RESEARCH ARTICLE

Yeast and yeast-like fungi associated with dry indehiscent fruits of *Nothofagus nervosa* in Patagonia, Argentina

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Abstract

Nothofagus nervosa (Raulí) is a native tree species that yields valuable timber. It was overexploited in the past and is currently included in domestication and conservation programs. Several research programs have focused on the characterization of epiphytic microorganisms because it has been demonstrated that they can affect plant–pathogen interactions and/or promote plant growth. Although the microbial ecology of leaves has been well studied, less is known about microorganisms occurring on seeds and noncommercial fruits. In this work, we analyzed the yeast and yeast-like fungi present on *N. nervosa* fruits destined for the propagation of this species, as well as the effects of fruit preservation and seed dormancy-breaking processes on fungal diversity. Morphological and molecular methods were used, and differences between fungal communities were analyzed using a similarity index. A total of 171 isolates corresponding to 17 species were recovered, most of which belong to the phylum *Ascomycota*. The majority of the species develop mycelia, produce pigments and mycosporines, and these adaptation strategies are discussed. It was observed that the preservation process considerably reduced yeast and yeast-like fungal diversity. This is the first study concerning microbial communities associated with this ecologically and economically important species, and the information presented is relevant to domestication programs.

Introduction

Microorganisms are capable of synthesizing metabolites that are of great relevance to industry, such as enzymes, fatty acids, pigments, and antibiotics. Microbial biodiversity is therefore considered one of the principal sources of innovation in biotechnology, and a variety of habitats have been screened to find biotechnologically important microorganisms (Bull *et al.*, 1992; Middelhoven, 1997; Bhadra *et al.*, 2008), such as those that could be of importance for plant improvement and the biocontrol of plant diseases.

There are approximately 200 000 species of vascular plants on record, and their surfaces constitute an important habitat for microorganisms, providing a wide range of microclimatic conditions for correspondingly diverse microbial communities (Andrews & Harris, 2000; Kowalchuk *et al.*, 2010). The phyllosphere is broadly defined as the surfaces and internal parts of aerial plant structures, including flowers, fruits, stems, and leaves (Timms-Wilson *et al.*, 2006; Whipps *et al.*, 2008). It is a harsh environment with reduced access to nutrients, high fluctuations in temperature and water availability, and exposure to wind and UV radiation (Kowalchuk *et al.*, 2010).

Microorganisms present in the phyllosphere are so numerous that they can influence plant fitness and contribute to important global processes, such as the carbon and nitrogen cycles (Andrews & Harris, 2000; Lindow & Brandl, 2003; Kowalchuk *et al.*, 2010). Consequently, they may affect the quality and productivity of agricultural crops (Whipps *et al.*, 2008), either by promoting plant growth (Glickmann *et al.*, 1998; Brandl *et al.*, 2001), increasing drought tolerance, and/or playing a major role in antagonizing pathogens. Hence, the phyllosphere represents a habitat with ecological and biotechnological significance, and a better understanding of this environment

may provide new insights into the development of control strategies for the management of plant diseases (Lindow & Brandl, 2003; Whipps *et al.*, 2008).

The most abundant inhabitants of the phyllosphere, and the most studied group, are the bacteria. Yeasts and yeast-like fungi (These fungi are also known as *dimorfic fungi* or *black yeasts*, both being technical terms to describe groups of fungi that are quite heterogeneous from a taxonomic and phylogenetic point of view, most of them being ascomycetous species. The former corresponds to those fungi that can change from the yeast form to the mycelial form in response to changes in environmental factors. *Black yeasts* have melanized cell walls and most of them exhibit mycelial growth and generate conidia (Sterflinger, 2006) are also active phyllosphere colonizers and belong to the largest group of fungi that grow in this environment, whereas filamentous fungi are transient inhabitants present mainly as dormant spores (Andrews & Harris, 2000; Lindow & Brandl, 2003; Whipps *et al.*, 2008). According to Glushakova & Chernov (2007), plant exudates are the main source of nutrients for yeasts and yeast-like fungi, and through consumption of these exudates, they can, in turn, stimulate plant metabolism. Some studies have also shown that selected members of the phyllosphere yeast community inhibit or limit infection of certain plant pathogens (e.g. Fokkema *et al.*, 1979; Janisiewicz, 1991; Elad *et al.*, 1994; Punja & Utkhede, 2003; Buck, 2004; Pusey *et al.*, 2009) through different modes of action, such as niche occupation, competition for nutrients, and antibiosis (Jacobsen, 2006). In fact, some species such as *Aureobasidium pullulans* (de Bary) G. Arnaud, *Kloeckera apiculata* (Reess) Janke, *Pichia guilliermondii* Wick. and *Sporobolomyces roseus* Kluyver & C.B. Niel have already been used as biocontrol agents to manage postharvest losses and control diseases on pear, citrus fruit, grape, peach, apple, sweet cherry, geranium, beans and tomato (McLaughlin *et al.*, 1992; Droby *et al.*, 1993; Elad *et al.*, 1994; Janisiewicz *et al.*, 1994; Chand-Goyal & Spotts, 1996a, b; Buck, 2004; Jacobsen, 2006; Elwakil *et al.*, 2009). Some yeasts have also been used for the biological control of different diseases associated with leaves and flowers (Dik & Fokkema, 1993; Urquhart & Punja, 1997; Tatagiba *et al.*, 1998; Paulitz & Bélanger, 2001; El-Mehalawy, 2004; Pusey *et al.*, 2009).

