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# Identity and pathogenicity of *Botryosphaeriaceae* and *Diaporthaceae* from *Juglans regia* in Chile

Israel JIMENEZ LUNA[1], Ximena BESOAIN[2], Sebastian SAA[2,3], Elena PEACH-FINE[2,4], Fabiola CADIZ MORALES[2], Natalia RIQUELME[2], Alejandra LARACH[2], Javiera MORALES[2], Exequiel EZCURRA[1], Vanessa E.T.M. ASHWORTH[1], Philippe E. ROLSHAUSEN[1,*]

[1] *Botany and Plant Sciences Department, University of California, Riverside, CA, USA*
[2] *Escuela de Agronomía, Pontificia Universidad Católica de Valparaíso, Chile*
[3] *Almond Board of California, Modesto, CA, USA*
[4] *School of Engineering and Computer Science, University of the Pacific, Stockton, CA, USA*
[*]Corresponding author. E-mail: philrols@ucr.edu

**Summary.** English walnut (*Juglans regia*) has become an important crop in Chile, representing 11.5% of the total area of fruit trees, surpassed only by grapevine. As the Chilean walnut industry rapidly expands, young orchards are at risk from the emergence of new fungal diseases. *Botryosphaeriaceae* and *Diaporthaceae* fungi have been recognized as main causes of wood diseases in walnut, with symptoms of dieback, canker, and blight. In winter 2017, samples were collected from different orchards in Valparaíso and Maule regions. Fungal isolates recovered were cultured, characterized morphologically, and identified using DNA sequence analyses. Three species (*Neofusicoccum parvum*, *Diplodia mutila*, *Diplodia seriata*) were characterized in *Botryosphaeriaceae* and two (*Diaporthe cynaroidis*, *Diaporthe australafricana*) in *Diaporthaceae*. Pathogenicity tests showed that *N. parvum* was the most aggressive species to walnut. This study confirmed the presence of pathogenic *Botryosphaeriaceae* and *Diaporthaceae* in *J. regia* that should be considered an increasing risk for the growing Chilean walnut industry.

**Keywords.** *Diaporthe*, *Diplodia, Neofusicoccum,* walnut, wood canker, host range.

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## INTRODUCTION

During the last two decades, production of English walnut (*Juglans regia* L.) has rapidly increased, with China as the main producer (369,000 metric tons), followed by the United States of America (USA) (250,389 metric tons) (www.nutfruit.org). Chile has also become a major producer, with approx. 49,000 planted ha mainly of cultivar 'Chandler' and 150,000 metric tons (Muñóz, 2017), positioning Chile as the third walnut exporting country worldwide.

*Botryosphaeriaceae* is one of the major fungal groups adversely affecting walnut production, including in California, USA (Chen *et al*., 2014),

Spain (López-Moral *et al*., 2020), China (Li *et al*., 2015) and Iran (Abdollahzadeh *et al*., 2013). Symptoms caused by these fungi include canker on trunks and scaffold branches and dieback of spurs and shoots resulting from previous infections of fruit peduncles and leaf scars (Moral *et al*., 2019b). Fungi reported as pathogenic to walnut include *Botryosphaeria dothidea*, *Diplodia mutila*, *Dip. seriata*, *Dothiorella iberica*, *Dot. omnivora*, *Dot. sarmentorum*, *Lasiodiplodia citricola*, *Las. pseudotheobromae*, *Las. theobromae*, *Neofusicoccum mediterraneum*, *Neof. nonquaesitum*, *Neof. parvum*, *Neof. ribis*, *Neof. vitifusiforme*, and *Neoscytalidium dimidiatum* (Haggag *et al*., 2007; Rumbos 2007; Chen *et al*., 2013a, 2014, 2019; Li *et al*., 2015; Eichmeier *et al*., 2020; Gusella *et al*., 2020; López-Moral *et al*., 2020). *Lasiodiplodia citricola* and *Neof. parvum* (Chen *et al*., 2013a, 2014) have been determined to be highly aggressive to English walnut, while *D. seriata* and *Dot. sarmentorum* are considered less aggressive (López-Moral *et al*., 2020). Furthermore, *Neof. parvum* has been reported to be widely distributed in over 90 hosts in more than 29 countries on six continents (Sakalidis *et al*., 2013; Gusella *et al*., 2020).

In contrast, fungi in the *Diaporthaceae* are less aggressive to English walnut than *Botryosphaeriaceae* species (Chen *et al*., 2014). Symptoms include stem dieback and branch canker, shoot blight, leaf spot and fruit rot (Chen *et al*., 2014). *Diaporthe amygdali, D. bicincta*, *D. eres*, *D. euonymi*, *D. juglandis*, *D. neotheicola*, *D. rhusicola*, *D. rostrata*, *D. rudis*, *Phomopsis albobestita*, and *P. arnoldiae* have been associated with *Juglans* spp. in America, Europe, and Asia, (Uecker 1988; Anagnostakis 2007; Udayanga *et al*., 2011, 2014; Gomes *et al*., 2013; Chen *et al*., 2014; Fan *et al*., 2015, 2018; López-Moral *et al*., 2020). *Diaporthe neotheicola* has been reported as the most widespread pathogen in several hosts including walnut (Chen *et al*., 2014; López-Moral *et al*., 2020).

In Chile, *Bot. dothidea*, *Dip. mutila*, *D. australafricana* and *D. cynaroidis* have been described as pathogens of walnut (Rina 2010; Díaz *et al*., 2018a; Jiménez Luna *et al*., 2020; ). Additionally, several studies have documented the presence of *Botryosphaeriaceae* and *Diaporthaceae* species on other cultivated crops or tree hosts, including grapevine (*Vitis vinifera*) (Auger *et al*., 2004; Morales *et al*., 2012; Valencia *et al*., 2015; Larach *et al*., 2020), apple (*Malus domestica*) (Diaz *et al*., 2018b, 2018c), avocado (*Persea americana*) (Valencia *et al*., 2019), highbush blueberry (*Vaccinium corymbosum*) (Guerrero *et al*., 1987; Espinoza *et al*., 2008, 2009; Elfar *et al*., 2013), kiwifruit (*Actinidia deliciosa*) (Díaz *et al*., 2017; Palma *et al*., 2000), hazelnut (*Corylus avellana*) (Guerrero and Pérez, 2013), and native forest trees including *Araucaria araucana*, *Drimys winter*, and *Aristotelia chilensis* (Besoain *et al*., 2019; Zapata *et al*., 2020).

A common avenue for species in both *Botryosphaeriaceae* and *Diaporthaceae* to infect trees is through spores depositing on tree wounds caused by pruning, mechanical trunk shakers and wind injuries (Agustí-Brisach *et al*., 2019; Moral *et al*., 2019a, 2019b). Implementing cost-effective preventative practices that limit the incidence of these pathogens is key to long-term profitability of walnut orchards. The Chilean Institute of Agricultural Research (INIA) has begun a research program to identify fungi involved in walnut dieback and canker, and conduct fungicide tests to develop disease management strategies (Gamalier and Valeria, 2019). Species in *Botryosphaeriaceae* and *Diaporthaceae* have been shown to be threats to walnut production in several countries, and many species have already been found on other crops in Chile. The goal of this study is to establish the baseline of infection through an extensive survey in new walnut production areas in Chile and identify the taxonomic names of the species associated with walnut wood diseases using phylogenetic analysis and confirm pathogenicity with standard plant bioassays.

## MATERIALS AND METHODS

### *Sampling locations and collection of fungi*

In the winter of 2017, 13 walnut orchards (5 to 15 years old) from the major Chilean production regions were surveyed. These were in the central zone of Chile, including two orchards in the Valparaíso Region, four in the O'Higgins Region and seven in the Maule Region. Only five of these orchards (all cultivar 'Chandler') showed symptoms of dieback, with four orchards in the Maule Region and one in the Valparaíso Region. Twenty-five symptomatic wood samples were collected (five symptomatic trees × five samples/tree) from each orchard. Fungal isolates obtained were from trees with diseased branches and twigs showing necrotic brown discolorations in the cortical and vascular tissues.

