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TAXONOMIC DESCRIPTION
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Polyphasic studies of new species of *Diaporthe* from native forest in Chile, with descriptions of *Diaporthe araucanorum* sp. nov., *Diaporthe foikelawen* sp. nov. and *Diaporthe patagonica* sp. nov.

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Abstract

During a survey of fungi in native forests in Chile, several unidentified isolates of *Diaporthe* were collected from different hosts. The isolates were characterized based on DNA comparisons, morphology, culture characteristics and host affiliation, in accordance with previous descriptions. Phylogenetic analysis of the ITS region, combined with partial *tub2* and *tef1* genes, showed that the isolates formed three distinct groups representing three new taxa. The three new species of *Diaporthe*, *Diaporthe araucanorum* on *Araucaria araucana*, *Diaporthe foikelawen* on *Drimys winteri* and *Diaporthe patagonica* on *Aristotelia chilensis* are described and illustrated in the present study.

INTRODUCTION

Chile has approximately 14.6 million hectares of native forests representing about 81.6% of the forest resources of the country [1], ranging from Mediterranean sclerophyllous formations, through sub-Mediterranean deciduous to temperate evergreen and deciduous forests [2], which provide a wide range of ecosystem services such as firewood, timber products, water supply, soil fertility, tourism, recreation, biodiversity conservation and carbon sequestration [3, 4].

Natural forests have been object of ongoing phytosanitary surveys by the Servicio Agrícola y Ganadero (SAG), the national plant protection organization of Chile. During surveillance activities carried out in 2016 to 2018, several *Diaporthe* isolates not identified at the species level were collected from *Araucaria araucana* (local name Araucaria), *Aristotelia chilensis* (Maqui) and *Drimys winteri* (Canelo). Based on colony features, morphology and ITS sequences, these isolates were classified into three groups. A posterior multi-locus phylogeny using ITS, β-tubulin (*tub2*) and translation elongation factor 1-α (*tef1*) genes revealed that these groups represent three undescribed *Diaporthe* species.

The genus *Diaporthe* comprises a large number of endophytic, saprotrophic and phytopathogenic species with diverse host associations and worldwide distribution [5–7]. Some species are responsible for severe diebacks, cankers, leaf-spots, blights, melanoses, stem-end rot and gummosis on different woody and herbaceous hosts [6, 8–10]. With the extensive use of DNA sequence-based methods for studying the genus in the last decade [11], the occurrence of *Diaporthe* species in natural forest ecosystems has increased noticeably, with new descriptions in Australia, Brazil, Canada, China, Japan, Portugal, La Rèunion, South Africa, Thailand, Tanzania and Zambia [12–18]. Among these, *Diaporthe virgiliae* is

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Abbreviations: APDA, potato dextrose agar amended with lactic acid; ITS, internal transcribed spacer; MEA, malt extract agar; OA, oatmeal agar; PDA, potato dextrose agar; WA, water agar.
The GenBank/EMBL/DDBJ accession numbers for the ITS, *tub2*, *tef1* and *cal* (only ex-type) sequences determined in this study for *Diaporthe araucanorum* sp. nov. strain RGM 2472 are MN509709, MN509720 and MN509731, for strain RGM 2405 they are MN509710, MN509721 and MN509732, for strain RGM 2546^T they are MN509711, MN509722, MN509733 and MN974277, and for strain RGM 2649 they are MN509712, MN509723 and MN509734; for *Diaporthe foikelawen* sp. nov. strain RGM 2539^T are MN509713, MN509724, MN509735 and MN974278, for strain RGM 2407 they are MN509714, MN509725 and MN509736, for strain RGM 2563 they are MN509715, MN509726 and MN509737, and for strain RGM 2621 they are MN509716, MN509727 and MN509738; and for *Diaporthe patagonica* sp. nov. strain RGM 2473^T are MN509717, MN509728, MN509739 and MN974279, for strain RGM 2666 they are MN509718, MN509729 and MN509740, and for strain RGM 2691 they are MN509719, MN509730 and MN509741.

an emerging disease causing the death of endemic *Virgilia oroboides* trees in South Africa [19].

Although several species of *Diaporthe* have been recorded in Chile [20], little is known about their occurrence in native trees. According to the most complete checklist of fungal phytoparasites in Chile compiled by Mujica and Vergara [21], no species of *Diaporthe* appear associated with Araucaria, Maqui or Canelo, nor to the forest environments where the isolates were collected. The aim of this study was thus to describe three novel *Diaporthe* species from Chilean native trees based on morphological characteristics and multigene phylogenetic analysis.

METHODS

Sample and fungal isolates

The SAG conducts annual surveys of the natural forests of Chile to detect recent tree mortality, tree damage and defoliation. From trees with suspect of fungal diseases, wood samples are collected from branches, stems and roots for general diagnosis within the SAG Laboratories network. In the laboratory, each sample is carefully examined and any visual damage is observed. Fungi are isolated from infected tissues routinely using potato dextrose agar amended with lactic acid (APDA) or 2% malt extract agar (MEA).

Between 2016–2018, several *Diaporthe* isolates were collected in field surveys from Araucaria, Maqui and Canelo trees between the Biobío and Aysén region in Chile. Isolates not identified at the species level using taxonomy and DNA analysis were conserved in the work culture collection of the laboratory for future taxonomic descriptions.

DNA extraction, PCR and DNA sequencing

Genomic DNA from aerial mycelium was extracted using the modified protocol of Cooke and Duncan [22]. Partial regions of four loci were amplified. The ITS region was amplified using universal primers ITS1 and ITS4 [23], a portion of *tub2* gene was amplified with primers TUB2Fd and TUB4Rd [24] and part of the *tef1* with primers EF1-728F [25] and EF2 [26]. For strains selected as type material, a part of the calmodulin (*cal*) gene was amplified using the primers CAL-228F and CAL-737R [25]. PCR amplifications were performed in 50 µl reactions (2.5 mM $MgCl_2$, 1×PCR buffer, 0.2 mM dNTP, 0.4 mM of each primer and 2.5 U Taq-polymerase enzyme). PCR conditions for amplification of these regions were similar to those described in the references used for primer design. Amplicons were sequenced by Macrogen (Republic of Korea) with both forward and reverse primers to ensure high-quality sequences.

Phylogenetic analyses

Initial BLAST analyses were performed on the NCBI databases to retrieve the closest matches in GenBank. Representative sequences of ex-type or authentic strains showing the highest similarity matches were downloaded and included in the datasets. Additionally, sequences used in recent phylogenetic studies of *Diaporthe* [12, 13, 27] were also included in the analyses to have a large vision of the Chilean isolates within the genus (Table 1). Sequences from individual loci were aligned using the online version of MAFFT version 7 [28] with the E-INS-i strategy and default settings. Ambiguous regions were trimmed using trimAL version 1.2 [29]. A partition homogeneity test (PHT=ILD=incongruency length difference test) was performed with PAUP version 4.0b10 [30, 31] to evaluate whether the individual datasets were congruent and could be combined. The dataset for the final phylogenetic analysis was created by combining the alignments of the ITS, *tub2* and *tef1*. *Diaporthella corylina* was chosen as the outgroup for rooting purposes. Maximum-parsimony (MP) analysis was performed with PAUP version 4.0b10 [31] using the heuristic search option and tree bisection and reconnection (TBR) as the branch-swapping algorithm, with maxtrees set at 1000. Maximum-likelihood analysis (ML) was conducted in RaxML-HPC BlackBox 8.2.12 [32] via the CIPRES Science Gateway version 3.3 [33], using a GTRGAMMA+I model of evolution. Branch support for ML and MP analyses were determined using 1000 bootstrap replicates. Alignment and phylogenetic tree were deposited in TreeBASE (s25782).