Many research programs have focused on the characterization of microorganisms that live epiphytically on leaves, the dominant aerial plant structure (Andrews & Harris, 2000; Lindow & Brandl, 2003; Jacobsen, 2006; Sláviková *et al.*, 2007; Pusey *et al.*, 2009). The microbiology of buds and flowers has also been well studied, mainly because this is the site of infection by several plant pathogens (Andrews & Harris, 2000; Pusey *et al.*, 2009). However, little is known about microbial populations on seeds and noncommercial fruits (Janisiewicz *et al.*, 2010), which might be also susceptible to deterioration caused by insects and different microorganisms, mainly during maturation and postharvest storage (Marchelli & Gallo, 1999; Hadanich *et al.*, 2008). Most of these microorganisms cause visible symptoms and/or reduce seed germination (Richarson, 1979; McGee, 1995). On the other hand, some studies have demonstrated that applying microorganisms to seeds may improve plant establishment, health and growth, particularly if they subsequently become established in the root zone (Wright *et al.*, 2003; Bennett & Whipps, 2008).

Several species belonging to the genus *Nothofagus* constitute the main component of South American temperate forests. *Nothofagus nervosa* (Phil.) Dim. et Mil. (Raulí) is one of the most economically important species of these forests. As it yields a highly valuable wood, resembling that of *Fagus sylvatica* L., it was overexploited in the past and natural populations were drastically reduced. This critical situation led to the implementation of conservation and domestication programs. The main purpose of these programs is to propagate *N. nervosa* in nurseries and use them subsequently for reforestation (Marchelli & Gallo, 1999; Gallo *et al.*, 2004). To accomplish this, seeds are needed, and they are collected directly from natural populations. All *Nothofagus* species have indehiscent dry fruits containing only one seed (nuts). As the pericarp does not split open, seedlings are cultivated directly from these fruits, which are not previously disinfected. It is, therefore, expected that the microbiota present on them would be carried through the cultivation system.

One of the most important diseases affecting *Nothofagus* propagation in nurseries is the damping-off caused by different species of *Fusarium*, *Rhizoctonia*, *Phytophthora,* and *Sclerotinia* (Azpilicueta *et al.*, 2010), which are common greenhouse pathogens worldwide (Paulitz & Bélanger, 2001; Azpilicueta *et al.*, 2010). These fungi can seriously reduce seed germination and seedling emergence, stand, and vigor. The use of fungicides is the conventional method for protecting plants against this disease, but this has resulted in resistance development in pathogens and most of them are environmentally harmful. Biocontrol methods provide an alternative to chemical fungicides (Berger *et al.*, 1996) and different microorganisms that are antagonistic against the fungi that cause damping-off have been found (Berger *et al.*, 1996; Mao *et al.*, 1997), including some yeast and yeast-like fungi (El-Tarabily, 2004; El-Mehalawy *et al.*, 2007; Elwakil *et al.*, 2009).

Our main objective was to determine the diversity of yeast and yeast-like fungi present on *N. nervosa* fruits prior to cultivation in nurseries and to evaluate how

previous manipulation (preservation and process used to break seed dormancy) influences the occurrence of these organisms on the fruits. Morphological and molecular tools were used to characterize these fungi, and differences in fungal diversity between treatments were evaluated. A long-term aim of this work is to identify potential biocontrol or growth promoting yeasts and yeast-like fungi that could be of importance for conservation and domestication programs.

Materials and methods

Fruit harvest

Fruits were collected from *N. nervosa* trees in a forest situated in the Yuco region (Lacar lake watershed, 40°07′48″ S, 71°34′48″W, Neuquén province, Patagonia, Argentina). The average age of the fruit-contributing trees was between 100 and 150 years.