Symptomatic wood samples were disinfected with 1% sodium hypochlorite for 30 s and then rinsed three times in sterile water. Five wood chips (≈3 × 3 ×3 mm) were placed in Petri dishes containing 2% potato dextrose agar (PDA; Difco Laboratories) supplemented with 0.2 g per L of tetracycline to suppress bacterial growth (Morales *et al*., 2012; Chen *et al*., 2014). Pure fungal cultures were grown on 2% agar with the addition of sterile grape leaves to stimulate formation of conidia so that cultures could be examined morphologically. Identification of morphotype isolates was based on morphological

characters as described by Phillips *et al.* (2013) for the *Botryosphaeriaceae*, and Udayanga *et al.* (2011) for the *Diaporthaceae*.

*DNA extraction, and PCR amplification and sequencing*

Young mycelium covering an area of approx. 2 $cm^2$ from each pure culture was removed for DNA extraction. DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. Each PCR amplification was performed in a 25 µL reaction volume on a T100 thermal cycler (BioRad). Each reaction consisted of 17.4 µL of sterile $H_2O$, 2.5 µL of PCR buffer, 1 µL dNTPs (10 mM), 0.5 µL of each primer (4 µM), 2 µL $MgCl_2$ (25 mM), and 0.1 µL of *Taq* DNA polymerase (5 u $\mu L^{-1}$), with DNA added at 1–2 µL (10-20 ng DNA $\mu L^{-1}$). The thermocycler setting consisted of initial denaturation at 94°C for 2 min, then 35 cycles of the following three steps: 1 min at 94°C for strand separation, 1 min at 58 to 65°C for primer annealing and 1 min at 72°C for amplification. The final extension step was for 3 min at 72°C. Amplicons were run on a 1% agarose gel using gel electrophoresis and were then stained with Gel Red dye (Biotium Inc.). The DNA regions amplified were the nuclear ribosomal internal transcribed spacer (ITS) region using the ITS1-ITS4 primer pair at 58°C annealing temperature (White *et al.*, 1990), the *translation elongation factor 1-α* (EF) gene using primers EF1-728F and EF1-986R at 58°C annealing temperature (Carbone *et al.*, 1999), and the *β-tubulin* (TUB) gene using primers Bt2a and Bt2b at 65°C annealing temperature (Glass and Donaldson, 1995). Resulting bands were visualized under UV light using a Gel Doc Imager (Bio-Rad), and PCR products were purified using the QiaQuick PCR Purification Kit (Qiagen). Forward and reverse reads were generated by Sanger sequencing, carried out at the UCR Institute of Integrative Genome Biology.

*Phylogenetic analyses*

Forward and reverse reads of each DNA sample were edited and combined into a consensus sequence using Sequencher v. 5.0.1 (Gene Codes Corporation). Sequences from each region were concatenated using Geneious v. 2020.1.1 (Biomatters Ltd) and aligned using ClustalW implemented in MEGA 7 (Kumar *et al.*, 2016) with manual adjustments. Sequence alignments and phylogenetic analyses were performed separately for *Botryosphaeriaceae* and *Diaporthaceae*. CBS and CMW type specimens and taxa identified from walnut and cultivated and wild plant hosts in Chile were used as reference sequences for phylogenetic reconstructions. Reference taxa were obtained from fungal culture collections, including the Westerdijk Institute/Centraalbureau voor Schimmelcultures, CBS-KNAW, Utrecht, The Netherlands, and the CMW collection of the Forestry and Agricultural Biotechnology Institute, Pretoria, South Africa. The nucleotide sequences of reference taxa were downloaded from the GenBank sequence database maintained by the National Center for Biotechnology Information (NCBI). All accession numbers are listed in Table 1.

The complete dataset of *Botryosphaeriaceae* consisted of three novel sequences and 28 reference sequences. The outgroup was *Cophinforma atrovirens* (Zhang *et al.*, 2021). The *Diaporthaceae* dataset consisted of 2 novel sequences and 35 reference sequences. The outgroup was *Diaporthella corylina* (Gomes *et al.*, 2013). Sequences were aligned using ClustalW implemented in MEGA 7 (Kumar *et al.*, 2016), with manual adjustments. Phylogenetic trees for the *Botryosphaeriaceae* and *Diaporthaceae* were constructed using Maximum Likelihood, with the optimal nucleotide substitution model determined by the corrected Akaike Information Criterion (AICc; Akaike, 1974; Hurvich and Tsai, 1989). Nodal support consisted of nonparametric bootstrapping with 1000 replicates. All positions containing gaps and missing data were eliminated. Single-gene phylogenies from each of the three gene partitions were also examined for the *Botryosphaeriaceae* and *Diaporthaceae* datasets to check for incongruence. Constructed trees are presented in Figure S1. Bootstrap support values for clades containing isolates obtained in the present study are shown in Table S1.

Separate phylogenetic analyses were performed for all fungal pathogens reported from alternative hosts (tree crops and wild hosts) in Chile for which DNA sequences were available in GenBank. These analyses were based on the ITS region alone because sequences for EF and TUB were not available for all the taxa in the NCBI database.

In planta *pathogenicity tests*

Although fungal isolates were collected in winter 2017, pathogenicity tests were carried out in winter 2019, when a completely randomized experimental design was set up for the morphotypes of each identified fungal species: *Neof. parvum*, *Dip. mutila*, *Dip. seriata*, *D. australafricana* and *D. cynaroidis*. The test was conducted on trees planted under field conditions at the Escuela de Agronomía, Pontificia Universidad Católica de Valparaíso, Chile. The cultivar used for inoculation was a 1.5-year-old 'Chandler' walnut

**Table 1.** GenBank accession numbers, hosts and species identity for all *Botryosphaeriaceae* and *Diaporthaceae* taxa used for phylogenetic analyses.