Morphological characterization

Morphological descriptions are based on colonies sporulating in culture. The isolates were subcultured on 2% WA supplemented with sterile pine needles [34], on PDA (DIFCO 213400), oatmeal agar (OA) [35] and 2% MEA (Oxoid LP0039), and incubated at 20 °C under a 12 h near-ultraviolet light/12 h dark cycle to induce sporulation, according to Gomes *et al.* [6]. Fungal structures were mounted on glass slides in clear lactic acid for microscopic examination after 28 days of incubation. Fifty measurements for conidia and 30 for other structures were recorded using the software Piximetre 5.9 [36]. The extreme measurements are given in parentheses with mean and standard deviation, with values rounded to 0.5 µm. Colony diameters were determined at 25 °C in darkness on PDA, OA and MEA after 14 days. Colony colours (surface and reverse), texture and the arrangement of the conidiomata were described. Nomenclatural novelties and descriptions were deposited in MycoBank [37].

RESULTS

Isolates

Eleven isolates of *Diaporthe* obtained from the Chilean native forest were included in this study. Symptoms observed in field samples were discoloration of leaves, necrotic leaf spots and branch or twig dieback, (Fig. 1). Based on morphological and molecular similarities, the isolates were divided into three groups. The first group consisted of four isolates associated with *Araucaria araucana*, collected in the natural distribution of the host, in both the Andes and Nahuelbuta mountains. This group produced dark brown slowly growing colonies on MEA. The second group consisted of four isolates and was associated with *Drimys winteri*. This group produced grey fast-growing colonies on MEA. Finally, the third group

Table 1. Isolates of *Diaporthe* species included in phylogenetic analyses in this study

Species	Strain	Country	Host	GenBank Accession No. (ITS, *tub2*, *tef1*)		
Diaporthe acaciigena	CBS 129521^T	Australia	*Acacia retinodes*	KC343005	KC343973	KC343731
Diaporthe alleghaniensis	CBS 495.72^T	Canada	*Betula alleghaniensis*	KC343007	KC843228	KC343733
Diaporthe alnea	CBS 146.46^T	Netherlands	*Alnus* species	KC343008	KC343976	KC343734
Diaporthe ambigua	CBS 114015; STE-U 2657^T	South Africa	*Pyrus communis*	KC343010	KC343978	KC343736
	CBS 187.87	Italy	*Helianthus annuus*	KC343015	KC343983	KC343741
Diaporthe ampelina	CBS 114016; STE-U 2660^T	France	*Vitis vinífera*	AF230751	JX275452	AY745056
	CBS 111888	USA	*Vitis vinífera*	KC343016	KC343984	KC343742
Diaporthe amygdali	CBS 126679^T	Portugal	*Prunus dulcis*	KC343022	KC343990	KC343748
Diaporthe anacardii	CBS 720.97^T	Eastern Africa	*Anacardium occidentale*	KC343024	KC343992	KC343750
Diaporthe angelicae	CBS 111592; AR 3724^T	Austria	*Heracleum sphondylium*	KC343026	KC343994	KC343752
Diaporthe araucanorum	**RGM 2472; CBS 145283**	**Chile**	***Araucaria araucana***	**MN509709**	**MN509720**	**MN509731**
	RGM 2405; CBS 145284	**Chile**	***Araucaria araucana***	**MN509710**	**MN509721**	**MN509732**
	RGM 2546; CBS 145285^T	**Chile**	***Araucaria araucana***	**MN509711**	**MN509722**	**MN509733**
	RGM 2649; CBS 145286	**Chile**	***Araucaria araucana***	**MN509712**	**MN509723**	**MN509734**
Diaporthe arecae	CBS 161.64^T	India	*Areca catechu*	KC343032	KC344000	KC343758
Diaporthe arengae	CBS 114979^T	Hong Kong	*Arenga engleri*	KC343034	KC344002	KC343760
Diaporthe asheicola	CBS 136967^T	Chile	*Vaccinium ashei*	KJ160562	KJ160518	KJ160594
Diaporthe australafricana	CBS 111886; STE-U 2676^T	Australia	*Vitis vinífera*	KC343038	KC344006	KC343764
Diaporthe baccae	CBS 136972^T	Italy	*Vaccinium corymbosum*	KJ160565	MF418509	KJ160597
Diaporthe beckhausii	CBS 138.27	Unknown	*Viburnum* species	KC343041	KC344009	KC343767
Diaporthe benedicti	BPI 893190	USA	*Salix* species	KM669929	–	KM669785
Diaporthe bicincta	CBS 121004^T	USA	*Juglans sp.*	KC343134	KC344102	KC343860
Diaporthe carpini	CBS 114437	Sweden	*Carpinus betulus*	KC343044	KC344012	KC343770
Diaporthe cassines	CBS 136440; CPC 21916^T	South Africa	*Cassine peragua*	KF777155	–	KF777244
Diaporthe celastrina	CBS 139.27^T	USA	*Celastrus scandens*	KC343047	KC344015	KC343773
Diaporthe citri	CBS 135422T; AR 3405	USA	*Citrus* species	KC843311	KC843187	KC843071
	CBS 134237	PR China	*Citrus reticulata*	JQ954660	KC357426	JQ954676
	AR 4469	USA	*Citrus* species	KC843321	KC843197	KC843081
Diaporthe citriasiana	CBS 134240^T	PR China	*Citrus unshiu*	JQ954645	KC357459	JQ954663
Diaporthe citrichinensis	CBS 134242^T	PR China	*Citrus* species	JQ954648	MF418524	JQ954666
Diaporthe cuppatea	CBS 117499^T	South Africa	*Aspalathus linearis*	AY339322	JX275420	AY339354
Diaporthe cynaroidis	CBS 122676; CPC 13180^T	South Africa	*Protea cynaroides*	KC343058	KC344026	KC343784
Diaporthe cytosporella	CBS 137020^T	Spain	*Citrus limon*	KC843307	KC843221	KC843116
Diaporthe decedens	CBS 109772	Austria	*Corylus avellana*	KC343059	KC344027	KC343785
Diaporthe detrusa	CBS 109770; AR 3424	Austria	*Berberis vulgaris*	KC343061	KC344029	KC343787
Diaporthe eleagni	CBS 504.72	Netherlands	*Eleangus* species	KC343064	KC344032	KC343790