Fruit collection was performed during the fruit-fall season (March–April) in 2002 and 2007 by placing nets below *N. nervosa* canopy at approximately 1.5 m above the ground. Nets were distributed to capture fruits from a minimum of 40 trees. Fruits were kept in plastic bags in a cold (2–4 °C), dry place until further procedures were performed (seeds are still viable after 6–9 years under these conditions) (Martinez & Schinelli, 2009). It is important to mention that, in this work, fruit collection and handling did not differ from the procedures commonly carried out for domestication programs.

Yeasts isolation and characterization

To evaluate whether preservation at 4 °C influences yeast and yeast-like fungal diversity, both fresh and preserved fruits were analyzed. Fresh fruits were collected some weeks before this work was performed (harvest 2007), while the preserved fruits were part of a harvest that had been stored at 4 °C for 5 years (harvest 2002). For yeast and yeast-like fungi isolation, 30 fresh and 30 preserved fruits were randomly selected from each pool.

For seed germination, it is first necessary to break seed dormancy by placing the fruits in cold running water (*c.* 5 °C) for 5 days (washing process). Another important requirement is that the seeds have to be sowed no more than 1 cm below the surface, in a porous substrate that allows hydration and avoids compaction. The optimum temperature for seed germination is 18–21 °C. In these conditions, seedlings emerge in 10–15 days (Schinelli & Martinez, 2010). To evaluate whether microbial composition changes after the washing process and to describe the yeast and yeast-like fungi present on the fruits at the time of sowing, 15 fresh and 15 preserved fruits of those previously selected were placed in cold running water for 5 days before isolation.

There were four treatments for yeast and yeast-like fungi isolation: fresh nonwashed (FnW), fresh washed (FW), preserved nonwashed (PnW), and preserved washed (PW) seeds. Each fruit was put into a sterile plastic tube containing 1 mL of sterile 0.9% NaCl solution and 0.15 g of sterile sand. Tubes were vortex-mixed at maximum speed for 2 min and centrifuged for 30 s. Aliquots of 100 μL of the supernatant were surface plated on MYP medium (malt extract 0.7, yeast extract 0.05, peptone-soytone 0.25 and agar 1.5% w/w) supplemented with 0.01% chloramphenicol. Plates were incubated at room temperature (20 °C) for 72 h and then at 4 °C for 48 h to enhance the development of characteristic colony color and other morphological features. Three representative colonies of each morphological type present were picked per plate, and pure isolates were obtained by repeated streaking on fresh MYP medium. All the isolates were first grouped according to their macromorphology (color, colony shape and texture, and presence of mycelia) and their ability to produce mycosporines. Mycosporine light induction, extraction, and analysis were assessed in collaboration with Dr. Martín Moliné (Laboratorio de Microbiología Aplicada y Biotecnología, Centro Regional Universitario Bariloche) and according to Moliné *et al.* (2011). Isolates were cryopreserved in 20% glycerol and stored at −80 °C.

Molecular analyses

DNA extraction and PCR fingerprinting

For total genomic DNA extraction, a loopful of a fresh culture of each isolate (cultivated in MYP – malt–yeast–peptone agar – at 20 °C for 72 h) was transferred to 100 μL of sterile pure water, frozen at −80 °C for an hour, and heated at 100 °C for 10 min. Tubes were centrifuged for 1 min at 13 200 ***g***, and the supernatant was transferred to a clean tube.

All the isolates within the different morphological groups were subjected to PCR fingerprinting, employing the mini/microsatellite-primed PCR technique (MSP-PCR) and the M13 primer (5′-GAGGGTGGCGGTTCT-3′, Sigma). PCR reaction was performed in a total volume of 25 μL containing 3 mM $MgCl_2$, 0.2 mM dNTPs, 0.8 μM primer, 1 U Taq polymerase (Invitrogen), 1× of the reaction buffer, and 5 μL of a 1 : 175 genomic DNA dilution (Libkind *et al.*, 2003; de García *et al.*, 2007). The amplification was carried out in a Multigene Labnet cycler according to the following PCR conditions: an initial denaturing step at 95 °C for 5 min, followed by 40 cycles of 45 s at 93 °C, 60 s at 55 °C and 60 s at 72 °C, and a final extension step

of 6 min at 72 °C. Amplified DNA fragments were separated by electrophoresis in 1.5% (w/v) agarose gels.

