (10 mg/ml) and stored at -20 ºC for future use.

DNA concentrations were measured using a UV-Vis Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington Delaware) and concentrations were then standardised at 5 ηg μL^{-1} in sterile distilled water. In addition, DNA quality was verified in a 1% (w/v) agarose gel using λ *Hind*III as a DNA ladder.

Internal transcribed spacer analysis

For the genetic analysis of the isolates, universal primers, specific to the internal transcribed spacer (ITS) regions of the Basidiomycota, ITS1F and ITS4B were used to amplify the ITS1-5.8S-ITS2 for which protocols were adjusted on two *Ch. purpureum* isolates collected on blueberry (Gardes & Bruns 1993).

Reaction conditions were performed in a 25 µL total volume made up of 0.2 mM dNTPs, 0.2 mM ITS1F and ITS4B primers, 1x of PCR buffer, 0.25 mM $MgCl_2$, 1 U *Taq* DNA polymerase (Invitrogen), and 10 ηg μL^{-1} of DNA template.

Amplification was performed in a DNA Engine DYAD™ thermocycler (MJ Research, Inc., Waltham Massachusetts) under the following conditions: 3 cycles of 1 min 20 s at 95 ºC, 1 min at 37 ºC, 1 min at 72 ºC, and 37 cycles of 35 s at 94 ºC, 40 s at 40 ºC, 1 min 20 s at 72 ºC with an elongation period of 10 min at 72 ºC, followed by a maintenance at 4 ºC.

Digestion of the amplified PCR products of four (4) randomly chosen strains was conducted in 20 µL total volume, containing: 3 µL amplified DNA product, 1x buffer (enzyme-specific buffer), and 5 to 8 units of restriction enzyme (made up to 20 µl with sterile molecular grade H_2O). Samples were incubated at 37 ºC for 2 h according to the manufacturer´s instructions (Thermo Scientific). A total of 32 restriction enzymes were used that recognize different nucleotide digestion sequences: *Acc*I, *Alu*I, *Ase*I, *Ava*II, *Bam*HI, *Bgl*II, *Bst*EII, *Dde*I, *Dra*I, *Eco*RI, *Eco*RV, *Hae*III, *Hha*I, *Hinf*III, *Hpa*II, *Mbo*I, *Mse*I, *Msp*I, *Nde*II, *Nde*I, *Pst*I, *Rsa*I, *Sau*3AI, *Sac*I, *Sac*II, *Spe*I, *Ssp*I, *Taq*I, *Sin*I, *Tru*9I, *Xba*I, and *Xho*I. Based on their performance, nine restriction enzymes were selected for genetic analysis of the 119 strains, using the reaction and amplification conditions as described previously.

Restriction fragments were separated on a 6% polyacrylamide gel (20x20 cm) in 1x TBE buffer at 100 V for 3 h (Cole-Parmer vertical dual adjustable slab gel system; Vermon, II). The gel was stained with GelRed (Biotium) to visualize the DNA fragments under ultraviolet light (G: Box ICHEMI XR image analyser; Syngene Co., Cambridge, United Kingdom). Fragment sizes were determined by visual comparison to 10 and 100 bp DNA ladders.

Data analysis

Band profiles were recorded for the 119 *Ch. purpureum* isolates. Polymorphic bands were considered as binary characters, and scored as "1" if present or "0" if absent, for each marker. Genetic similarity between pairs was estimated by Jaccard´s coefficient to transform values into genetic distance, which was defined as the square root of the one-complement of the similarity (√1-S). The distance matrix was analysed by metric multidimensional scaling (MDS) as the grouping technique (PC analysis). A hierarchical clustering technique using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method was used; it was selected because it provided the highest cophenetic correlation. Genetic distances were subjected to an analysis of molecular variance (AMOVA) to determine the statistical significance of the data (Excoffier *et al*. 1992). The existence of specific band patterns was determined with the ITS-RFLPs to identify some isolates.

Statistical analysis of the data was performed with the InfoGen version 2013 FCA statistical programme (National University of Cordoba, Argentina) (Balzarini & Di Rienzo 2013).

RESULTS

Diversity parameters of *Chondrostereum purpureum* collected in blueberry

The ITS1F and ITS4B primers were used to amplify the ITS region in 100 *Ch. purpureum* isolates collected only from blueberry. In each case only a single fragment was obtained. Nine out of 32 enzymes detected different levels of polymorphism and they were selected for the analysis. These nine restriction enzymes were: *AluI, DdeI, DraI, EcoRI, HhaI, MboI, MseI, SpeI,* and *Taq*.

These nine (9) restriction enzymes detected a total 67 bands from 100 *Ch. purpureum* isolates, of which 90% were polymorphic. A total of 70 isolates presented an identical pattern for the ITS/restriction enzyme combinations used in this study.

The *Hha*I restriction enzyme generated the highest number of bands (13) and banding pattern (8), followed by *Mbo*I (9), *Eco*RI (Fig. 1) and *Taq*I (8) (Table 1). The highest values of polymorphic information content was obtained by the restriction enzymes *Mse*I (PIC= 0.15) and *Mbo*I (PIC= 0.10); both being the most informative restriction enzymes in this study. The lowest probability that two isolates share the same band (PRSA) value was 1x 10^{-4} for the restriction enzymes *Mse*I and 4.9 x 10^{-3} for *Spe*I. These markers had a high confidence level for identifying *Ch. purpureum* strains. On the contrary, it was shown that the restriction enzyme *Alu*I has no discriminatory ability (PMF= 0) and its polymorphic information content was very low for the isolates collected on blueberry (PIC= 0.02) (Table 1).

Cluster analysis based on genetic distance of *Chondrostereum purpureum* isolates

The PC analysis using the Jaccard´s similarity coefficient (Fig. 2) explained 52% of total variability. The PC1 and PC2, explains 33.5% and 18.5% of the variability, respectively, for the nine polymorphic restriction enzymes.