Continued

Table 1. Continued

Species	Strain	Country	Host	GenBank Accession No. (ITS, *tub2*, *tef1*)		
Diaporthe endophytica	CBS 133811; CPC 20292^T	Brazil	*Schinus terebinthifolius*	KC343065	KC344033	KC343791
Diaporthe eres	CBS 138594; AR 5193^T	Germany	*Ulmus* species	KJ210529	KJ420799	KJ210550
	CBS 101742	Netherlands	*Fraxinus* species	KC343073	KC344041	KC343799
	CBS 439.82	Netherlands	*Fraxinus* species	KC343090	KC344058	KC343816
	CBS 109767	Austria	*Acer campestre*	KC344043	KC343801	KC343801
	MFLUCC 17–0997	Italy	*Juglans regia*	KY964202	KY964086	KY964158
Diaporthe fibrosa	CBS 109751; AR 3425	Austria	*Rhamnus cathartica*	KC343099	KC344067	KC343825
Diaporthe foeniculina	CBS 111553^T	Spain	*Foeniculum vulgare*	KC343101	KC344069	KC343827
	CBS 187.27	Italy	*Camellia sinensis*	KC343107	KC344075	KC343833
	CBS 111554	Portugal	*Foeniculum vulgare*	KC343102	KC344070	KC343828
	CBS 123208	Portugal	*Foeniculum vulgare*	KC343104	KC344072	KC343830
	CBS 123209	Portugal	*Foeniculum vulgare*	KC343105	KC344073	KC343831
Diaporthe foikelawen	**RGM 2539; CBS 145289^T**	**Chile**	***Drimys winteri***	**MN509713**	**MN509724**	**MN509735**
	RGM 2407; CBS 145287	**Chile**	***Drimys winteri***	**MN509714**	**MN509725**	**MN509736**
	RGM 2563; CBS 145290	**Chile**	***Drimys winteri***	**MN509715**	**MN509726**	**MN509737**
	RGM 2621; CBS 145288	**Chile**	***Drimys winteri***	**MN509716**	**MN509727**	**MN509738**
Diaporthe helianthi	CBS 592.81^T	Serbia	*Helianthus annuus*	KC343115	KC344083	KC343841
	CBS 344.94	Unknown	*Helianthus annuus*	KC343114	KC344082	KC343840
Diaporthe helicis	CBS 138596; AR 5211^T	Germany	*Hedera hélix*	KJ210538	KJ420828	KJ210559
Diaporthe heterophyllae	CBS 143769; CPC 26215^T	France	*Acacia heterophylla*	MG600222	MG600226	MG600224
Diaporthe hongkongensis	CBS 115448^T	PR China	*Dichroa febrifuga*	KC343119	KC344087	KC343845
Diaporthe impulsa	CBS 114434	Sweden	*Sorbus aucuparia*	KC343121	KC344089	KC343847
Diaporthe inconspicua	CBS 133813^T	Brazil	*Maytenus ilicifolia*	KC343123	KC344091	KC343849
Diaporthe infecunda	CBS 133812^T	Brazil	*Schinus terebinthifolius*	KC343126	KC344094	KC343852
Diaporthe infertilis	CBS 230.52^T	Suriname	*Citrus sinensis*	KC343052	KC344020	KC343778
Diaporthe melonis	CBS 507.78^T	USA	*Cucumis melo*	KC343142	KC344110	KC343868
Diaporthe neilliae	CBS 144.27^T	USA	*Spiraea sp*	KC343144	KC344112	KC343870
Diaporthe nothofagi	BRIP 54801^T	Australia	*Nothofagus cunninghamii*	JX862530	KF170922	JX862536
Diaporthe novem	CBS 127271^T	Croatia	*Glycine max*	KC343157	KC344125	KC343883
Diaporthe ocoteae	CBS 141330^T	France	*Ocotea bullata*	KX228293	KX228388	–
Diaporthe oncostoma	CBS 589.78	France	*Robinia pseudoacacia*	KC343162	KC344130	KC343888
Diaporthe patagonica	**RGM 2473; CBS 145291^T**	**Chile**	***Aristotelia chilensis***	**MN509717**	**MN509728**	**MN509739**
	RGM 2666; CBS 145755	**Chile**	***Aristotelia chilensis***	**MN509718**	**MN509729**	**MN509740**
	RGM 2691; CBS 145756	**Chile**	***Aristotelia chilensis***	**MN509719**	**MN509730**	**MN509741**
Diaporthe perjuncta	CBS 109745; AR 3461^T	Austria	*Ulmus glabra*	KC343172	KC344140	KC343898
Diaporthe perseae	CBS 151.73	Netherlands: Antilles	*Persea americana*	KC343173	KC344141	KC343899

Continued

Table 1. Continued

Species	Strain	Country	Host	GenBank Accession No. (ITS, *tub2*, *tef1*)		
Diaporthe pseudomangiferae	CBS 101339^T	Dominican Republic	*Mangifera indica*	KC343181	KC344149	KC343907
Diaporthe pseudophoenicicola	CBS 462.69T	Spain	*Phoenix dactylifera*	KC343184	KC344152	KC343910
Diaporthe pseudotsugae	MFLU 15-3228^T	Italy	*Pseudotsuga menziesii*	KY964225	KY964108	KY964181
D. pulla	CBS 338.89^T	Croatia	*Hedera hélix*	KC343152	KC344120	KC343878
Diaporthe racemosae	CBS 143770; CPC 26646^T	South Africa	*Euclea racemosa*	MG600223	MG600227	MG600225
Diaporthe rudis	CBS 113201; STE-U 5683^T	Portugal	*Vitis vinífera*	KC343234	KC344202	KC343960
	CBS 114436	Sweden	*Sambucus cf. racemosa*	KC343236	KC344204	KC343962
	AR 3422	Austria	*Laburnum anagyroides*	KC843331	KC843177	KC843090
	CBS 109492	Austria	*Laburnum anagyroides*	KC343232	KC344200	KC343958
Diaporthe saccarata	CBS 116311^T	South Africa	*Protea repens*	KC343190	KC344158	KC343916
Diaporthe salicicola	BRIP 54825^T	Australia	*Salix purpurea*	JX862531	KF170923	JX862537
Diaporthe schini	CBS 133181^T	Brazil	*Schinus terebinthifolius*	KC343191	KC344159	KC343917
Diaporthe sojae	CBS 139282; FAU635^T	USA	*Glycine max*	KJ590719	KJ610875	KJ590762
	AR 3602	Japan	*Cucumis melo*	KJ590714	KJ610870	KJ590757
	CBS 116019	USA	*Caperonia palustris*	KC343175	KC344143	KC343901
	DP 0601	USA	*Glycine max*	KJ590706	KJ610862	KJ590749
Diaporthe sterilis	CBS 136969^T	Italy	*Vaccinium corymbosum*	KJ160579	KJ160528	KJ160611
Diaporthe subcylindrospora	KUMCC 17-0151^T	PR China	Dead wood	MG746629	MG746631	MG746630
Diaporthe terebinthifolii	CBS 133180; CPC 20290^T	Brazil	*Schinus terebinthifolius*	KC343216	KC344184	KC343942
Diaporthe toxica	CBS 534.93; ATCC 96741^T	Australia	*Lupinus angustifolius*	KC343220	KC344188	KC343946
Diaporthe unshiuensis	CGMCC3.17569^T	PR China	*Citrus unshiu*	KJ490587	KJ490408	KJ490466
Diaporthe vaccinii	CBS 160.32^T	USA	*Vaccinium macrocarpon*	AF317578	KC344196	GQ250326
	CBS 118571	USA	*Vaccinium corymbosum*	KC343223	KC344191	KC343949
	CBS 122114	USA	*Vaccinium corymbosum*	KC343225	KC344193	KC343951
Diaporthella corylina	CBS 121124^T	PR China	*Corylus* species	KC343004	KC343972	KC343730