Sequencing and phylogenetic analysis

Isolates with identical DNA banding patterns were grouped together, and at least two representatives of each group were sequenced. For DNA sequencing, the D1/D2 domain of the 26S rRNA gene was amplified using the NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) primers (Genbiotech, Argentina). PCR was performed with 0.5 mM $MgCl_2$, 0.8 mM dNTPs, 0.2 μM each primer, 1 U Taq polymerase, and 1× reaction buffer (Invitrogen), and 3.8 μL of the 1:500 genomic DNA dilution was added to the reaction. PCR conditions were as follows: a denaturing step at 95 °C for 2 min, 35 cycles of 15 s at 95 °C, 25 s at 54 °C and 20 s at 72 °C, and a final extension step of 10 min at 72 °C. Sequencing was performed from purified PCR products (Wisard kit; Promega) using BigDye chemistry (Applied Biosystems) and analyzed on ABI3130XL automatic sequencers (Genomic Unit, CNIA INTA Castelar facilities, Argentina).

Sequences were manually corrected, aligned, and subjected to phylogenetic analysis using the Molecular Evolutionary Genetics Analysis software (MEGA4). Two phylogenetic trees, one for Ascomycetes and the other for Basidiomycetes, were constructed using the neighbor-joining algorithm. Bootstrap values were calculated from 1000 replicates and the Kimura 2-parameter model to estimate evolutionary distance. If several strains of a single species were sequenced, only four were taken into account for constructing the tree (if there were sequences belonging to the same species but with some nucleotidic differences – 5 or less – those with the higher number of differences were selected). On the phylogenetic tree, all the species were clustered with their nearest phylogenetic relatives according to the NCBI database (http://www.ncbi.nlm.nih.gov) and with type strains if they were available. All the nucleotide sequences obtained in this work were deposited in the NCBI GenBank database and are available for comparative purposes under the accession numbers HQ629551–HQ629619 (Table S1). The Index Fungorum database was used for nomenclature and classification of the species (www.indexfungorum.org).

Community analysis

Beta-diversity is defined as the variation of species composition over space and time (Anderson *et al.*, 2006) and is crucial to the understanding of how environmental factors affect biodiversity, even in microbial populations. The modified Jaccard index described by Chao *et al.* (2005) takes species abundance into account, being better suited than the corresponding classic index for the assessment of compositional similarity between samples containing numerous rare species (Chao *et al.*, 2005), which is the case in this work. This index was selected for measuring beta-diversity and for evaluating how the preservation and washing processes affected yeast and yeast-like fungal composition associated with *N. nervosa* fruits. Differences in yeast and yeast-like fungal communities were analyzed between: (a) fresh and preserved fruits, (b) FnW and FW fruits, and (c) PnW and PW fruits.

Statistical analysis

A chi-squared analysis was carried out to test the association between the categorical variables of preservation (fresh and preserved) and washing process (nonwashed and washed). To evaluate whether these treatments modify the yeast and yeast-like fungi abundance on the fruits, two Paired *t*-tests were also conducted (fresh vs. conserved and nonwashed vs. washed).

Results

A total of 171 isolates were recovered from the 60 *N. nervosa* fruits examined: 94% from fresh and 6% from preserved fruits (Table 1). Yeast and yeast-like fungi were present in all the fresh but only in 20% of the preserved fruits. The percentage of isolates recovered from nonwashed and washed treatments were 47% and 53%, respectively. The chi-squared test showed that there was no association between these variables (preservation and washing) ($\chi^2 = 0.69$, $P < 0.05$), and the Paired *t*-test indicated that the preservation process significantly reduced the quantity of yeast and yeast-like fungi present on the fruits analyzed ($P = 0.013$), whereas the washing treatment did not bring about a change ($P = 0.170$).

Seventeen yeasts and yeast-like fungi species were identified using a four-step strategy that involved: (i) allocation of isolates into distinct morphological groups, (ii) genomic profiling within each morphological group using MSP-PCR fingerprinting (M13 primer), (iii) sequencing of the D1/D2 domain of 26S rRNA gene for representatives of each genomic group, and (iv) species allocation of isolates by phylogenetic positioning, using sequences of reference strains available in databases. Yeasts belonging to the phylum *Ascomycota* were dominant, both at an isolate (78%) and a species level (61%), being five of them new *Ascomycetous* species. The remaining species belong to the phylum *Basidiomycota* (Table 1).