| Identity | Collection code | Host | Country of Origin | ITS | EF | TUB |
|---|---|---|---|---|---|---|
| ***Botryosphaeriaceae*** | | | | | | |
| *Botryosphaeria dothidea* | CMW 8000 | *Prunus* sp. | Switzerland | AY236949 | AY236898 | AY236927 |
| *Bot. dothidea* | CMW 7780 | *Fraxinus excelsior* | Switzerland | AY236947 | AY236896 | AY236925 |
| *Cophinforma atrovirens* | CBS 117451 | *Eucalyptus* sp. | Venezuela | KX464086 | KX464556 | KX464782 |
| *Diplodia africana* | RGM 2718 | *Araucaria araucana* | Chile | MN046380 | - | - |
| *Dip. africana* | CBS 120835 | - | South Africa | MH863094 | - | - |
| *Dip. mutila* | CBS 112553 | *Vitis vinifera* | Portugal | AY259093 | AY573219 | DQ458850 |
| *Dip. mutila* | CBS 230.30 | *Phoenix dactylifera* | USA | DQ458886 | DQ458869 | DQ458849 |
| *Dip. mutila* | 4D33 | *Persea americana* | CA, USA | KF778789 | KF778979 | KF778884 |
| *Dip. mutila* | PALUC1M | *Persea americana* | Chile | MF568683 | - | - |
| *Dip. mutila* | DMnog4 | *Juglans regia* | Chile | MG386824 | - | - |
| *Dip. mutila* | Mz-F22 | *Malus domestica* | Chile | MG450386 | - | - |
| *Dip. mutila* | Sample 301 | *Juglans regia* | Chile | MW412902 | MW574125 | MW596891 |
| *Dip. pinea* | CMW 39341 | *Cedrus deodara* | Montenegro | KF574998 | KF575028 | KF575094 |
| *Dip. pinea* | CMW 39338 | *Cedrus atlantica* | Serbia | KF574999 | KF575029 | KF575095 |
| *Dip. sapinea* | CMW 190 | *Pinus resinosa* | USA | KF766159 | AY624251 | AY624256 |
| *Dip. seriata* | CBS 112555 | *Vitis vinifera* | Portugal | AY259094 | AY573220 | DQ458856 |
| *Dip. seriata* | CBS 119049 | *Vitis* sp. | Italy | DQ458889 | DQ458874 | DQ458857 |
| *Dip. seriata* | PALUC14M | *Persea americana* | Chile | MF578223 | - | - |
| *Dip. seriata* | KJ 93.56 | *Vitis vinifera* | Chile | AF027759 | - | - |
| *Dip. seriata* | Mz-F1 | *Malus domestica* | Chile | KU942427 | - | - |
| *Dip. seriata* | Sample 105 | *Juglans regia* | Chile | MW412901 | MW574124 | MW596890 |
| *Dip. scrobiculata* | CBS 109944 | *Pinus greggii* | Mexico | DQ458899 | DQ458884 | DQ458867 |
| *Dip. scrobiculata* | CBS 113423 | *Pinus greggii* | Mexico | DQ458900 | DQ458885 | DQ458868 |
| *Dothiorella iberica* | CBS 115041 | *Quercus ilex* | Spain | AY573202 | AY573222 | EU673096 |
| *Dot. iberica* | CBS 113188 | *Quercus suber* | Spain | AY573198 | EU673278 | EU673097 |
| *Dot. iberica* | PALUC3M | *Persea americana* | Chile | MF578225 | - | - |
| *Dot. sarmentorum* | CBS 115038 | *Malus pumila* | The Netherlands | AY573206 | AY573223 | EU673101 |
| *Lasiodiplodia citricola* | 6I34 | *Juglans regia* | CA, USA | KF778809 | KF778999 | KF778904 |
| *Las. citricola* | CBS 124707 | *Citrus* sp. | Iran | GU945354 | GU945340 | KU887505 |
| *Las. citricola* | IRNKB3 | *Juglans regia* | Iran | MN634040 | MN633994 | MN633442 |
| *Las. pseudotheobromae* | CBS 116459 | *Gmelina arborea* | Costa Rica | EF622077 | EF622057 | EU673111 |
| *Las. theobromae* | CBS 164.96 | Fruit along coral reef coast | Papua New Guinea | AY640258 | AY640255 | EU673110 |
| *Las. theobromae* | PALUC449F | *Persea americana* | Chile | MF578754 | - | - |
| *Neofusicoccum arbuti* | B03-07 | Blueberry 'Aurora' | Chile | EU856061 | - | - |
| *Neof. arbuti* | UW01 | *Arbutus menziesii* | WA, USA | AY819720 | - | - |
| *Neof. australe* | CMW 6837 | *Acacia* sp. | Australia | AY339262 | - | - |
| *Neof. australe* | CAP258 | *Olea europaea* | Italy | EF638778 | - | - |
| *Neof. australe* | PALUC439F | *Persea americana* | Chile | MF578755 | - | - |
| *Neof. australe* | B1-05 | Blueberry 'Duke' | Chile | EU856059 | - | - |
| *Neof. australe* | vid-1559 | *Vitis vinifera* | Chile | JX290091 | - | - |
| *Neof. mediterraneum* | 6I29 | *Juglans regia* | CA, USA | KF778849 | KF779039 | KF778944 |
| *Neof. nonquaesitum* | UCR2733 | *Persea americana* | USA | KT965281 | | - |
| *Neof. nonquaesitum* | PALUC4M | *Persea americana* | Chile | MF578228 | | - |
| *Neof. nonquaesitum* | CABI IMI-500168 | *Vaccinium corymbosum* | Chile | JX217819 | - | - |
| *Neof. nonquaesitum* | 4L78 | *Juglans regia* | CA, USA | KF778851 | KF779041 | KF778946 |
| *Neof. nonquaesitum* | PD90 | *Prunus dulcis* | CA, USA | GU251157 | GU251289 | GU251817 |
| *Neof. parvum* | CBS 110301 | *Vitis vinifera* | Portugal | AY259098 | AY573221 | EU673095 |

(Continued)

**Table 1.** (Continued).

| Identity | Collection code | Host | Country of Origin | ITS | EF | TUB |
|---|---|---|---|---|---|---|
| *Neof. parvum* | CMW9080 | *Populus nigra* | New Zealand | AY236942 | - | - |
| *Neof. parvum* | CMW 9081 | *Populus nigra* | New Zealand | AY236943 | AY236888 | AY236917 |
| *Neof. parvum* | PALUC16M | *Persea americana* | Chile | MF578229 | - | - |
| *Neof. parvum* | B1-06 | Blueberry 'Mistry' | Chile | EU856063 | - | - |
| *Neof. parvum* | Sample 172 | *Juglans regia* | Chile | MW412903 | MW574126 | MW596892 |
| *Neof. vitifusiforme* | 5H02 | *Juglans regia* | CA, USA | KF778868 | KF779058 | KF778963 |
| *Neof. vitifusiforme* | CBS 110881 | *Vitis vinifera* | South Africa | AY343383 | AY343343 | KX465061 |
| *Neoscytalidium dimidiatum* | CBS 499.66 | *Mangifera indica* | Mali | FM211432 | EU144063 | FM211167 |
| ***Diaporthaceae*** | | | | | | |
| *Diaporthe ambigua* | CBS 114015 | *Pyrus communis* | South Africa | KC343010 | - | - |
| *D. ambigua* | 6-KF | *Actinidia deliciosa* | Chile | KJ210025 | - | - |
| *D. ambigua* | 5.5.4r1(2) | *Vaccinium* sp. | Chile | KC143171 | - | - |
| *D. ampelina* | CBS 111888 | *Vitis vinifera* | USA | KC343016 | KC343742 | KC343984 |
| *D. amygdali* | CBS 115620 | *Prunus persica* | GA, USA | KC343020 | KC343746 | KC343988 |
| *D. amygdali* | CBS 126679 | *Prunus dulcis* | Portugal | KC343022 | KC343748 | KC343990 |
| *D. amygdali* | ColPat-533 | *Juglans regia* 'Chandler' | Spain | MK447999 | MK490937 | MK522117 |
| *D. araucanorum* | RGM 2472 | *Araucaria araucana* | Chile | MN509709 | - | - |
| *D. asheicola* | CBS 136968 | *Vaccinium ashei* | Chile | KJ160563 | KJ160595 | KJ160519 |
| *D. asheicola* | CBS 136967 | *Vaccinium ashei* | Chile | KJ160562 | KJ160594 | KJ160518 |
| *D. australafricana* | CBS 111886 | *Vitis vinifera* | Australia | KC343038 | KC343764 | KC344006 |
| *D. australafricana* | CBS 113487 | *Vitis vinifera* | South Africa | KC343039 | KC343765 | KC344007 |
| *D. australafricana* | 16-KF | *Actinidia deliciosa* | Chile | KX999702 | - | - |
| *D. australafricana* | Pho73-07 | *Vaccinium* sp. | Chile | KC143190 | - | - |
| *D. australafricana* | 15.2.2(4) | *Vaccinium* sp. | Chile | KC143175 | - | - |
| *D. australafricana* | Sample 302 | *Juglans regia* | Chile | MW407063 | MW574121 | MW574123 |
| *D. beckhausii* | CBS 138.27 | *Viburnum* sp. | - | KC343041 | KC343767 | KC344009 |
| *D. chamaeropis* | CBS 454.81 | *Chamaerops humilis* | Greece | KC343048 | KC343774 | KC344016 |
| *D. chamaeropis* | CBS 753.70 | *Spartium junceum* | Croatia | KC343049 | KC343775 | KC344017 |
| *D. cynaroidis* | CBS 122676 | *Protea cynaroidis* | South Africa | KC343058 | KC343784 | KC344026 |
| *D. cynaroidis* | Sample 102 | *Juglans regia* | Chile | MW407062 | MW574120 | MW574122 |
| *D. eres* | CBS 101742 | *Fraxinus* sp. | The Netherlands | KC343073 | KC343799 | KC344041 |
| *D. eres* | CPC 16510 | *Vaccinium corymbosum* | Chile | KJ160572 | - | - |
| *D. foeniculina* | CBS 117166 | *Aspalathus linearis* | South Africa | DQ286286 | - | - |
| *D. foikelawen* | RGM 2539 | *Drimys winteri* | Chile | MN509713 | - | - |
| *D. neotheicola* | CBS 123208 | *Foeniculum vulgare* | Portugal | EU814480 | GQ250315 | JX275464 |
| *D. neotheicola* | 6I30 | *Juglans regia* | CA, USA | KF778871 | KF779061 | KF778966 |
| *D. neotheicola* | 3.4.4r1(1) | *Vaccinium* sp. | Chile | KC143192 | - | - |
| *D. neotheicola* | ColPat-445 | *Juglans regia* 'Tulare' | Spain | MK447993 | MK490932 | MK522106 |
| *D. neotheicola* | ColPat-448 | *Juglans regia* 'Serr' | Spain | MK447994 | MK490939 | MK522107 |
| *D. neotheicola* | ColPat-450 | *Juglans regia* 'Vina' | Spain | MK447996 | MK490934 | MK522109 |
| *D. neotheicola* | ColPat-532 | *Juglans regia* 'Chandler' | Spain | MK447998 | MK490936 | MK522111 |
| *D. neotheicola* | ColPat-551 | *Juglans regia* 'Hartley' | Spain | MK448000 | MK490940 | MK522112 |
| *D. nobilis* | CBS 200.39 | *Laurus nobilis* | Germany | KC343151 | KC343877 | KC344119 |
| *D. novem* | CBS 127271 | *Glycine max* | Croatia | KC343157 | - | - |
| *D. novem* | 1-KF | *Actinidia deliciosa* | Chile | KJ210020 | - | - |
| *D. passiflorae* | CPC 19183 | *Passiflora edulis* | South America | JX069860 | - | - |
| *D. passiflorae* | 15.3.1r1 | *Vaccinium* sp. | Chile | KC143196 | - | - |