Isolates collected from blueberries were widely distributed on PC1, about half were located on the negative side and the rest at the positive PC1 coordinate, thus two main groups can be identified. In addition, a group of 48 *Ch. purpureum* isolates were located at the same point along with Cho 391, used as a reference strain; indicating the genetic identity of these isolates in this study. All these isolates came from the central-south and south zone of the country. For the PC2, the isolates were also widely distributed along the axis.

On the other hand, cluster analysis using the UPGMA method led to a dendrogram with a cophenetic correlation index of 0.99, which indicates that the dendrogram distances reflected original distances (Fig. 3). The observed genetic distances among isolates ranged from 0 to 1 (mean 0.33). Two main groups can be observed in the dendrogram, the lower cluster included 51 isolates, which come from the central (2%), central-south (22%) and south zone (76%), clustered at the same genetic distance, along with the reference strain Cho-391. Most of them (49) had the same molecular profile for the nine restriction enzymes evaluated. Only two strains Cho-816 and Cho-882 seemed to be slightly different (Fig. 3).

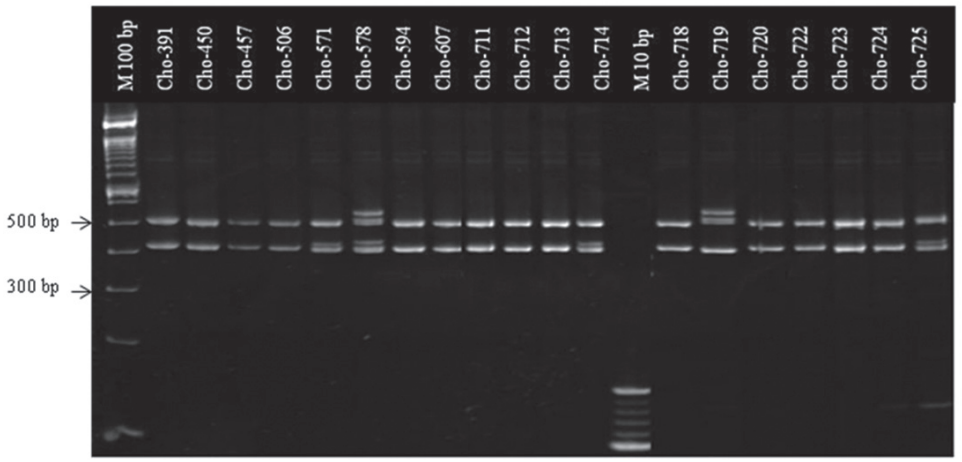

Figure 1. Example of ITS-RFLP band patterns from 19 *Chondrostereum purpureum* isolates generated by *Eco*RI restriction enzyme. M100bp and 10bp= Standard molecular weight. / Ejemplo de patrón de bandas ITS-RFLP band de 19 aislados de *Chondrostereum purpureum* detectados con la enzima de restricción *Eco*RI. Estándares de peso molecular: M100bp y 10bp.

Table 1. Total (TB), polymorphic (PB) and monomorphic bands (MB), proportion of polymorphic loci (PPL), polymorphic information content (PIC) and probability of two individuals randomly sharing the same alleles detected by ITS-RFLP characterisation of *Chondrostereum purpureum* collected from blueberry. / Número de bandas total (TB), polimórficas (PB), monomórficas (MB), proporción de loci polimórficos (PPL), contenido de información polimórfica (PIC) y probabilidad de que dos individuos compartan alelos al azar (PRSA), detectados por la caracterización de *Chondrostereum purpureum* recolectado en arándano, mediante ITS-RFLP.

Restriction enzyme	TB	PB	MB	PPL (95%)	PIC	Bands present (%)	PRSA
*Alu*I	7	7	0	0.00	0.02	43.00	$3{,}7 \times 10^{-1}$
*Dde*I	4	4	0	0.25	0.07	28.04	$3{,}0 \times 10^{-2}$
*Dra*I	7	7	0	0.29	0.08	31.14	$1{,}0 \times 10^{-2}$
*Eco*RI	8	7	1	0.25	0.07	28.75	$3{,}4 \times 10^{-2}$
*Hha*I	13	11	2	0.23	0.07	25.62	$5{,}5 \times 10^{-2}$
*Mbo*I	9	6	3	0.22	0.10	26.44	$2{,}5 \times 10^{-2}$
*Mse*I	7	6	1	0.43	0.15	52.57	$1{,}0 \times 10^{-4}$
*Spe*I	4	4	0	0.25	0.06	50.88	$4{,}9 \times 10^{-3}$
*Taq*I	8	8	0	0.13	0.05	26.02	$2{,}0 \times 10^{-3}$
Total	67	60	7			33.01	$5{,}5 \times 10^{-18}$

TB: Total bands, PB: Polymorphic bands, MB: Monomorphic bands, PPL (95%): Proportion of polymorphic loci at 95%, PIC: Polymorphic information content, BP (%): Bands present, PRSA: Probability of two individuals randomly sharing the same allele (band). / TB: Bandas totales, PB: Bandas polimórficas, MB: Bandas monomórficas, PPL (95%): Proporción de loci polimórficos a 95%, PIC: Contenido de información polimórfica, BP (%): Bandas presentes, PRSA: Probabilidad de dos individuos compartiendo aleatoriamente el mismo alelo (banda).

The second group showed a higher genetic distance for the ITS-RFLP evaluated. This group clustered 49 isolates. The majority of the strains came from the southern (88%) and the central-southern region (12%). Within this group, the isolates presented several molecular profiles. This clustering of isolates based on Jaccard´s coefficient corroborates the results found in the PC analysis of isolates represented in the biplot graph (Fig. 2).

Finally, the analysis of molecular variance (AMOVA) showed no statistical differences between *Ch. purpureum* collection zones ($p = 0.425$) and between the locations within the same zone ($p = 0.803$) (Table 2).