All the *Ascomycetous* species isolated in this work are dark pigmented (green, brown, and black), melanin-containing yeast-like fungi capable of forming conspicuous

Table 1. Taxa and number of strains isolated per species in each treatment

Taxa	Fresh		Preserved		Total (%)	Pig	Myc	Mcl
	nW	W	nW	W				
Ascomycota								
Dothideales								
Aureobasidium pullulans (de Bary) G. Arnaud	15	14	1	1	31 (18%)	$+^{M}$	+	+
Dothichiza sp. 1	26	21	–	–	47 (27%)	$+^{M}$	+	+
Dothichiza sp. 2	1	–	–	–	1 (< 1%)	$+^{M}$	+	+
Coniochaetales								
Lecythophora mutabilis (J.F.H. Beyma) W. Gams & McGinnis	1	–	2	–	3 (2%)	$+^{M}$	–	+
Taphrinales								
Taphrina wiesneri (Ráthay) Mix	–	1	–	–	1 (< 1%)	$+^{M}$	+	+
Chaetothyriales								
Phaeomoniella zymoides Hyang B. Lee, J.Y. Park, Summerb. & H.S. Jung	1	–	–	–	1 (< 1%)	$+^{M}$	+	+
Phaeomoniella sp.	1	–	–	–	1 (< 1%)	$+^{M}$	+	+
Incertae sedis								
Ascomycetous yeast sp. 1	19	27	–	–	46 (27%)	$+^{M}$	+	+
Ascomycetous yeast sp. 2	–	–	–	1	1 (< 1%)	$+^{M}$	–	+
Coniozyma leucospermi (Crous & Denman) Crous	–	1	–	–	1 (< 1%)	$+^{M}$	+	+
Basidiomycota								
Tremellales								
Cryptococcus adeliensis Scorzetti, I. Petrescu, Yarrow & Fell	–	2	–	–	2 (1%)	–	–	–
Cryptococcus diffluens (Zach) Lodder & Kreger-van Rij	1	–	–	–	1 (< 1%)	–	+	–
Cryptococcus wieringae Á. Fonseca, Scorzetti & Fell	1	–	–	–	1 (< 1%)	–	+	–
Cryptococcus heveanensis (Groen.) Baptist & Kurtzman	11	8	–	1	20 (12%)	–	+	–
Trichosporon dulcitum (Berkhout) Weijman	–	1	–	–	1 (< 1%)	–	+	+
Sporidiobolales								
Rhodotorula colostri (T. Castelli) Lodder	–	1	–	3	4 (2%)	$+^{C}$	–	–
Rhodotorula fujisanensis (Soneda) E.A. Johnson & Phaff	–	8	–	1	9 (5%)	–	–	–
Strains isolated per treatment	77	84	3	7				
	161		10		171			

W, washed; nW, nonwashed; %, percentage of the total number of isolates; Pig, pigments; ^{M}, melanin; ^{C}, carotenes; Myc, mycosporines; Mcl, mycelia.

mycelium. Most of the basidiomycetous isolates are nonpigmented and lack mycelia, with the exception of *Rhodotorula colostri* that is a pink carotenogenic yeast, and *Trichosporon dulcitum*, which develops mycelia. Seventy-one percent of the total number of species produces mycosporines, including the most abundant ones (Table 1).

Dothichiza sp. 1, *Ascomycetous* yeast sp. 1, *A. pullulans* (*Ascomycota*) and *Cryptococcus heveanensis* (*Basidiomycota*) were the most abundant species (Table 1). Altogether, these four species accounted for 84% of the total number of isolates. *Aureobasidium pullulans* was the only species present in all the treatments, and *C. heveanensis* was found in three of them. Both species were present more frequently on fresh fruits. *Dothichiza* sp. 1 and *Ascomycetous* yeast sp. 1 were recovered only from fresh fruits. *Lecythophora mutabilis* was present only on nonwashed fruits, while *R. colostri* and *Rhodotorula fujisanensis* appeared exclusively on washed fruits (Table 1). The remaining species (59%), were represented by no more than one or two isolates and were found in only one treatment, mostly corresponding to fresh fruits.

The different clusters on the phylogenetic trees correspond to the orders to which the yeasts and yeast-like fungi analyzed in this study belong (Fig. 1). Figure 1a shows that the yeast-like fungi *Ascomycetous* yeast sp. 2 is close to the *L. mutabilis* cluster (Fig. 1a), but there are 10 nucleotidic differences distinguishing it from this species, so that it represents a new species. Both species lack the ability to produce mycosporines (Table 1). *Ascomycetous* yeast sp. 1 is also a new species, and there are eight nucleotidic differences between it and its closest relative (Fungal Endophyte 9096), which is also an undescribed species. *Aureobasidium pullulans* strains are situated in different subclusters on the same branch, suggesting that there are different varieties of this species among the strains recovered in this work. *Dothichiza* sp. 1 and *Dothichiza* sp. 2 are separated from *Dothichiza pithyophila* by 13 and 17 nucleotidic differences, respectively, thus clearly phylogenetically separated from this species and from each other. In the tree corresponding to the phylum *Ascomycota*, it can also be observed that *Phaeomoniella* sp. 1 is separated from the *Phaeomoniella zymoides* cluster,