(Continued)

**Table 1.** (Continued).

| Identity | Collection code | Host | Country of Origin | ITS | EF | TUB |
|---|---|---|---|---|---|---|
| *D. rudis* | 10-KF | *Actinidia deliciosa* | Chile | KJ210029 | - | - |
| *D. rudis* | CBS 449.82 | *Lupinus* sp. | The Netherlands | KC343240 | KC343966 | KC344208 |
| *D. rudis* | CBS 100170 | *Fraxinus excelsior* | The Netherlands | KC343230 | KC343956 | KC344198 |
| *D. rudis* | CBS 114011 | *Vitis vinifera* | Portugal | KC343235 | KC343961 | KC344203 |
| *D. rudis* | CBS 113201 | *Vitis vinifera* | Portugal | KC343234 | KC343960 | KC344202 |
| *D. rhusicola* | CBS 129528 | *Rhus pendulina* | South Africa | JF951146 | KC843100 | KC843205 |
| *D. rhusicola* | 6I14 | *Prunus dulcis* | CA, USA | KF778872 | KF779062 | KF778967 |
| *D. rhusicola* | 6I31 | *Juglans regia* | CA, USA | KF778874 | KF779064 | KF778969 |
| *D. rhusicola* | ColPat-444 | *Juglans regia* 'Tulare' | Spain | MK447992 | MK490931 | MK522105 |
| *D. sterilis* | CBS 136969 | *Vaccinium corymbosum* | Italy | KJ160579 | KJ160611 | KJ160528 |
| *D. sterilis* | CBS 136970 | *Vaccinium corymbosum* | Italy | KJ160580 | KJ160612 | KJ160529 |
| *D. toxica* | CBS 534.93 | *Lupinus angustifolius* | Western Australia | KC343220 | KC343946 | kC344188 |
| *D. toxica* | CBS 546.93 | *Lupinus* sp. | Western Australia | KC343222 | KC343948 | KC344190 |
| *D. vaccinia* | CBS 160.32 | *Vaccinium macrocarpon* | USA | KC343228 | KC343954 | KC344196 |
| *D. amygdali* | ColPat-533 | *Juglans regia* 'Chandler' | Spain | MK447999 | MK490937 | MK522117 |
| *D. amygdali* | CBS 126679 | *Prunus dulcis* | Portugal | KC343022 | KC343748 | KC343990 |
| *D. amygdali* | CBS 115620 | *Prunus persica* | GA, USA | KC343020 | KC343746 | KC343988 |
| *D. cf. heveae* | CBS 852.97 | *Hevea brasiliensis* | Brazil | KC343116 | KC343842 | KC344084 |
| *Diaporthella corylina* | CBS 121124 | *Corylus* sp. | China | KC343004 | KC343730 | KC343972 |

scion grafted to a Vlach clonal rootstock. Pathogenicity tests were conducted on two sets of plants inoculated at different times for logistical reasons, with one set used for the three *Botryosphaeriaceae* species and one set for the two *Diaporthe* species. A total of 70 trees were inoculated, with ten trees for each treatment. The experiment was repeated twice. Inoculations were each conducted by using 3 mm diam. mycelium/PDA plugs from a 7-d-old pure culture. Each stem wound was produced 30 cm above ground (half-way up the stem), and a 3 mm diam. hole was produced with a cork borer to insert an agar plug bearing mycelia. Negative controls were inoculated with sterile 2% PDA plugs. After inoculation, the wounds were wrapped with parafilm. Data were recorded 3 months after inoculation by measuring canker lengths in the host xylem tissues. To complete Koch's postulates, pathogens were re-isolated and cultured in 2% PDA medium, and presence of each pathogen was confirmed morphologically.

### *Statistical analyses*

The data collected were analyzed using R studio and depicted as box and whisker plots. The data were subjected to analysis of variance, and treatment means were compared using Tukey's least significant difference test at $P \leq 0.05$.

## RESULTS

Five samples collected from one orchard in the Valparaíso Region were infected with *Botryosphaeriaceae* fungi. All these isolates developed gray mycelium which then became dark green with fusoid, hyaline and thin-walled conidia. The isolates were keyed as *Neofusicoccum* according to Phillips *et al.* (2013). Of the 20 samples collected from the four orchards in the Maule Region, 14 samples were infected with *Botryosphaeriaceae* fungi, and six samples were infected with *Diaporthaceae* fungi. *Botryosphaeriaceae* isolates had abundant aerial and initially white to white-gray fast-growing mycelium that turned dark green with time. Conidia were thick-walled and aseptate. Isolates keyed as *Diplodia* according to Phillips *et al.* (2013), were of two morphotypes which were separated on the basis on conidium pigmentation, one with hyaline conidia and the other with brown conidia (Phillips *et al.*, 2013). *Diaporthe* isolates were characterized by production of black conidiomata with alpha conidia in cultures (Udayanga *et al.*, 2011). Two morphotypes were separated on the basis of production of beta conidiospores with only one morphotype producing these conidiophores. Three DNA loci (ITS, TUB, and EF) were sequenced for species identification of the five selected morphotypes, including three *Botryosphaeriaceae* (one *Neofusicoccum* sp. and two *Diplodia* spp.) and two *Diaporthaceae*.