Genetic relationships between *Chondrostereum purpureum* collected from blueberry and other hosts

The dendrogram of the complete collection of the 119 *Ch. purpureum* isolates that came from blueberry and 10 woody species, clustered all together the isolates with a cophenetic correlation index of 0.99 (Fig. 4). Most of the isolates collected from blueberry presented same genetic distance compared with those collected in other hosts (Fig. 3). Thus, the lower branch of the dendrogram showed 63 out of 119 *Ch. purpureum* isolates with the same genetic profile. Twelve of these isolates came from different hosts, such as poplar, apricot, peach, eucalyptus, apple, pistachio and rose. Again, isolates Cho-816 and 882, collected from blueberry were grouped at a slightly greater genetic distance (Fig. 4) from the others. In the upper cluster of the dendrogram, seven isolates collected on apple, cherry, peach and *Acacia* were widely distributed along with the isolates collected from blueberry (Fig 4).

For the group of isolates collected in blueberry, the confidence interval of the Shannon-Weaver (ShaW) genetic diversity index, using the percentile method, was 3.3-3.4 at a fixed 95% confidence level. The estimated ShaW index for the group of isolates from other host species (not blueberry) was 3.3; this indicated that this group has about the same diversity than the blueberry isolates.

The AMOVA performed for the molecular data coming from the two fungal host categories (blueberry and other species) (Table 3) did not show significant statistical difference ($p = 0.960$).

The PC analysis of the types of *Ch. purpureum* host based on the ITS-RFLP molecular data, showed 56.8% that the total variability was explained by PC1; this indicated that the molecular profiles of the blueberry isolates were different from the molecular profiles of isolates from species such as *Acacia dealbata*, *Populus nigra*, *Prunus armeniaca*, *Prunus persica*, *Eucalyptus globulus*, *Pistacia vera* L., *Rosa chinensis*, and *Prunus avium*, which were very similar to each other. On the other hand, PC 2 explained a 26% of the variability and allowed to separate the isolates collected from *Malus x domestica* Borkh from the other *Ch. purpureum* hosts.

Table 2. Analysis of molecular variance (AMOVA) for ITS data between collection zones of *Chondrostereum purpureum* from blueberry plants. / Análisis de varianza molecular (AMOVA) para los datos de ITS entre zonas de recolección de *Chondrostereum purpureum* desde plantas de arándano.

Source of variation	df	SS	MS	P value	Variance components	% variation
Zones	2	147.50	73.75	0.425	0	0
Zones> Region	3	105.54	35.18	0.803	0	0
Within-zone	94	8601.56	91.51	0.810	91.51	100
Total	99	8854.59	89.44		91.51	100

df: degrees of freedom, SS: sum of squares, MS: mean squares. P≤ 0.05. / df: grados de libertad, SS: suma de cuadrados, MS: cuadrados medios. P≤ 0,05.

Table 3. Analysis of molecular variance (AMOVA) of ITS-RFLP from *Chondrostereum purpureum* collected from blueberry and other woody species. / Análisis de la varianza molecular (AMOVA) de ITS-RFLP de *Chondrostereum purpureum* recolectado desde arándano y otras species leñosas.

Source of variation	df	SS	MS	P value	Variance components	% variation
Host	1	0.56	0.56	0.960	0	0
Within-host	117	1062.52	9.08	0.845	9.08	100
Total	118	1063.08	9.01		9.08	100

df: degrees of freedom, SS: sum of squares, MS: mean squares. P≤ 0.05. / df: grados de libertad, SS: suma de cuadrados, MS: cuadrados medios. P≤ 0,05.

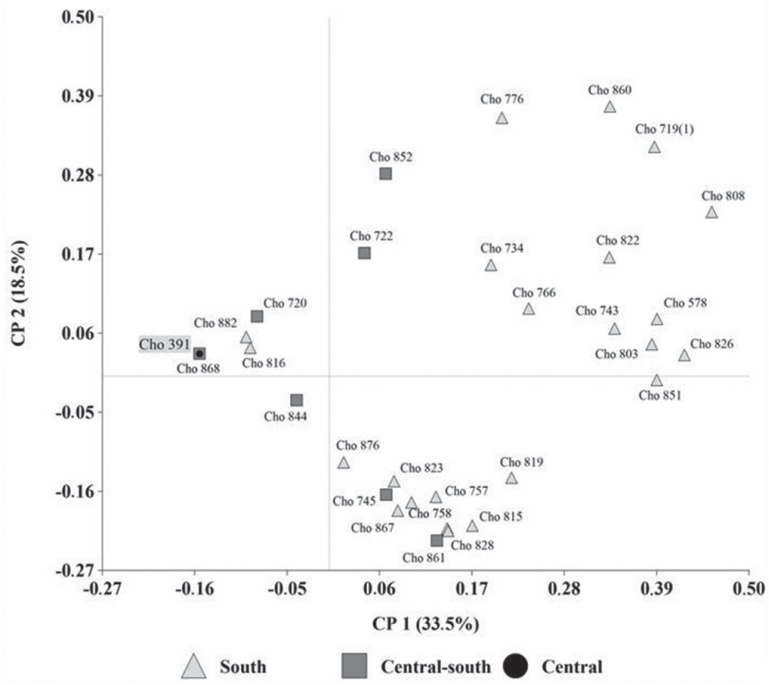

Figure 2. Principal Coordinates Analysis from ITS-RFLP evaluation of Chilean *Chondrostereum purpureum* isolates collected from blueberry plants. Jaccard´s coefficient. / Análisis de Coordenadas Principales de ITS-RFLP de aislados *Chondrostereum purpureum* recolectados desde plantas de arándano. Coeficiente de Jaccard.