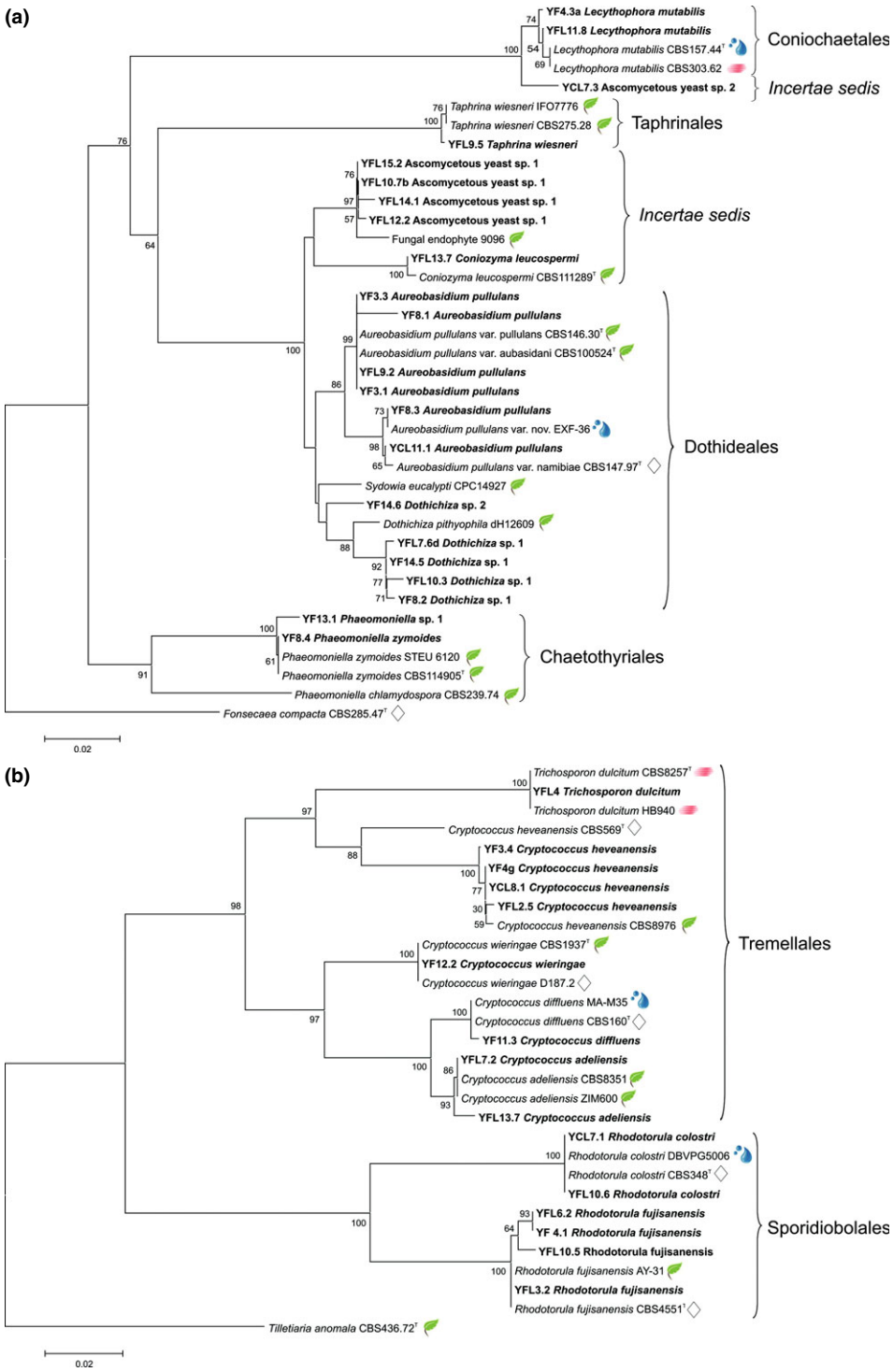

Fig. 1. Phylogenetic tree obtained by neighbor-joining and the Kimura 2-parameter analysis of the D1/D2 domains of the 26S rRNA gene. The numbers on the branches are the frequencies with which a given branch appeared in 1000 bootstrap replications (values smaller than 50% are not shown). Sequences determined in this study are in bold. Additional sequences were retrieved from the GenBank. *Fonsecaea compacta* and *Tilletiaria anomala* were used as outgroups for the yeasts and yeast-like fungi present on *Nothofagus nervosa* fruits, belonging to the (a) phylum *Ascomycota* and (b) phylum *Basidiomycota*, respectively. The following symbols indicate the substrate from which each strain was isolated: = plant associated, = soil associated, = aquatic environments, ◇ = others, T = Type strain.

which suggests that it is a different species (Fig. 1a). The phylogenetic tree corresponding to the phylum *Basidiomycota* (Fig. 1b) shows that *C. heveanensis* is closer to *T. dulcitum* than to the other *Cryptoccocus*. *Rhodotorula colostri* and *R. fujisanensis* are included in the same order and do not produce mycosporines (Table 1). It can be observed in these trees that most of the nearest phylogenetic neighbors were also isolated from plants or soil.