Alignment of 32 DNA sequences from species in the *Botryosphaeriaceae* resulted in a dataset of 1308 nucleotide positions (557 positions in the ITS partition, 330 in the EF partition and 421 in the TUB partition). These included 871 conserved sites (ITS = 436, EF = 108, TUB = 327), 425 variable sites (ITS = 121, EF = 201, TUB = 103), 371 parsimony-informative sites (ITS = 106, EF = 182, TUB = 83), and 54 singleton sites (ITS = 15, EF = 19, TUB = 20). The optimum model of nucleotide substitution inferred using the AICc was the Tamura-Nei model (Tamura and Nei, 1993), with a discrete Gamma distribution and a proportion of invariant sites (TN93 + G + I). The tree with the greatest log likelihood (-3242.47) is shown in Figure 1. The phylogenetic analyses supported with strong bootstrap values the placement of the three *Botryosphaeriaceae* samples 301, 105 and 172 in, respectively, the *Dip. mutila*, *Dip. seriata* and *Neof. parvum* clades.

A separate phylogenetic analysis including ITS sequences of fungal samples previously identified from alternate hosts in Chile was also generated (Figure 2). Alignment of 54 DNA sequences of species in *Botryosphaeriaceae* resulted in a dataset of 1211 nucleotide positions. These included 408 conserved sites, 139 variable sites, 120 parsimony-informative sites, and 19 singleton sites. The optimum model of nucleotide substitution inferred using the AICc was the Tamura-Nei model (Tamura and Nei, 1993), with a discrete Gamma distri-

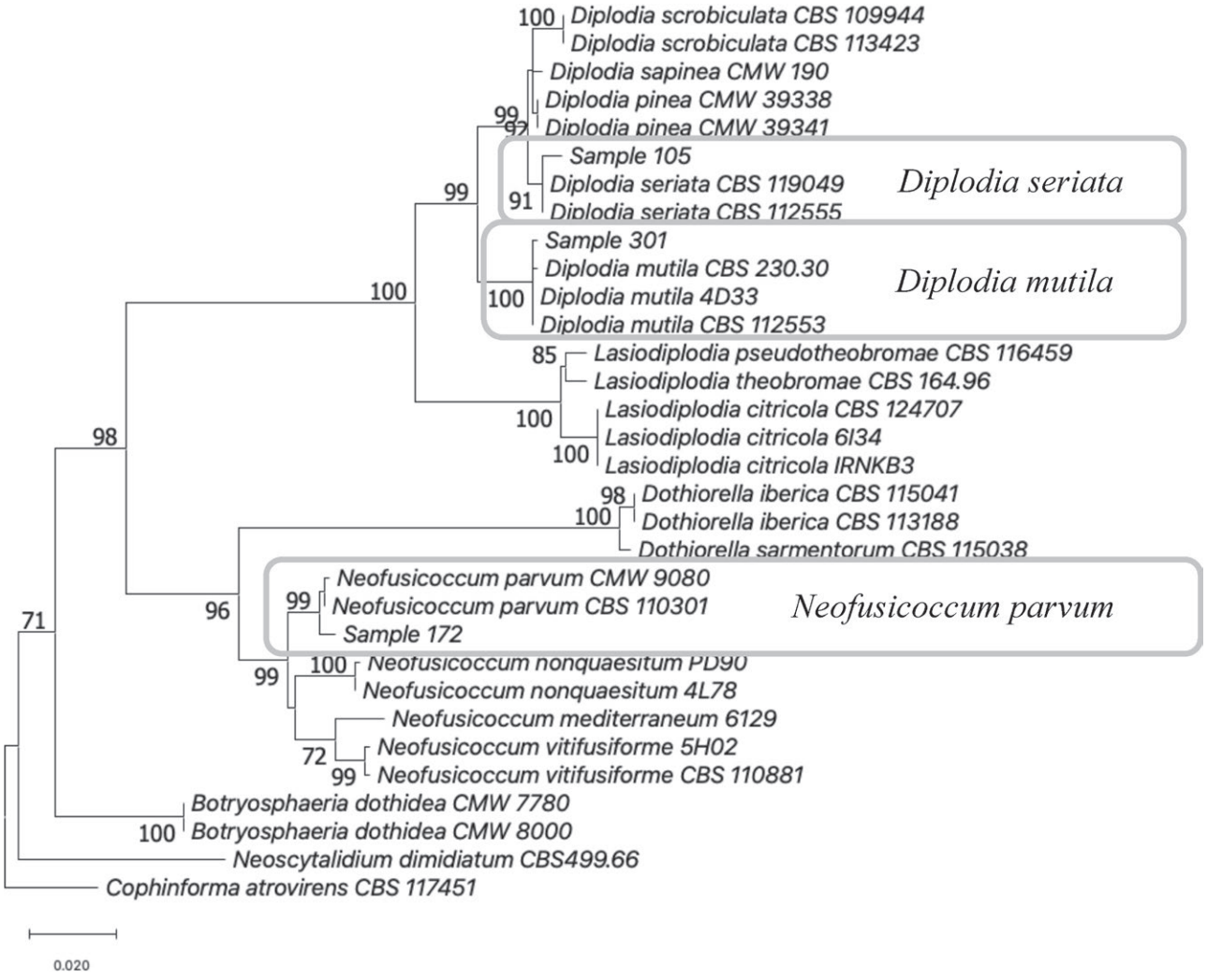


**Figure 1.** Phylogenetic tree reconstructed by maximum likelihood analysis from the concatenated sequences of the nuclear ribosomal internal transcribed spacer, translation elongation factor and beta-tubulin for three *Botryosphaeriaceae* species isolated from three walnut orchards in Chile and 29 *Botryosphaeriaceae* reference sequences retrieved from the GenBank database. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Numbers on branches are bootstrap support values.