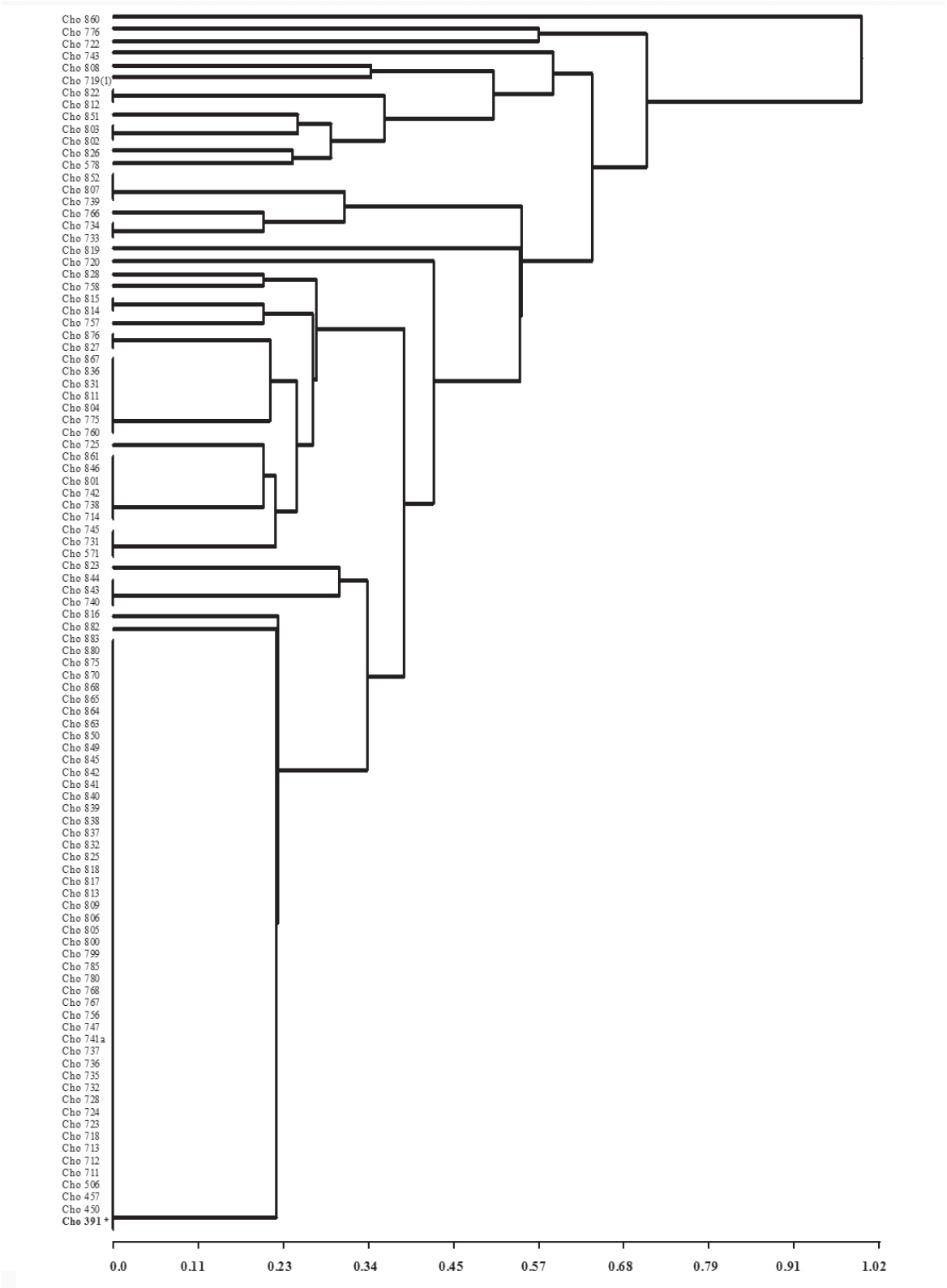

FIGURE 3. Dendrogram from ITS-RFLP analysis of Chilean *Chondrostereum purpureum* isolates collected from blueberry plants. Jaccard´s coefficient. *Cho 391: reference *C. purpureum* isolate. / Dendrograma del análisis ITS-RFLP de aislados *Chondrostereum purpureum* recolectados desde plantas de arándano. Coeficiente de Jaccard. *Cho 391: Aislado de referencia.