The modified Jaccard index showed low values when the comparisons between fresh/preserved and PnW/PW fruits were made (0.29 and 0.11, respectively), indicating that the yeast communities found on the fruits were very different following these treatments. In contrast, this abundance-based index indicated that fungal communities associated with FnW and FW fruits were similar (78%) (Table 2).

Discussion

Yeast and yeast-like fungus diversity

The method used in this work for assessing yeast and yeast-like fungus diversity on *N. nervosa* fruits consisted of: isolating these fungi according to standard techniques and sorting all the isolates into groups based on macroscopic characteristics and mycosporine production; each group was further characterized using the MSP-PCR fingerprinting method and the M13 primer, which usually generates species-specific patterns; finally, representative strains of the different MSP-PCR groups were selected for DNA sequencing. This strategy has been used in other studies on genetic diversity among species and genera (Balefires Couto *et al.*, 1996; Meyer *et al.*, 2001; Gadanho & Sampaio, 2002; Libkind, 2007) and also as a tool for yeast diversity description in natural environments (Gadanho *et al.*, 2003; Libkind *et al.*, 2003; de García *et al.*, 2007; Muñoz, 2010; Mestre *et al.*, 2011). One of the main reasons this strategy is suitable for this type of study, and so widely used, is that it allows rapid and inexpensive genetic characterization of several isolates at the same time (Libkind, 2007).

Table 2. Community analysis of the yeast and yeast-like fungi present on fruits before and after the preservation and washing processes

Beta indices	Fresh (16)–Preserved (6)	Fresh nW(10)–W(10)	Preserved nW(2)–W(5)
No. shared species	5	4	1
No. unique species	11–1	6–6	1–4
Shared species frequencies	0.35–0.60	0.92–0.83	0.33–0.14
Modified Jaccard index	0.29	0.78	0.11

nW, nonwashed fruits; W, washed fruits; (no.), number of species per treatment.

Some authors observed that *Ascomycetous* and basidiomycetous yeasts and yeast-like fungi were present in approximately equal frequencies on leaves (Slávikoá *et al.*, 2007) and bark (Bhadra *et al.*, 2008) of different tree species. However, Fonseca & Inácio (2006) suggested that one of the most significant trends to emerge from the analysis of several studies carried out in the phyllosphere is the clear dominance of Basidiomycetes. These authors argue that the microenvironments present on aerial plant organs are analogous (leaves, stems, most fruits, and flowers), so it is not surprising that the dynamics and composition of the respective yeast communities are also similar. Nevertheless, in this work, 17 species of yeasts and yeast-like fungi were isolated from *N. nervosa* fruits, the Ascomycetes being dominant both at isolate (78%) and species level (61%). These fruits are not fleshy and do not have stomata or water retaining structures, so they have lower humidity conditions than other plant organs. These results are in agreement with Middelhoven (1997) and Bhadra *et al.* (2008), who also found that *Ascomycetous* yeast and yeast-like fungi were dominant in the phyllosphere of 24 plant species in an arid climate and in different species of tree bark from a forest in India. Taking this information into account, plus the fact that Ascomycetes seem to be more stress resistant than Basidiomycetes (Baar *et al.*, 1999; Gorbushina & Broughton, 2009), it can be suggested that *Ascomycetous* yeast and yeast-like fungi are better adapted to low water availability and desiccation than the basidiomycetous (Bhadra *et al.*, 2008), thus explaining the higher incidence of ascomycetes in the microbiota of *N. nervosa* indehiscent dry fruits.

Most of the species present on *N. nervosa* fruits have been previously found on plants (Fig. 1; Weber *et al.*, 2002; Glushakova & Chernov, 2004; Muñoz, 2010), except for *L. mutabilis* and *T. dulcitum*, which have been isolated from soil. Most of the *T. dulcitum* strains found in the CBS database and in other studies (Mestre *et al.*, 2011) have also been isolated from soil, suggesting that this is the primary habitat of this species and that it reaches aerial plant parts by vectors (small mammals, birds, and insects) and/or wind. Bhadra *et al.* (2008) also found that most of the nearest phylogenetic neighbors to the yeast groups isolated from tree barks were isolated in association with plants and their environment (fruits, fruit juices, plant extracts, insects, and soil). The occurrence of soil yeasts in the phyllosphere is not rare, according to Andrews & Harris (2000), who stated that species found in the rhizosphere are commonly present in the phylloplane.