New species are in bold. AR, Collection of A.Y. Rossman; ATCC, American type culture collection; Manassas, Virginia, USA; BRIP, Plant Pathology Herbarium, Dutton Park, Queensland, Australia; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CPC, Collection Pedro Crous, housed at CBS; CGMCC, China General Microbiological Culture Collection, Beijing, China; KUMCC, Culture collection of Kunming Institute of Botany, Kunming, China; MFLU, Mae Fah Luang University Herbarium, Thailand; MFLUCC, Mae Fah Luang University Culture Collection, Thailand; RGM, Chilean Microbial Genetic Resources Collection, INIA Quilamapu, Chillán, Chile; STE-U, Stellenbosch University culture collections, South Africa; T, ex type, ex-epitype, ex-paratype, ex-neotype or ex-isotype.

consisted of three isolates associated with *Aristotelia chilensis*, collected in the Chilean Patagonia area. This group produced white fast-growing colonies on MEA.

Cultures included in this study were deposited at the Chilean Microbial Genetic Resources Collection (RGM bank), INIA Quilamapu, Chillán, Chile and at the Westerdijk Fungal Biodiversity Institute (CBS collection), Utrecht, The Netherlands. Details of the specimens used are listed in Table 1.

Phylogenetic analyses

The combined alignment of ITS, *tub2* and *tef1* sequences used for both MP and ML analyses comprised 97 taxa, including

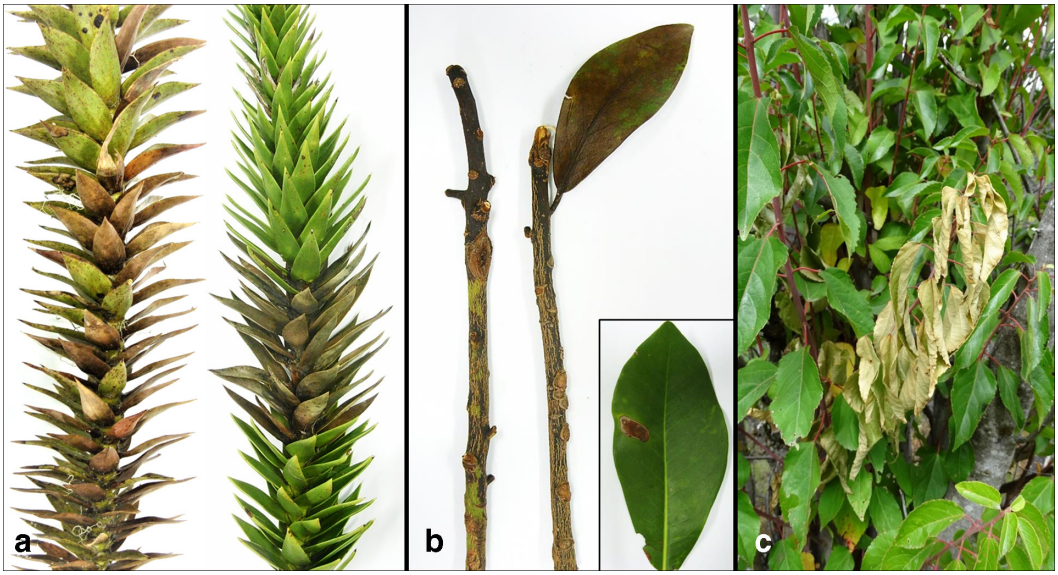

Fig. 1. Symptoms on plant tissues with associated *Diaporthe* species. (a) Branches of *Araucaria araucana* showing foliar discoloration and necrosis. (b) Twig of *Drimys winteri* showing die-back and defoliation; (insert to a) leaf spots symptom. (c) Twig of *Aristotelia chilensis* showing chlorosis and die-back.

our strains and the outgroup (*Diaporthella corylina* CBS 121124). The final dataset consisted of 1290 characters (ITS, 1–573; *tub2*,574–1058; *tef1*,1059–1290) including gaps. The PHT resulted in a low *P*-value (*P*=0.01), probably due to the little variation within the ITS region. Although the *P*-value was low, studies [38, 39] suggest that the data could still be combined.

The combined dataset contained 605 constant, 213 parsimony-uninformative and 472 parsimony-informative characters. The MP analysis generated 1000 equally most parsimonious trees of 2533 steps with: consistency index (CI), 0.353; retention index (RI), 0.794; homoplasy index (HI), 0.647; and rescaled consistency index (RC), 0.280. The ML analysis yielded a best-scoring tree with a final ML optimization likelihood value of −15655896. The parameters for the GTRGAMMA+I model were as follows: estimated base frequencies (A), 0.225008; (C), 0.304907; (G), 0.239868; (T): 0.230217; substitution rates A↔C, 1.170985; A↔G, 3.029823; A↔T, 1.072746; C↔G, 0.847702; C↔T, 4.526916; G↔T, 1.000000; proportion of invariable sites, 0.298122; and gamma distribution shape parameter, 0.712925.

Phylogenetic trees obtained from MP and ML analyses produced trees with similar overall topologies at the species levels. The tree with the highest log likelihood for the ML analysis is shown in Fig. 2. Isolates obtained from the Chilean native forest were accommodated in three distinct clades representing separate taxa, all with bootstrap support of 100%. The clades formed by isolates from *Araucaria araucana*, *Drimys winteri* and *Aristotelia chilensis* are described as new species.

Taxonomy

Morphological observations, supported by phylogenetic analysis of the ITS, *tub2* and *tef1* sequences, revealed three novel species of *Diaporthe* occurring in native forest in Chile, which are described below.

DESCRIPTION OF *DIAPORTHE ARAUCANORUM* SP. NOV.

Diaporthe araucanorum (a.rau.ca.no'rum. N.L. gen. pl. n. *araucanorum* of the 'Araucanos', a group of Amerindian tribes of central Chile) Fig. 3.

MycoBank no. MB832751.

Typus. Chile, Curanilahue county (37° 42′ 14″ S, 73° 07′ 08″ W), from *Araucaria araucana* in October 2017, collected by G. Hinojosa F74316-17 (holotype RGM 2546, preserved in a metabolically inactive state at the Chilean Microbial Genetic Resources Collection, INIA Quilamapu, Chillán, Chile). Ex-type culture CBS 145285, deposited at the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands.