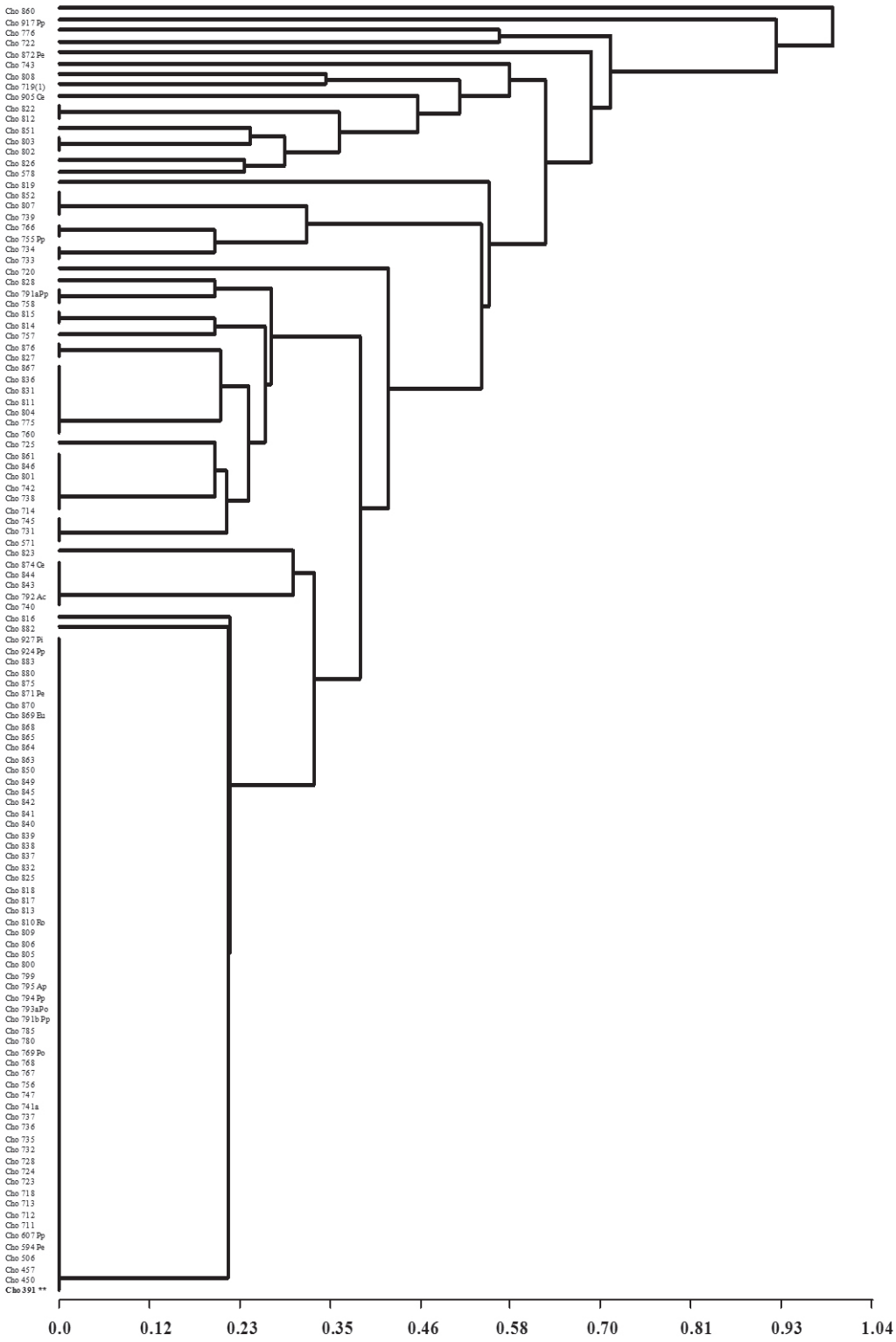

Figure 4. Dendrogram from ITS-RFLP analysis of Chilean *Chondrostereum purpureum* isolates collected from blueberry plants and other woody plants. Jaccard´s coefficient. / Dendrograma del análisis ITS-RFLP de aislados *Chondrostereum purpureum* recolectados desde plantas de arándano y de otras especies leñosas*. Coeficiente de Jaccard.

*Hosts: Po: poplar, Ar: acacia, Ce: cherry, Ap: Apricot, Pe: peach, Eu: eucalyptus, Pp: apple, Pi: pistachio, Ro: rose. Huésped: Po: álamo, Ar: aromo, Ce: cereza, Ap: albaricoque, Pe: durazno, Eu: eucalipto, Pp: manzana, Pi: pistacho, Ro: rosa.

** Cho 391: reference *C. purpureum* isolate. / Cho 391: Aislado de referencia.

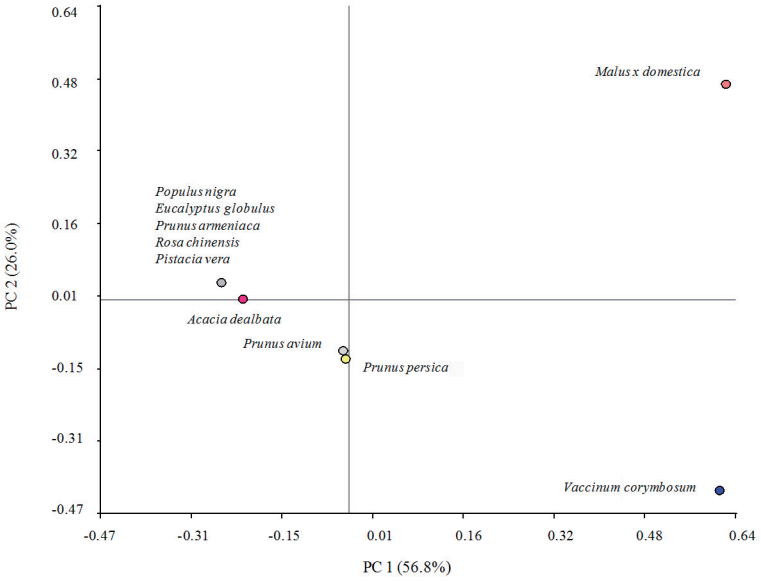

FIGURE 5. Principal coordinates analysis obtained with ITS markers for isolates of *Chondrostereum purpureum* collected in different woody species. / Análisis de Coordenadas Principales obtenido con marcadores ITS en aislados de *Chondrostereum purpureum* recolectados en diferentes especies leñosas.

DISCUSSION

This is the first study to examine Chilean genetic diversity of *Ch. purpureum* using ITS-RFLP of the ribosomal DNA. The isolates of this fungus included in this study had a good representation because they were collected from orchards of the whole area where the specie is planted in Chile and the pathogen was present. To have a clear identification of the Chilean isolates, the strain IMI 394944 (Cho-391) of *Ch.purpureum* identified by CABI (UK), was used as a reference.

The ITS-RFLP markers showed a low level of diversity in Chilean populations of *Ch. purpureum* and only nine (9) out of 32 ITS-/restriction enzymes combinations were able to differentiated about half of the isolates analyzed in this study.

In spite of this, the isolates were collected in the wide range of geographic zone, including south, central south and central zone of the country, sixty eight (68) out of one hundred nineteen (119) isolates showed the same genetic profile (genetic distance), indicating the low variability of the isolated that were attacking the blueberry plants. These results were confirmed by the low values of polymorphic information content and number of banding patterns present in the population studied.

Although the diversity is low, the amplification of the rDNA conserved region using specific primers for Basidiomycetes combined with the nine restriction enzymes detected some degree of polymorphisms, that were able to characterize genetically of Chilean *Ch. purpureum* population attacking blueberry orchards in Chile. On the other hand, when it was characterized the *Ch. purpureum* populations attacking other species different from blueberry, it was found that 12 isolates had identical banding profile compared with isolates collected from blueberry. These isolates were grouped together, at distance "0" including Cho-391 (reference strain). Therefore, more than half of the isolates collected in other species could not be differentiated from the isolates attacking blueberry.

On the contrary, some differentiation was possible to found among isolates collected in different hosts, and were located in the second cluster of the dendrogram (Fig. 4). In this group, some isolates coming from different hosts showed also identical profile with blueberry. This could mean that, with the combination for ITS-RFLP and restriction enzymes used in this study, was not possible to distinguish between populations coming from different hosts or these isolates are not specific for attacking different species.