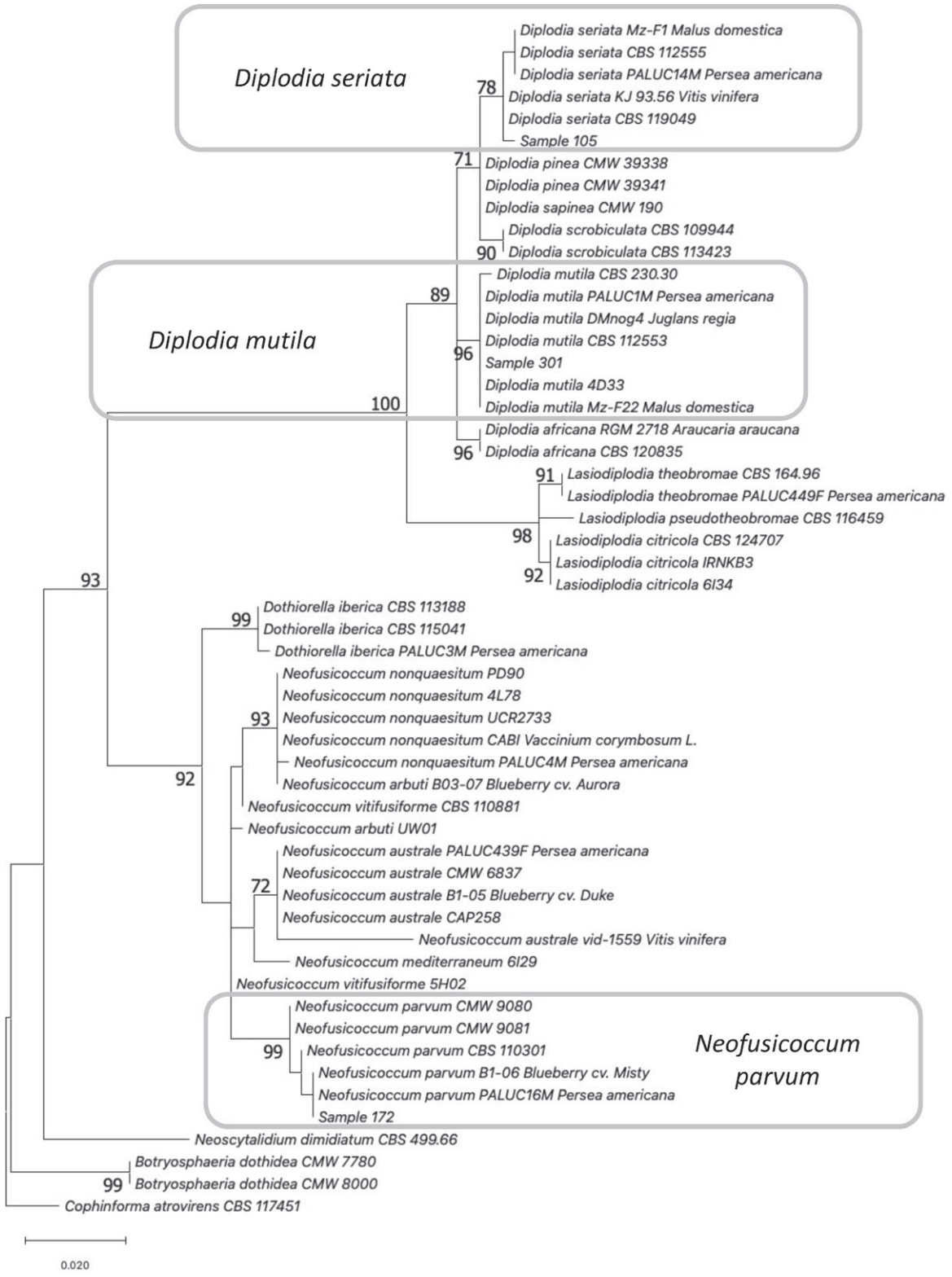


**Figure 2.** Phylogenetic tree reconstructed by maximum likelihood analysis from the sequences of the nuclear ribosomal internal transcribed spacer. The dataset included three novel *Botryosphaeriaceae* taxa isolated from three walnut orchards in Chile and 51 GenBank sequences of *Botryosphaeriaceae* that included reference sequences as well as sequences of previously reported Chilean walnut pathogens. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Numbers on branches are bootstrap support values.

bution and a proportion of invariant sites (TN93 + G + I). The tree with the greatest log likelihood (-1363.49) is shown in Figure 2. The results illustrated the diversity of pathogens in *Botryosphaeriaceae* present in Chile and the range of crops affected by these pathogens. Sample 301 was one of four *Dip. mutila* isolates reported in Chile, with the three others originating from apple, avocado and walnut (Díaz *et al*., 2018b; Valencia *et al*., 2019). Sample 105 was one of four *Dip. seriata* isolates, and the other three were reported from apple, avocado, and grapevine (Morales *et al*., 2012; Díaz *et al*., 2018c). Sample 172 was one of three *Neof. parvum* isolates, the

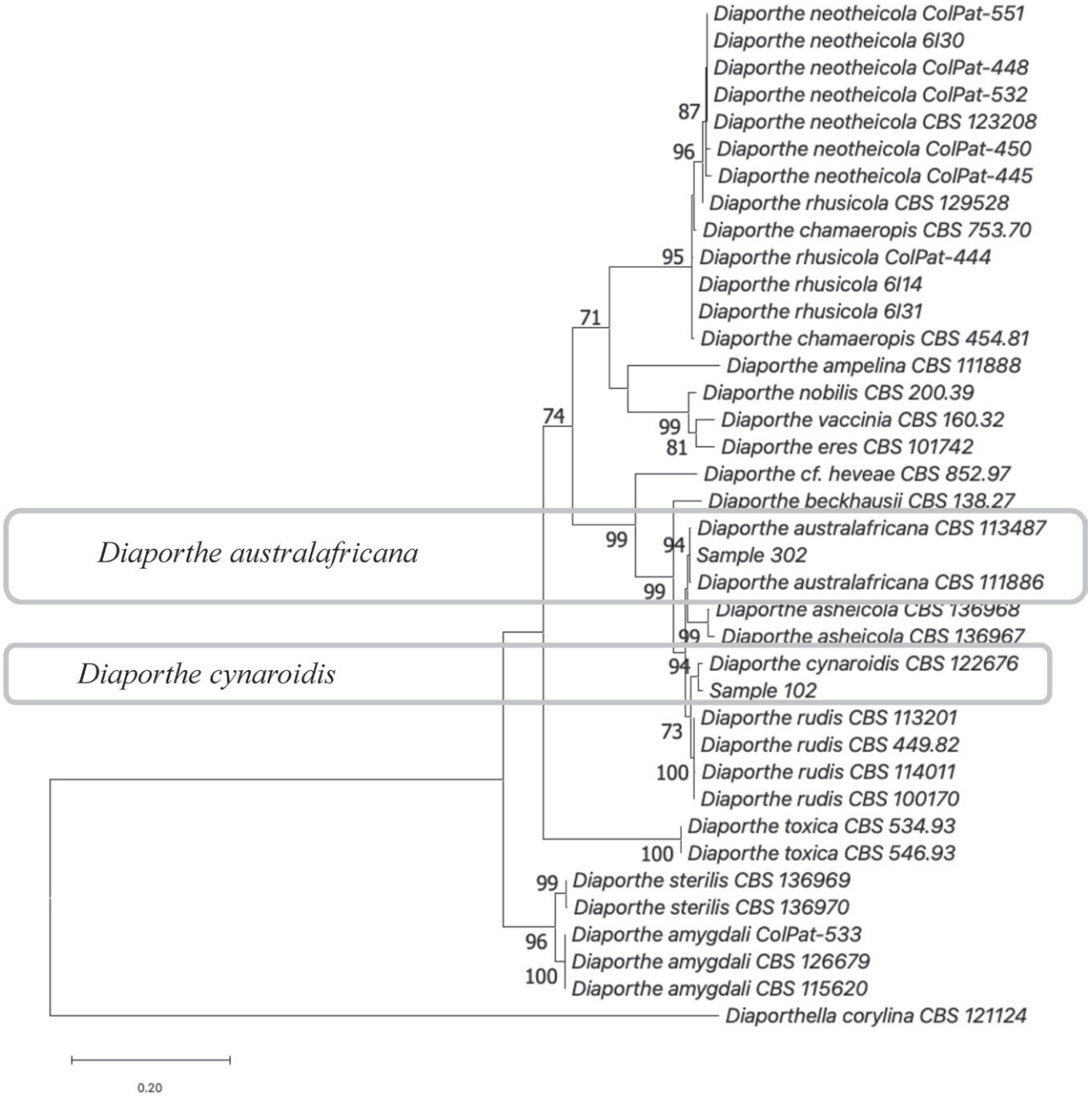


**Figure 3.** Phylogenetic tree reconstructed by maximum likelihood analysis from the concatenated sequences of the nuclear ribosomal internal transcribed spacer, translation elongation factor and beta-tubulin for two *Diaporthaceae* taxa recovered from two walnut orchards in Chile, and 36 *Diaporthe* reference sequences retrieved from the GenBank database. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Numbers on branches are bootstrap support values.