Sexual morph not observed. *Conidiomata* pycnidial on pine needles on WA, unilocular, globose to subglobose, up to 550 µm wide, mainly solitary, mostly embedded, erumpent, with short black neck, often with white conidial droplets exuding from ostiole; pycnidal wall consisting of 4–7 layers of brown cell of *textura angularis*. *Conidiophores* hyaline, smooth, 1–2 septate, rarely ramificate 15–31×1–2 µm. *Conidiogenous cells* phialidic, cylindrical, hyaline, mainly terminal, tapering towards the apex, without visible periclinal thickening. *Paraphyses* not observed. *Alpha conidia* aseptate, hyaline, smooth,

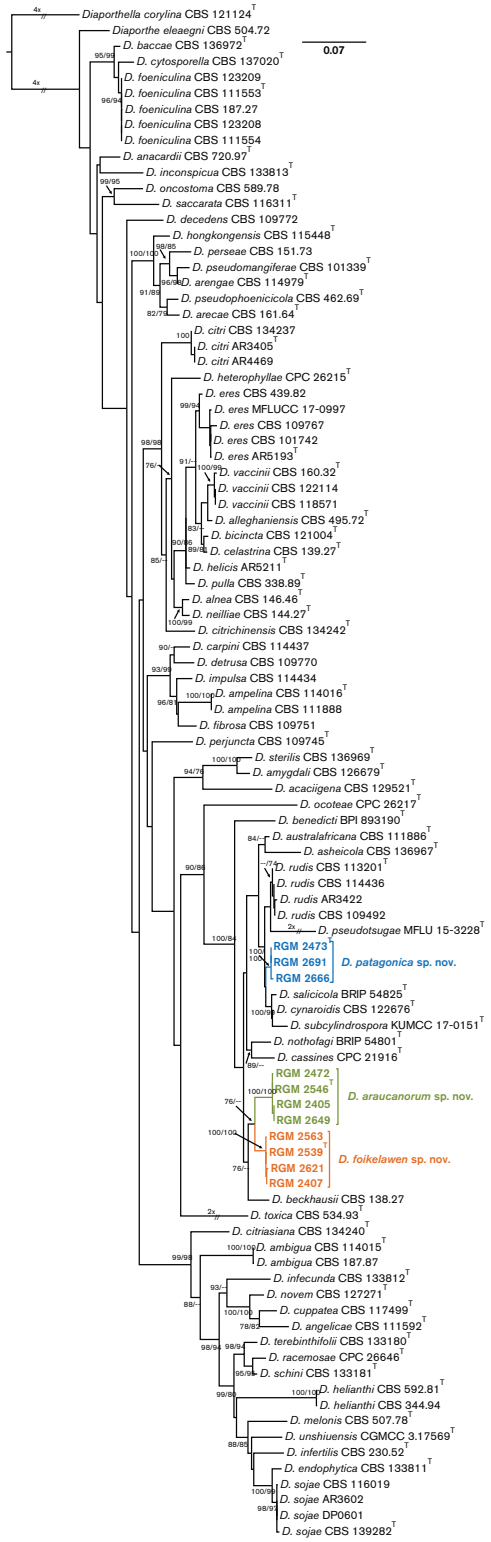

Fig. 2. RAxML phylogram obtained from the combined ITS (573 bp), *tub2* (485 bp) and *tef1* (232 bp) sequence alignment of selected species of *Diaporthe*. The tree was rooted to *Diaporthella corylina* CBS 121124. The novel species described in this study are shown in colour. Bootstrap values above 75% are recorded at nodes as ML/MP.

fusiform, mainly 2 guttulate, (7–)8.5–10(–10.5) × (2–)2.5(–3) µm (av. 9.3±0.8×2.6±0.3 µm). *Beta conidia*, hyaline, aseptate, smooth, filiform, straight or curved, eguttulate, (13–)15.5–22.5(–26)×1–1.5(–2) µm (av. 19.3±2.8×1.4±0.2 µm). *Gamma conidia* not observed.

Culture characteristics. Colony on MEA reaching 50.6±5.1 mm, with a lobate edge, appressed, almost without aerial mycelium, dark brown in the centre and amber to light brown at the periphery. On PDA slow-growing reaching 11.4±0.9 mm diam., low, compact, cream-coloured (except isolates RGM 2405, RGM 2472 and RGM 2649 that covered almost the entire plate after 2 weeks). On OA covering entire plate after 2 weeks, appressed, dark brown-colored with felty cream mycelium patches.

Additional specimens examined. Chile, Curacautin, Conguillío National Park (38° 38′ 28″ S, 71° 29′ 41″ W), from *Araucaria araucana*, in June 2016, collected by P. Pulgar, living culture RGM 2472=CBS 145283. Chile, Curanilahue county, Trongol Alto (37° 37′ 15″ S, 73° 09′ 34″ W), from *Araucaria araucana*, in March 2017, collected by G. Hinojosa, living culture RGM 2405=CBS 145284. Chile, Melipeuco county, Laguna Carilafquen (38° 55′ 21″ S, 71° 29′ 15″ W), from *Araucaria araucana*, March 2018, collected by D. Rupaillan, living culture RGM 2649=CBS 145286.

Diaporthe araucanorum has conidia that are similar in size to *Phomopsis araucariae*, which was originally described on the fallen scale-leaves of *Araucaria imbricata* in England [40]. *D. araucanorum* was isolated from leaves of *Araucaria araucana* growing in its natural habitat in Chile, a different host and eco-region, and morphologically differs from *P. araucariae* in its larger conidiomata (up to 550 vs 200–300 µm) and its shorter beta conidia (mostly 15.5–22.5 vs 22–25 µm). *D. araucanorum* is phylogenetically close but clearly differentiated from the new described *D. foikelawen* based on ITS [identities=549/564 (97%), five gaps], *tub2* [identities=516/530 (97%), no gaps] and *tef1* [identities=557/598 (93%), two gaps] sequences, and from *D. beckhausii* based on ITS [GenBank KC343041; identities=542/560 (97%), five gaps), *tub2* [GenBank KC344009; identities=469/490 (96%), three gaps] and *tef1* [GenBank KC343767; identities=294/330 (89%), four gaps) sequences. An additional *cal* sequence for ex-type was deposited in GenBank with the number MN974277.

DESCRIPTION OF *DIAPORTHE FOIKELAWEN* SP. NOV.

Diaporthe foikelawen (foi.ke.la'wen. N.L. n. *foikelawen*, from the host plant name *Drimys winteri*, in Mapudungun language, pronounced as foikelawen) Fig. 4.

MycoBank no. MB832752.

Typus. Chile, Freire county, Los Yugos (38° 56′ 55″ S, 72° 47′ 20″ W, from *Drimys winteri*, June 2016, collected by M. Espinoza F55605-16 (holotype RGM 2539, preserved in a metabolically inactive state at the Chilean Microbial Genetic Resources Collection, INIA Quilamapu, Chillán, Chile).