According to the statistical analysis, there was not a statistical difference between hosts and the region where the isolates were collected. Furthermore, these results indicated that the higher percentage of variability is within each host category and collection region. These results agree with Gosselin *et al.* (1996), using RAPDs who did not find any relationship between ecological or host specialization, in spite of that a high variation detected within isolates. Based on an analysis with Random Amplified Microsatellite (RAMS) markers, Vartiamäki *et al*. (2008) found similar results analyzing five *Ch. purpureum* populations from Finland and Lithuania. The results showed a higher

variation within the studied populations (98.76%) compared with the variation obtained among populations (1.24%) and no statistical differences between them. In this study, a low differentiation in *Ch. purpureum* populations that attack blueberry could be related to the fact that this disease can be considered as a recent disease, and the technique used was not able to detect genetic variations among isolates within and between species.

The first symptoms of xylem necrosis was detected in the blueberry cultivar 'Brigitta Blue' during the 2005-2006 season in Los Lagos Region located in the south zone (France *et al*. 2009), and 20% of the analysed isolates in this study came from this region. It was supposed that the pressure or variability of the isolates on blueberries in this zone must be greater than the other zones, because of the origin of the symptoms of the disease, a high number orchards and a better environment for the development of the disease.

Based on these results, further studies based on other genetic analysis and identification methods of the pathogen needs to be done. For example, the sequencing of the amplified PCR, using ITS primers, their blast analysis and comparison with other species sequences deposited in a GenBank (Itoo *et al*. 2015). This will allowed us to confirm the identification of species and to determine its genetic structure.

Silverleaf is a potential disease that can have serious consequences for blueberry orchards and should be included in the strategic blueberry disease control programmes because the macropropagated plants in nurseries, the most common way to multiply plants, could facilitate the spread of this disease within the zone and other areas of the country.

Moreover, silverleaf could be a serious problem, since this disease has been reported in Oregon, zone of origin and natural distribution of the blueberry crop, where the plant coexists with this fungus (France *et al*. 2009).

ACKNOWLEDGEMENTS

The authors wish to thanks the Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT), Project N° 1120978, for financial support of this work. This study is part of this project and it corresponds to a thesis work for a Magister degree.

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Annex 1. Isolates of *Chondrostereum purpureum* (Cho), host and cultivar, region and zone of origin, geographic location and cluster group generated by the ITS-RFLP analysis. / Aislados of *Chondrostereum purpureum* (Cho), huésped y cultivar, región y zona de origen, localidad geográfica y agrupamiento basado en el análisis de ITS-RFLP.

N°	Identification	Host	Cultivar	Region	Zone	Coordinates		Molecular grouping
						Longitude	Latitude	
1	Cho-391	Blueberry	Brigitta	Los Lagos	South	73°03'11''W	40°31'20''S	1
2	Cho-450	Blueberry		Maule	Central-south	71°49'27.99''W	36°08'23.85''S	1
3	Cho-457	Blueberry		Maule	Central-south	71°49'27.99''W	36°08'23.85''S	1
4	Cho-506	Blueberry	Legacy	Los Lagos	South	73°07'54.9''W	40°34'28.22''S	1
5	Cho-571	Blueberry	Misty	Biobío	Central-south	72°30'02.04''W	37°27'35.81''S	2
6	Cho-578	Blueberry	Brigitta	La Araucanía	South	72°40'34.90"W	39°06'02.02"S	2
7	Cho-594	Peach		L.B. O'Higgins	Central	70°44'20.27''W	34°10'04.99''S	1
8	Cho-607	Apple		Biobío	Central-south	72°21'16.03''W	37°28'22.34''S	1
9	Cho-711	Blueberry	Brigitta	Los Ríos	South	72°22'51.06''W	40°07'28.35''S	1
10	Cho-712	Blueberry	Brigitta	Los Ríos	South	72°22'51.06''W	40°07'28.35''S	1
11	Cho-713	Blueberry	Brigitta	Los Ríos	South	72°25'45,5''W	40°07'10.4''S	1
12	Cho-714	Blueberry	Brigitta	Los Ríos	South	73°30'12''W	40°18'58.3''S	2
13	Cho-718	Blueberry	Brigitta	Los Lagos	South	73°0'59.5''W	40°57'04.4''S	1
14	Cho-719(1)	Blueberry	Brigitta	Los Ríos	South	72°38'47.5''W	40°12'53.5''S	2
15	Cho-720	Blueberry	Brigitta	Biobío	Central-south	72°21'38.48''W	36°31'06.42''S	2
16	Cho-722	Blueberry	Brigitta	Biobío	Central-south	71°44'48.81''W	36°33'27.5''S	2
17	Cho-723	Blueberry	Brigitta	Biobío	Central-south	72°19'35.4''W	36°51'55''S	1
18	Cho-724	Blueberry	Brigitta	La Araucanía	South	72°21'03.67"W	38°13'53.28"S	1
19	Cho-725	Blueberry	Brigitta	La Araucanía	South	72°21'03.67"W	38°13'53.28"S	2
20	Cho-728	Blueberry	Brigitta	La Araucanía	South	72°23'50.6"W	38°50'59.2"S	1
21	Cho-731	Blueberry	Brigitta	La Araucanía	South	72°18'36.9''W	38°37'54''S	2
22	Cho-732	Blueberry	Brigitta	La Araucanía	South	72°19'23''W	38°25'5.9''S	1
23	Cho-733	Blueberry	Brigitta	La Araucanía	South	72°18'36.9''W	38°37'54''S	2
24	Cho-734	Blueberry	Brigitta	La Araucanía	South	72°15''48.2''W	38°0'12.3''S	2
25	Cho-735	Blueberry	Brigitta	La Araucanía	South	72°29'30.64''W	38°20'14.88''S	1
26	Cho-736	Blueberry	Brigitta	Biobío	Central-south	71°58'59.72''W	36°52'21.29''S	1