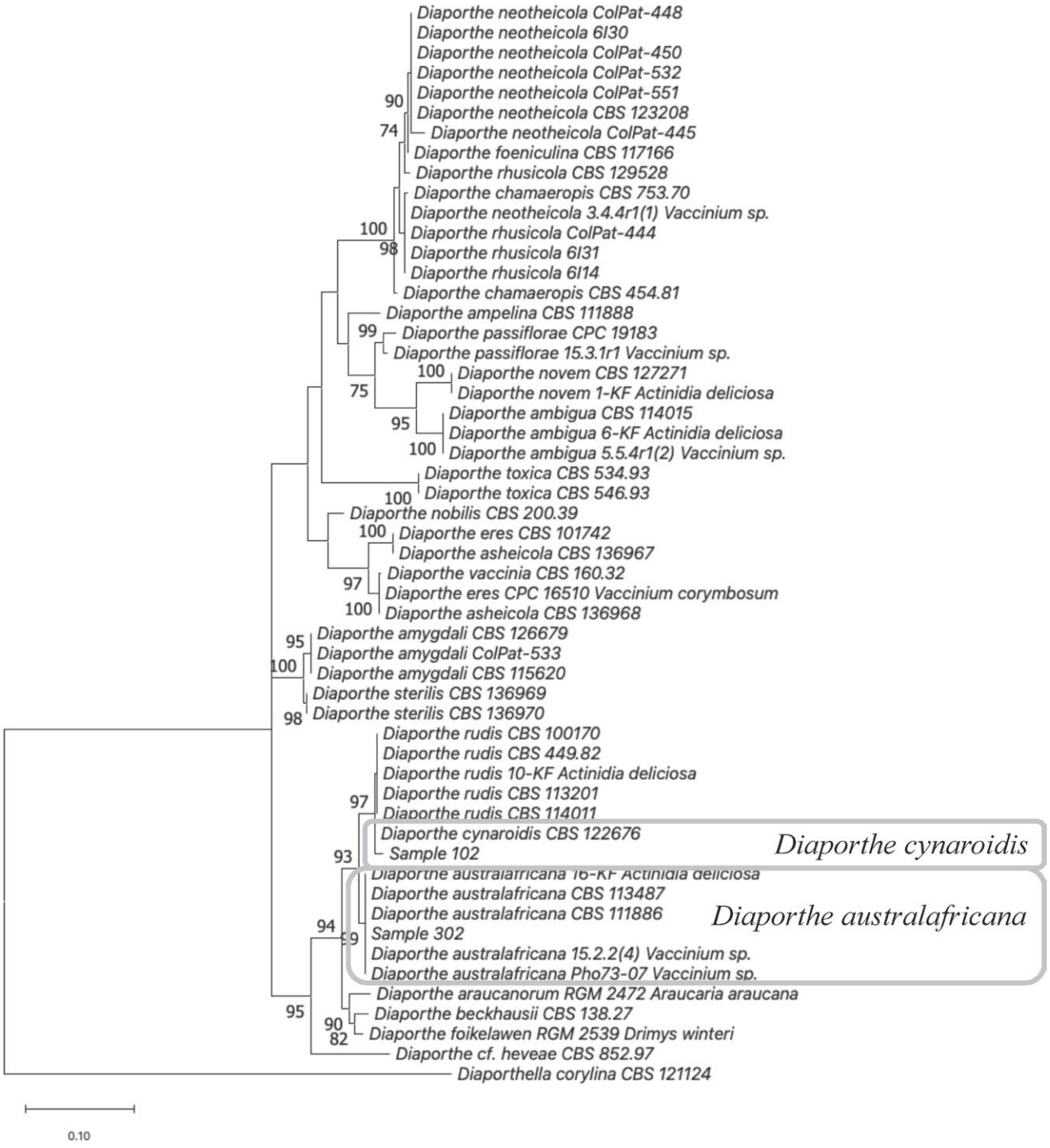


**Figure 4.** Phylogenetic tree reconstructed by maximum likelihood analysis from the sequences of the nuclear ribosomal internal transcribed spacer for two novel *Diaporthaceae* taxa recovered from two walnut orchards in Chile, and 51 GenBank sequences of *Diaporthaceae* that included reference sequences as well as sequences of previously reported Chilean walnut pathogens. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Numbers on branches are bootstrap support values

other two were from avocado and blueberry (Espinoza *et al*., 2008, 2009; Valencia *et al*., 2019).

The alignment of 38 *Diaporthaceae* DNA sequences comprised 1791 nucleotide positions (610 ITS, 403 EF and 778 TUB), of which 794 were conserved (ITS = 403, EF = 81, TUB = 310), 872 were variable (ITS = 176, EF =

287, TUB = 409), 575 were parsimony informative (ITS = 93, EF = 224, TUB = 258), and 292 were singleton sites (ITS = 83, EF = 62, TUB = 147). The AICc-inferred optimal model of nucleotide substitution was a General Time Reversible model (Nei and Kumar 2000) with a discrete Gamma distribution (GTR + G). The tree with the greatest log likelihood (-6640.99) is shown in Figure 3. Phylogenetic reconstruction placed our samples 302 and 102 in clades of, respectively, *Diaporthe australafricana* and *D. cynaroidis*, with strong bootstrap support.

A separate phylogenetic analysis including ITS sequences of *Diaporthaceae* previously identified from alternative hosts in Chile was also generated because the sequences for all three loci were not available in the NCBI database (Figure 4). This analysis was based on an alignment of 54 *Diaporthaceae* DNA sequences comprising 564 nucleotide positions in the ITS region, of which 351 were conserved, 181 were variable, 117 were parsimony-informative, and 64 were singleton sites. The AICc-inferred optimal model of nucleotide substitution was a General Time Reversible model (Nei and Kumar 2000) with a discrete Gamma distribution and a proportion of invariant sites (TN93 + G + I). The tree with the greatest log likelihood (-2291.02) is shown in Figure 4. Sample 102 was the only *D. cynaroidis* isolate reported in Chilean walnut at this time, and sample 302 was one of four *D. australafricana* isolates, the other three originating from blueberry and kiwifruit (Espinoza *et al*., 2008; Elfar *et al*., 2013; Díaz *et al*., 2017).

Phylogenies derived from the individual gene partitions did not reveal incongruence, although bootstrap support was less than in the concatenated trees (Table S1). Of the three partitions, the ITS region provided the strongest support for clades harboring four of the novel samples. Only Sample 102 received strongest support from EF for its affiliation with the *D. cynaroidis* clade. This is consistent with the results of Santos *et al*. (2010), who recommended the EF region for use in *Diaporthe*.

All five species caused wood lesions on inoculated walnut stems compared to the mock-inoculated control plants, but there were some differences in virulence (Figure 5) ($P < 0.05$; One-way ANOVA followed by Tukey test for multiple comparison of means). *Neofusicoccum parvum* was the most aggressive species causing larger lesions ($P = 0.0045$) than *Dip. mutila*. *Diplodia seriata* gave intermediate lesion lengths, but these were not significantly different from either *Neof. parvum* or *Dip. mutila* ($P = 0.0764$) (Figure 5A). In addition, mean lesion lengths were not significantly different ($P > 0.05$) between *D. australafricana* and *D. cynaroidis* but were greater than those from the mock-inoculated controls (Figure 5B).

## DISCUSSION

The survey indicated that incidence of wood diseases in Chilean walnut orchards was low, as only five of the thirteen assessed orchards were symptomatic. This is probably because that commercial walnut production is a young industry in Chile, coupled with the long incubation period required for wood pathogens to cause wood dieback (Duthie *et al*., 1991). The industry was first established in the late 1970's with approx. 4000 ha planted in the Valparaíso Region. After 2000, Chile walnut production grew ten-fold to over 43,000 ha (INC, 2021), but the bulk of the new planted area was further south in the O'Higgins and Maule Regions that have wetter and cooler weather patterns than the Valparaíso Region. The dryer Valparaíso Region displayed low wood disease incidence, and *Neof. parvum* was the only pathogen isolated. In contrast, a broader diversity of pathogenic species and greater disease incidence was recorded in the two major walnut producing regions in central Chile, where two *Diplodia* and two *Diaport*he species were identified. These results were similar to those of Larach *et al*. (2020), who also identified greater incidence of disease in wetter and cooler Chilean vineyards in comparison to dryer areas. This suggests that pathogenic fungi causing wood disease may become increasingly problematic as orchards age, as plantation size expands, and also possibly because environmental conditions in wetter areas are more suitable for the pathogens to spread and cause wood diseases. Overall, this study has confirmed the results of Díaz *et al*. (2018a) and Jiménez Luna *et al*. (2020), who found *Dip. mutila*, *D. australafricana* and *D. cynaroidis* in walnut orchards. The present results also expand the host range of *Dip. seriata* and *Neof. parvum* to walnut in Chile.