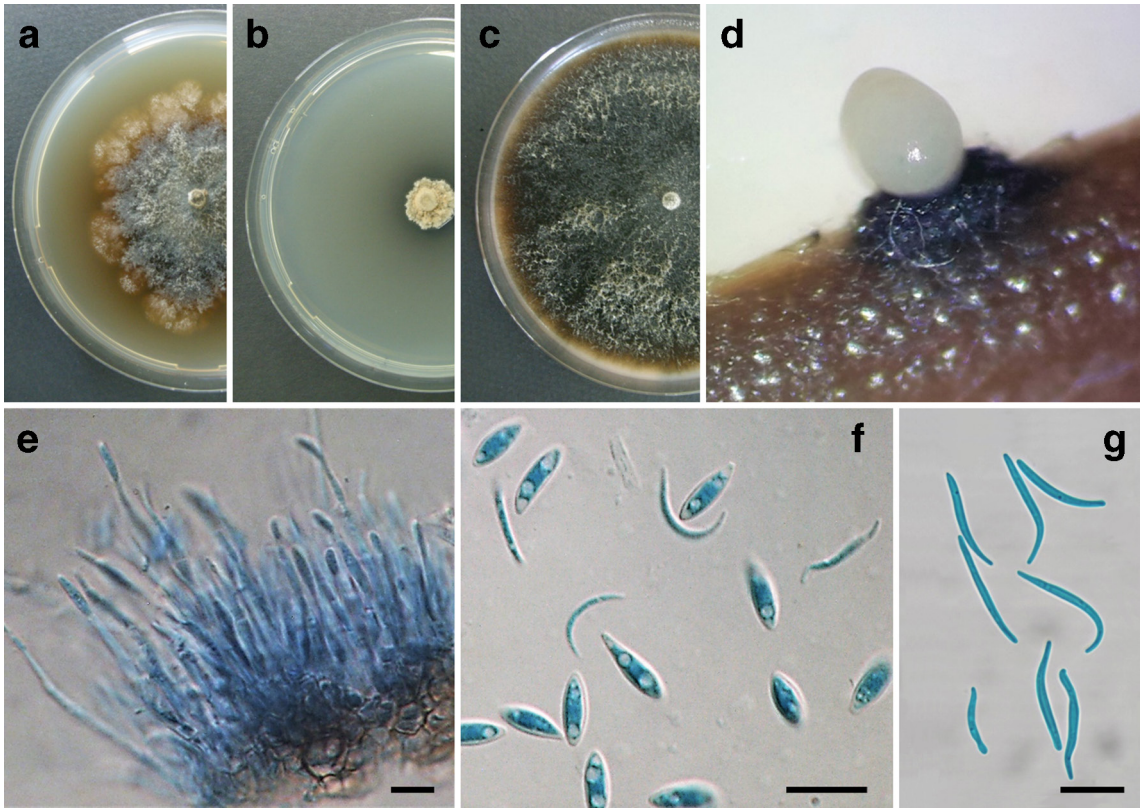

Fig. 3. *Diaporthe araucanorum* (RGM 2546). (a–c) Colonies at 2 weeks on MEA, PDA and OA, respectively. (d) Conidiomata sporulating on sterilized pine needle on WA. (e) Transverse section of conidioma with conidiophores. (f) Alpha conidia. (g) Beta conidia. Bars, 10 µm.

Ex-type culture CBS 145289, deposited at the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands.

Sexual morph not observed. *Conidiomata* pycnidial on pine needles on WA, eustromatic, immerse to erumpent, brown to black, scattered or aggregated, globose, flask-shaped to conical, unilocular or convoluted up to 450 µm wide, outer surface smooth, including short necks up to 650 µm, ostiole single, or several in conidiomata complex; pycnidal walls consisting of two regions of *textura angularis*; the outer region brown, 4–6 layers, inner region light to dark brown towards the outer region with more compressed cells; conidial mass globose to conical or exuding in cirrhi, light yellow to cream. *Conidiophores* hyaline smooth, 1–2 septate, rarely branched, densely aggregated, cylindrical, straight, 15–26×1–2.2 µm. *Conidiogenous cell* phialidic, cylindrical to cymbiform, terminal and lateral with slight taper towards apex, 5–17×1–1.7 µm, with inconspicuous periclinal thickening. *Paraphyses* not observed. *Alpha conidia* aseptate, hyaline, smooth guttulate, fusoid to ellipsoide, tapering towards both ends, straight, (7.5–)9.5–11(–12.5)×(2.5–)3–3.5(–4) µm (av. 10.1±0.7×3.1±0.4 µm). *Beta conidia* spindle shaped, aseptate, smooth, hyaline, apex subacutate, base lightly truncate, some straight mostly curved towards one end, (13–)15–19(–20.5)×1–1.5 (av. 17±1.6×1±0.2 µm). *Gamma conidia* not observed.

Culture characteristics. Colonies covering entire plate after 2 weeks. On MEA with moderate aerial mycelium, grey surface, reverse olivaceous-grey. On PDA with moderate aerial mycelium and a main concentric ring, grey surface with light brown patches, reverse iron-grey. On OA white sparse aerial mycelium, surface and reverse olivaceous.

Additional specimens examined. Chile, Tortel county (47° 49′ 04″ S, 73° 26′ 20″ W), from *Drimys winteri*, in April 2017, collected by O. Ojeda, living culture RGM 2407=CBS 145287. Chile, Vilcún county, Ruta S-39 (38° 42′ 52″ S, 72° 14′ 17″ W), from *Drimys winteri*, Nov 2017, collected by M. Espinoza, living culture RGM 2563=CBS 145290. Chile, Tucapel county, Quilleco (37° 28′ 08″ S, 71° 58′ 57″ W), from *Drimys winteri*, in April 2018, collected by D. Figueroa, living culture RGM 2621=CBS 145288.

Spegazzini [41] described *Diaporthe winteri* on dead bark on *Drimys winteri* in Staten Island, Argentina, which later was renamed as *D. drimydis* by Saccardo and Sydow [42]. This description was based on the sexual morph and the type material (herbarium) deposited at the Museo La Plata (Argentina). *D. foikelawen* did not produce a sexual morph in culture, or when incubated on sterile twigs of *Drimys winteri* after 3 months (at 20 °C, with near-UV light), even though all the specimens examined were crossed against each other.