N°	Identification	Host	Cultivar	Region	Zone	Coordinates		Molecular grouping
						Longitude	Latitude	
27	Cho-737	Blueberry	Brigitta	Biobío	Central-south	72°05’41.39”W	36°53’0.62”S	1
28	Cho-738	Blueberry	Brigitta	Biobío	Central-south	72°7’26.73”W	36°50’35.11”S	2
29	Cho-739	Blueberry	Brigitta	Biobío	Central-south	72°1’40.86”W	36°51’21.29”S	2
30	Cho-740	Blueberry	Brigitta	Biobío	Central-south	72°7’1.64”W	36°29”9.95”S	2
31	Cho-741a	Blueberry	Brigitta	Los Ríos	South	72°51’26,4”W	40°20’20.5”S	1
32	Cho-742	Blueberry	Brigitta	La Araucanía	South	72°40’59.88”W	38°15’00.00”S	2
33	Cho-743	Blueberry	Brigitta	Los Lagos	South	73°5’11.3”W	40°54’30.2”S	2
34	Cho-745	Blueberry	Brigitta	Los Ríos	South	72°16’59.1”W	39°34’12.5”S	2
35	Cho-747	Blueberry	Drapper	Los Lagos	South	73°5’14.6”W	40°54’51”S	1
36	Cho-755	Apple		Biobío	Central-south	72°05’15”W	36°35’47”S	2
37	Cho-756	Blueberry	Brigitta	Los Ríos	South	73°3’49.6”W	39°36’57.5”S	1
38	Cho-757	Blueberry	Brigitta	Los Ríos	South	72°52’36.9”W	40°7’73”S	2
39	Cho-758	Blueberry	Brigitta	Los Lagos	South	73°5’11.3”W	40°54’30.2”S	2
40	Cho-760	Blueberry	Brigitta	Los Ríos	South	72°53’54.6”W	40°13’16.5”S	2
41	Cho-766	Blueberry	Brigitta	La Araucanía	South	72°21’37.55”W	38°43’27”S	2
42	Cho-767	Blueberry	Brigitta	La Araucanía	South	72°35’31.14”W	38°57’43.6”S	1
43	Cho-768	Blueberry	Brigitta	Biobío	Central-south	71°46’46.89”W	36°32’39.84”S	1
44	Cho-769	Poplar		Biobío	Central-south	71°56’49.62”W	36°37’49.34”S	1
45	Cho-775	Blueberry	Blue Heaven	La Araucanía	South	72°39’6.8”W	39°6’7.5”S	2
46	Cho-776	Blueberry	Brigitta	La Araucanía	South	72°21’22.68”W	38°43’35.6”S	2
47	Cho-780	Blueberry	Brigitta	Biobío	Central-south	72°22’52.0”W	37°20’34.1”S	1
48	Cho-785	Blueberry	Brigitta	Biobío	Central-south	71°56’49.62”W	36°37’49.34”S	1
49	Cho-791a	Apple		Maule	Central-south	71°44’37.63”W	35°40’34.43”S	2
50	Cho-791b	Apple		Maule	Central-south	71°44’37.63”W	35°40’34.43”S	1
51	Cho-792	Acacia		Biobío	Central-south	72°05’15”W	36°35’47”S	2
52	Cho-793a	Poplar		Biobío	Central-south	72°30’02.04”W	37°27’35.81”S	1
53	Cho-794	Apple		La Araucanía	South	72°31’09.28”W	37°37’44.91”S	1
54	Cho-795	Apricot		Biobío	Central-south	72°05’15”W	36°35’47”S	1
55	Cho-799	Blueberry	Liberty	Los Ríos	South	72°41’56”W	40°19’52”S	1
56	Cho-800	Blueberry	Brigitta	La Araucanía	South	72°01’40.74”W	38°55’55.40”S	1
57	Cho-801	Blueberry	Brigitta	La Araucanía	South	72°23’45”W	38°51’22”S	2
58	Cho-802	Blueberry	Brigitta	La Araucanía	South	72°23’45”W	38°51’22”S	2
59	Cho-803	Blueberry	Brigitta	La Araucanía	South	72°23’45”W	38°51’22”S	2
60	Cho-804	Blueberry	Brigitta	La Araucanía	South	72°15’18.1”W	39°0’12”S	2
61	Cho-805	Blueberry	Brigitta	La Araucanía	South	72°15’18.1”W	39°0’12”S	1
62	Cho-806	Blueberry	Brigitta	La Araucanía	South	72°15’18.1”W	39°0’12”S	1
63	Cho-807	Blueberry	Brigitta	La Araucanía	South	72°13’06.42”W	38°58’13.86”S	2
64	Cho-808	Blueberry	Brigitta	La Araucanía	South	72°01’40.74”W	38°55’55.40”S	2
65	Cho-809	Blueberry	Brigitta	Biobío	Central-south	72°00’47.36”W	37°07’19.26”S	1
66	Cho-810	Rose	Queen Elizabeth		Central-south	72°15’39.85”W	36°36’29.03”S	1
67	Cho-811	Blueberry	Brigitta	Los Lagos	South	73°09’57.99”W	40°54’30.35”S	2
68	Cho-812	Blueberry	Drapper	Los Lagos	South	73°09’57.99”W	40°54’30.35”S	2
69	Cho-813	Blueberry	Liberty	La Araucanía	South	71°47’14.19”W	40°05’05.19”S	1
70	Cho-814	Blueberry	Brigitta	La Araucanía	South	72°15’37.5”W	39°11’46”S	2
71	Cho-815	Blueberry	Brigitta	La Araucanía	South	72°15’37.5”W	39°11’46”S	2
72	Cho-816	Blueberry	Brigitta	La Araucanía	South	72°15’37.5”W	39°11’46”S	1
73	Cho-817	Blueberry	Brigitta	La Araucanía	South	72°15’19”W	38°40’17,4”S	1
74	Cho-818	Blueberry	Brigitta	La Araucanía	South	72°15’19”W	38°40’17.4”S	1