Infection of young orchards with both *Botryosphaeriaceae* and *Diaporthe* species could be attributed to different infection routes for these fungi. Infections may have initially come from plant nursery materials, as has been reported for several crop types (Smit *et al*., 1996; Espinoza *et al*., 2009; Chen *et al*., 2013b; Whitelaw-Weckert *et al*., 2013; Tennakoon *et al*., 2017), including walnut (Chen *et al*., 2013a). However, inoculum originated most likely from alternative hosts grown in proximity to walnut orchards, either following wet events (rainfall or sprinkler or furrow irrigation), which aid pathogen infection by dispersing inoculum (Valencia et al., 2019) to exposed tree wounds caused by pruning or mechanical harvesters (Michailides and Morgan 1993; Luo *et al*., 2020). In Chile, all three *Botryosphaeriaceae* species isolated from walnut have also been shown to cause branch canker and dieback in avocado trees, and walnuts are

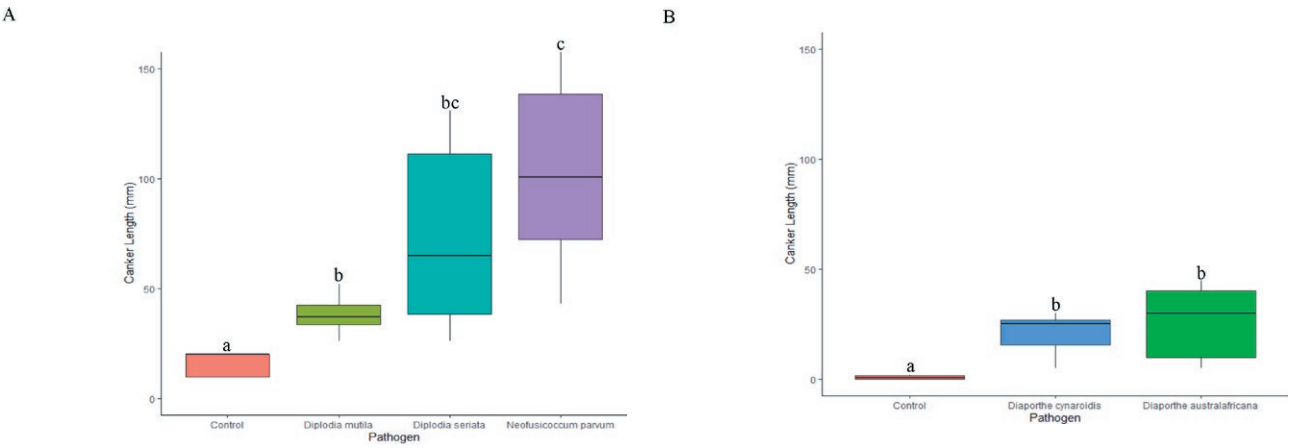


**Figure 5.** Average canker length (mm) after 12 weeks caused by three *Botryosphaeriaceae* species (**A**) and two *Diaporthe* species (**B**) on walnut stems inoculated with mycelial plugs. Bars topped with different letters indicate treatment means that are significantly different for *Botryosphaeriaceae* and *Diaportheaceae* ($P < 0.05$) species.

often planted near avocado orchards in the regions surveyed (Valencia *et al*., 2019). The pathogens have also been found on apple, blueberry, and grapevine (Auger *et al*., 2004; Espinoza *et al*., 2009; Morales *et al*., 2012; Díaz *et al*., 2018b; Larach *et al*., 2020), all of which have been grown locally, and infected hosts could become the inoculum sources for neighboring walnut orchards. Similarly, *D. australafricana* and *D. cynaroidis* have been identified in European hazelnut, blueberry, and kiwifruit (Elfar *et al*., 2013; Guerrero *et al*., 2013; Díaz *et al*., 2017). The phylogenetic analyses of the present study indicated that several additional *Botryosphaeriaceae* and *Diaporthe* species known to be pathogenic to walnut (*Las. theobromae*, *Dot. iberica*, or *D. neotheicola*; Chen *et al*., 2014; Lopez-Moral *et al*., 2020; Sohrabi *et al*., 2020) have been reported in Chile on avocado (Valencia *et al*., 2019) and blueberry (Espinoza *et al*., 2008), and these inoculum sources could potentially become threats to local walnut production.

The present study has demonstrated that *Neof. parvum*, *Dip. seriata*, *Dip. mutila*, *D. australafricana* and *D. cynaroidis* isolated from walnut wood diseases were all pathogenic. This study gave similar results to previous studies indicating that *Neof. parvum* is one of the most aggressive wood pathogens to many crops in addition to English walnut (Chen *et al*., 2014; López-Moral *et al*., 2020), including almond (Inderbitzin *et al*., 2010; Holland *et al*., 2021), avocado (McDonald *et al*., 2009), citrus (Adesemoye and Eskalen, 2011), and grapevine (Úrbez-Torres *et al*., 2009). The broad incidence and high virulence of *Neof. parvum* indicates that this fungus is one of the main pathogens of walnut in Chile causing trunk and limb cankers, eventually resulting in decline of affected trees. *Diplodia. seriata* and *Dip. mutila* were weakly virulent with respect to wood lesions caused to walnut branches compared to *Neof. parvum*, and these results are similar to those in other reports (Chen *et al*., 2014; López-Moral *et al*., 2020). *Diaporthe australafricana* and *D. cynaroidis* were also in the same range of virulence as *Dip. seriata* and *Dip. Mutila*, and were comparable to previous reports of mild aggressiveness of species in the *Diaporthe* group including *D. rhusicola* and *D. neotheicola* (Chen *et al*., 2014; López-Moral *et al*., 2020). Fungus genomics have showed that *Diaporthe* species and *Diplodia* species have limited enzymatic capabilities to colonize woody tissues and break down cell wall lignin (Morales-Cruz *et al*., 2015; Garcia *et al*., 2021), and that these fungi may be more responsible for shoot/fruit blights and twig dieback symptoms than capable of causing cankers on tree trunks and scaffolds, as reported with *Neof. parvum*.

Protecting host wounds with fungicide applications is the best strategy for preventing fungal infections, as has been demonstrated in other pathosystems (Rolshausen *et al*., 2010; Díaz and Latorre, 2013). In Chile, applications of lime sulfur are currently used in walnut to control the development of *Botryosphaeriaceae* and *Diaporthaceae* (http://www.sag.cl/ambitos-de-accion/plaguicidas-y-fertilizantes). Integrated disease management remains effective for control of fungi causing wood diseases. Pruning in dry weather, managing canopy size allowing ventilation and sunlight exposure, and maintaining low tree planting densities are recommended practices to minimize the risks and severity of infections (Moral *et al*., 2019a, 2019b). In addition, pruning and removal of dead and infected tissues, and avoiding excessive wetting of host trunks or canopies is strongly encouraged,

to limit the build-up and spread of pathogen inoculum and extend crop longevity and productivity, as has been shown in pistachio orchards and vineyards (Michailides and Morgan 1993; Gispert *et al*., 2020). To date there are no walnut cultivars known to be resistant to the causal agents of wood disease, although cultivar 'Chandler' has been reported to be more tolerant to infections, followed by 'Tulare' and 'Vina' (Chen *et al*., 2014). As Chile looks to expand walnut production to meet global market demand, management of these diseases will be key to sustaining the longevity and productivity of walnut orchards.

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