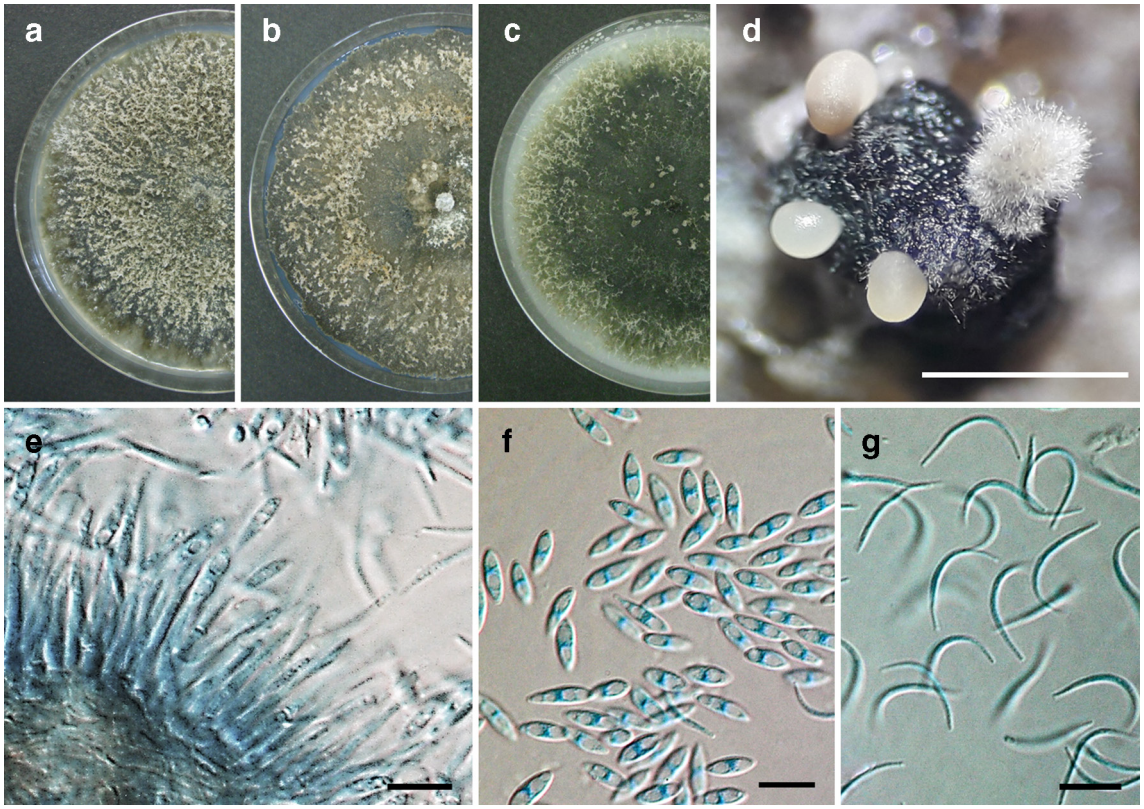

Fig. 4. *Diaporthe foikelawen* (RGM 2539). (a–c) Colonies at 2 weeks on MEA, PDA and OA, respectively. (d) Conidiomata sporulating on MEA. (e) Transverse section of conidioma with conidiophores. (f) Alpha conidia. (g) Beta conidia. Bars: (d), 500 µm; (e–f), 10 µm.

With the only sexual morph of *D. drimydis* described in the literature and the asexual morph observed in *D. foikelawen*, it is not possible to link morphologically both species. Because the herbarium material (LPS – Spegazzini) is currently not available for loan, we could not try a DNA comparison.

Diaporthe foikelawen is phylogenetically close but clearly differentiated from the new described *D. araucanorum* based on ITS [identities 549/564 (97%), five gaps], *tub2* [identities 516/530 (97%), no gaps] and *tef1* [identities 557/598 (93%), two gaps] sequences, and from *D. beckhausii* based on ITS [GenBank KC343041; identities=550/559 (98%), two gaps], *tub2* [GenBank KC344009; identities=470/489 (96%), one gap] and *tef1* [GenBank KC343767; identities=304/331 (92%), four gaps] sequences. *D. foikelawen* is easily differentiable from *D. araucanorum* by colony morphology and from *D. beckhausii* in the beta conidia size, being longer in *D. foikelawen* (mostly 15–19 µm) than *D. beckhausii* (8–13 µm). An additional *cal* sequence for ex-type was deposited on GenBank with the number MN974278.

DESCRIPTION OF *DIAPORTHE PATAGONICA* SP. NOV.

Diaporthe patagonica (pa.ta.go'ni.ca. N.L. fem. adj. *patagonica* pertaining to Patagonia) Fig. 5.

MycoBank no. MB832753.

Typus. Chile, Aysén county (45° 27′ 37″ S, 72° 19′ 55″ W), from *Aristotelia chilensis*, in September 2016, collected by M. González F72203-16 (holotype RGM 2473, preserved in a metabolically inactive state at the Chilean Microbial Genetic Resources Collection, INIA Quilamapu, Chillán, Chile). Ex-type culture CBS 145291, deposited at the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands.

Sexual morph not observed. *Conidiomata* pycnidial on pine needles on WA, eustromatic, convoluted to unilocular, immerse to erumpent, brown to black, scattered or aggregated, globose, flask-shaped, outer surface smooth, up to 550 µm wide, with black cylindrical ostiolate necks up to 1 mm; pycnidal wall consisting of two regions of *textura angularis*, outer region brown, 6–9 layers, inner region light to dark brown towards the periphery; conidial mass poorly exuded, light yellow to cream. *Conidiophores* hyaline, smooth, densely aggregated, cylindrical, straight, reduced to conidiogenous cell. *Conidiogenous cells* phialidic, cylindrical, terminal and lateral, with slight taper towards apex, with inconspicuous periclinal thickening, 10.5–22×1–2.5 µm. *Paraphyses* not observed. *Alpha conidia* aseptate, hyaline, smooth, mostly biguttulate, fusoid to ellipsoide, tapering towards both ends, straight, (5.5–)6–7.5(–10)×(2–)2.5(–3)