N°	Identification	Host	Cultivar	Region	Zone	Coordinates		Molecular grouping
						Longitude	Latitude	
75	Cho-819	Blueberry	Brigitta	La Araucanía	South	72°15’19’’W	38°40’17,4’’S	2
76	Cho-822	Blueberry	Liberty	La Araucanía	South	72°23’45’’W	38°51’2.2’’S	2
77	Cho-823	Blueberry	Brigitta	La Araucanía	South	72°40’34.90’’W	39°06’02.02’’S	2
78	Cho-825	Blueberry	Brigitta	Los Lagos	South	73°07’54.90’’W	40°34’28.22’’S	1
79	Cho-826	Blueberry	Liberty	La Araucanía	South	71°47’14.19’’W	40°05’05.19’’S	2
80	Cho-827	Blueberry	Brigitta	Los Lagos	South	73°07’54.90’’W	40°34’28.22’’S	2
81	Cho-828	Blueberry	Brigitta	Los Lagos	South	73°3’36.1’’W	40°31’51.7’’S	2
82	Cho-831	Blueberry	Brigitta	La Araucanía	South	72°40’34.90’’W	39°06’02.02’’S	2
83	Cho-832	Blueberry	Brigitta	La Araucanía	South	72°33’21.6’’W	39°22’19.3’’S	1
84	Cho-836	Blueberry	Brigitta	La Araucanía	South	72°37’53.45’’W	39°22’05.23’’S	2
85	Cho-837	Blueberry	Brigitta	La Araucanía	South	72°13’27.99’’W	38°40’03.79’’S	1
86	Cho-838	Blueberry	Brigitta	La Araucanía	South	72°13’27.99’’W	38°40’03.79’’S	1
87	Cho-839	Blueberry	Brigitta	Los Lagos	South	73°07’54.90’’W	40°34’28.22’’S	1
88	Cho-840	Blueberry	Brigitta	Los Lagos	South	73°07’54.90’’W	40°34’28.22’’S	1
89	Cho-841	Blueberry	Brigitta	La Araucanía	South	72°40’34.90’’W	39°06’02.02’’S	1
90	Cho-842	Blueberry	Brigitta	La Araucanía	South	72°01’40.74’’W	38°55’55.40’’S	1
91	Cho-843	Blueberry	Brigitta	Los Lagos	South	73°07’54.90’’W	40°34’28.22’’S	2
92	Cho-844	Blueberry	Brigitta	Los Lagos	South	73°07’54.90’’W	40°34’28.22’’S	2
93	Cho-845	Blueberry	Duke	Biobío	Central-south	72°06’04.75’’W	36°50’41.97’’S	1
94	Cho-846	Blueberry	Brigitta	Biobío	Central-south	72°06’04.75’’W	36°50’41.97’’S	2
95	Cho-849	Blueberry	Brigitta	Los Ríos	South	72°22’51.06’’W	40°07’28.35’’S	1
96	Cho-850	Blueberry	Brigitta	Los Ríos	South	72°22’51.06’’W	40°07’28.35’’S	1
97	Cho-851	Blueberry	Brigitta	Los Ríos	South	72°22’51.06’’W	40°07’28.35’’S	2
98	Cho-852	Blueberry	Brigitta	Los Ríos	South	71°47’14.19’’W	40°05’05.19’’S	2
99	Cho-860	Blueberry	Brigitta	La Araucanía	South	72°13’27.99’’W	38°40’03.79’’S	2
100	Cho-861	Blueberry	Brigitta	Los Lagos	South	73°5’11.3’’W	40°54’30.2’’S	2
101	Cho-863	Blueberry	Brigitta	Los Lagos	South	73°5’11.3’’W	40°54’30.2’’S	1
102	Cho-864	Blueberry	Brigitta	La Araucanía	South	72°13’27.99’’W	38°40’03.79’’S	1
103	Cho-865	Blueberry	Bluecrop	Los Lagos	South	73°5’11.3’’W	40°54’30.2’’S	1
104	Cho-867	Blueberry	Brigitta	Los Lagos	South	73°5’11.3’’W	40°54’30.2’’S	2
105	Cho-868	Blueberry	Brigitta	Valparaíso	Central	71°23’49.02’’W	33°17’ 38.17’’S	1
106	Cho-869	Eucalyptus	E. globulus	Biobío	Central-south	71°47’00.79’’W	36°42’02.34’’S	1
107	Cho-870	Blueberry	Brigitta	Biobío	Central-south	73°22’43.7’’W	37°43’25’’S	1
108	Cho-871	Peach	Canning	Valparaíso	Central	71°13’46.87’’W	32°49’20.71’’S	1
109	Cho-872	Peach	Nectarine	Valparaíso	Central	71°13’46.87’’W	32°49’20.71’’S	2
110	Cho-874	Cherry		La Araucanía	South	72°22’38.90’’W	38°40’38.49’’S	2
111	Cho-875	Blueberry	Brigitta	La Araucanía	South	72°35’4.19’’W	38°44’2.07’’S	1
112	Cho-876	Blueberry	Brigitta	La Araucanía	South	72°35’4.19’’W	38°44’2.07’’S	2
113	Cho-880	Blueberry	Drapper	Los Lagos	South	73°03’11’’W	40°31’20’’S	1
114	Cho-882	Blueberry	Brigitta	La Araucanía	South	72°37’53.45’’W	39°22’05.23’’S	1
115	Cho-883	Blueberry	Brigitta	La Araucanía	South	72°37’53.45’’W	39°22’05.23’’S	1
116	Cho-905	Cherry	Lapins	Biobío	Central-south	72°06’08.72’’W	36°36’33.67’’S	2
117	Cho-917	Apple	Fuji	Biobío	Central-south	72°06’08.72’’W	36°36’33.67’’S	2
118	Cho-924	Apple	Granny Smith	L.B. O’Higgins	Central	70°43’59.88’’W	34°04’00.12’’S	1
119	Cho-927	Pistachio		L.B. O’Higgins	Central	70°50’04.95’’W	34°20’06.13’’S	1

Recibido: 05.01.2016
Aceptado: 20.05.2017