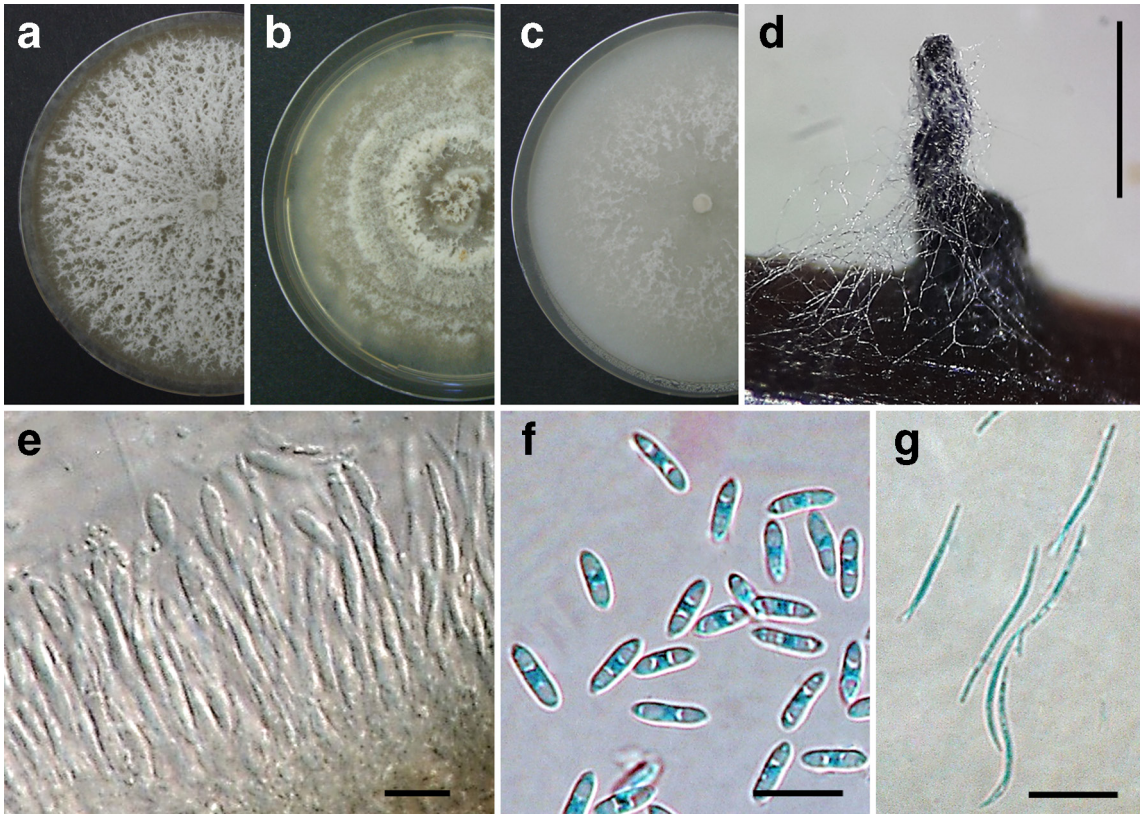

Fig. 5. *Diaporthe patagonica* (RGM 2473). (a–c) Colonies at 2 weeks on MEA, PDA and OA, respectively. (d) Conidiomata on sterilized pine needle on WA. (e) Transverse section of conidioma with conidiophores. (f) Alpha conidia. (g) Beta conidia. Bars: (d) 500 µm; (e–g) 10 µm.

µm (av. 7.0±0.7×2.4±0.2 µm). *Beta conidia*, hyaline, spindle shaped, aseptate, smooth, hyaline, apex subacutate, base lightly truncate, some straight, mostly curved towards one end, (12–)16–22(–28.5)×1–1.5 µm (av. 18.8±2.7×1.3±0.2 µm). *Gamma conidia* scarcely observed.

Culture characteristics. Colonies covering entire plate after 2 weeks. On MEA with profuse aerial mycelium, white surface, reverse amber. On PDA with fluffy aerial mycelium and concentric rings, dirty white surface, reverse ochreous with brown rings. On OA white sparse aerial mycelium, surface and reverse white.

Additional specimens examined. Chile, Aysén county (45° 05′ 30″ S, 72° 08′ 08″ W), from *Aristotelia chilensis*, in October 2018, collected by R. Vidal, living culture RGM 2666=CBS 145755. Chile, Aysén county (45° 12′ 49″ S, 72° 12′ 11″ W), from *Aristotelia chilensis*, in October 2018, collected by R. Vidal, living culture RGM 2691=CBS 145756.

Diaporthe patagonica was collected from *Aristotelia chilensis* trees showing branch die-back symptoms in the Region of Aysén, Chilean Patagonia. This species is phylogenetically related to *D. salicicola*, *D. cynaroidis* and *D. subcylindrospora*. *D. patagonica* is separated from these and other *Diaporthe* species using *tub2*, *tef1* and *cal* genes, with *tef1* and *cal* performing best as a diagnostic sequence. Based on a megablast search of NCBIs GenBank nucleotide database restricted to ex-type strains, the closest hits using the *tef1* sequence were *D. asheicola* [GenBank KJ160594; identities=576/595 (97%), one gap], *D. salicicola* [GenBank JX862537; identities=572/592 (97%), four gaps] and *D. nothofagi* [GenBank JX862536; Identities=550/597 (97%), six gaps). Closest hits using the *cal* sequence were *D. cynaroidis* (GenBank KC343300, identities=435/467 (93%), three gaps], *D. asheicola* [GenBank KJ160542; identities=431/467 (98%), three gaps] and *D. rudis* [GenBank KC843146; identities=431/467 (92%), three gaps). An additional *cal* sequence for the ex-type was deposited in GenBank with the number MN974279.

DISCUSSION

Diaporthe species records for the Chilean native forest date from before 1928, mostly associated with sclerophyllous trees [20, 21]. The current study introduced three novel species of *Diaporthe* associated with evergreen species in the central-southern area of Chile. The species were identified on the basis of morphological and phylogenetic criteria.

DNA sequence analyses were performed using four loci (ITS, *tub2*, *tef1* and *cal*) reported as useful markers to identify species of *Diaporthe* [43]. The novel species can be distinguished from the other species of *Diaporthe* by all

genes studied, but most effectively using *tef1* and *cal*. The multi-locus phylogenetic analysis grouped the isolates from *Araucaria araucana*, *Drimys winteri* and *Aristotelia chilensis* in three distinct clades with a very strong bootstrap value at the node (MP/ML=100/100), which supports the introduction of three new species.

Diaporthe araucanorum was isolated from branches of *Araucaria araucana*, a relict conifer of the temperate latitudes of southern Chile and southwestern Argentina. Araucaria forests have a relatively limited natural distribution spanning only three grades of latitude from 37° 20′ to 40° 20′ S [44]. The multilocus phylogenetic analysis showed *D. araucanorum* clustered close to *D. foikelawen* and *D. beckhausii*. It morphologically differs from *D. foikelawen* in the cultural characteristics, with *D. araucanorum* producing dark brown colonies of slow-growth on MEA and *D. foikelawen* grey colonies of fast-growth; and differing from *D. beckhausii* in the beta conidia size, with beta conidia longer in *D. araucanorum* (mostly 15.5–22.5 µm) than *D. beckhausii* (8–13 µm) [45].

Diaporthe foikelawen was isolated from leaves and twigs of *Drimys winteri* var. *chilensis*, a small evergreen tree commonly found in the swamp forests and lowlands of Chile and Argentina. The variety chilensis occurs from Salamanca to Aysén (32–42°S), including Chiloé Island [46]. The multilocus phylogenetic analysis showed *D. foikelawen* close to *D. araucanorum* and *D. beckhausii*. Morphologycally it differs from *D. beckhausii* in the beta conidia size, with beta conidia longer in *D. foikelawen* (mostly 15–19 µm) than *D. beckhausii* (8–13 µm).

Diaporthe patagonica was isolated from branches of *Aristotelia chilensis*, another evergreen tree of the sub Antarctic forests of Chile and Argentina, widely distributed between the Limarí and Aysén provinces (31–42°S) [47]. All the isolates were collected in the Chilean Patagonia in Aysén. *D. patagonica* is phylogenetically close to *D. salicicola, D. cynaroides* and *D. subcylindrospora*. Morphologically *D. patagonica* resembles *D. salicicola*, but it differs in the beta and gamma conidia production (*D. salicicola* produces only alfa conidia) [48].

The utility of host association for species recognition is limited in *Diaporthe* because several species have wide host ranges and multiple species can be recovered from a single host plant [5, 8, 49]. Thus, the identification and description of new species must be a polyphasic approach, with morphological, cultural, ecological and molecular characteristics that support the novelties. Although it is unlikely that the species described here are host-specific, to our knowledge, they have not been isolated from a different host in Chile until now.

Diaporthe includes several endophytic, saprotrophic and phytopathogenic species on a wide range of hosts [5–7]. Pathogenic species on forest and ornamental trees usually have been reported causing canker and dieback diseases [15, 50]. Although the novel species described here were isolated from necrotic leaf spots and branch showing dieback, their pathogenicity should be evaluated.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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