Aureobasidium pullulans was the only species present in all the treatments. This is a cosmopolitan yeast-like fungus that has been isolated from diverse substrates and seems to be ubiquitous on aerial plant surfaces worldwide (Fonseca & Inácio, 2006; Janisiewicz *et al.*, 2010; Muñoz, 2010). Some species found in this study have been previously recorded in other substrates from Patagonia. *Rhodotorula colostri*, *R. fujisanensis,* and *T. dulcitum* have also been reported from *Nothofagus* forest soil (Mestre *et al.*, 2011), while *R. colostri* and *Cryptococcus adeliensis* have been found in aquatic environments of glacial origin in northern Andean Patagonia (de García *et al.*, 2007).

Only four of the species identified in this work (24%) were dominant at community level. This observation is in accordance with different authors who stated that populations in the phyllosphere are commonly dominated by a few species. Rare species account for a significant proportion of the species richness present in this environment, and many novel taxa are present among them (Lindow & Brandl, 2003; Glushakova & Chernov, 2004; Fonseca & Inácio, 2006; Bhadra *et al.*, 2008; Whipps *et al.*, 2008; Janisiewicz *et al.*, 2010). Five of the species isolated from *N. nervosa* fruits (29%) have not been previously described; two of which are the most abundant species (*Dothichiza* sp. 1 and *Ascomycetous* yeast sp. 1). This information suggests that different plant species harbor highly adapted microorganisms, which might have novel biotechnological capacities (Kowalchuk *et al.*, 2010).

Effects of the preservation and washing processes

Following the arrival of microbial cells in the phyllosphere, a variety of factors determine whether they are able to colonize and survive on the plant surface, mostly substrate characteristics and environmental conditions (Whipps *et al.*, 2008). For example, the growth of microorganisms in this environment is supported by nutrients leaking from the plant, as well as external sources, like pollen deposits, organic debris, and honeydew (Glushakova & Chernov, 2007; Whipps *et al.*, 2008). If the supply of nutrients is insufficient, most of the microorganisms present on the plant organ will not be able to survive for a long period of time. Temperature and water availability are also important factors that regulate microbial diversity. Some authors observed that fungal diversity decreases during the winter and reaches a minimum at the beginning of spring, probably as a consequence of microbial intolerance to low temperatures and desiccation, and/or to a decrease in airborne inoculum and nutrients (Buck *et al.*, 1998; Glushakova & Chernov, 2007). When *N. nervosa* fruits are preserved, microorganisms living on them are exposed to low temperatures for long periods of time, without nutrient, water, or inoculum input. Under these conditions, it is to be expected that preservation will influence the abundance and composition of fruit-borne yeast and yeast-like fungi, as observed in this study, where significantly lower numbers of isolates and species were registered on preserved than on fresh fruits (Table 2).

A conspicuous feature of aerial plant surfaces is that free moisture is quite transient, mainly associated with rain and dew. Consequently, water availability significantly influences microbial communities in the phyllosphere. For instance, when the fern *Polypodium polypodioides* (L.) Watt is exposed to rainfall after a period of desiccation, the complex phyllosphere community undergoes changes in overall structure and activity (Jackson *et al.*, 2006). In *N. nervosa,* we observed that yeast and yeast-like fungal communities tended to differ between PnW and PW fruits (only two species were isolated before the washing treatment and five after it, with only one in common). When FnW and FW fruits are compared, we see that species composition also differs (six species disappeared during the washing process but another six emerged), although communities are similar because the most abundant species are the same for both treatments (*A. pullulans*, *Dothichiza* sp. 1, *Ascomycetous* yeast sp. 1, and *C. heveanensis*) (Tables 1 and 2). One possible explanation for this phenomenon is that species better adapted to high moisture conditions or those that need high water availability emerge (e.g. *R. colostri* and *R. fujisanensis* that were recovered from fresh and preserved fruits only after the washing treatment), while those not tolerant to this condition do not survive (e.g. *L. mutabilis* that was present in both types of fruits before the washing treatment, but not after it). It is also possible that the washing treatment removed some competitive species, so others were able to emerge. These findings suggest that the washing process alter the yeast and yeast-like fungal composition present on the fruits, but not the overall community structure.

These results indicate that customary procedures during *N. nervosa* propagation (such as storage at 4 °C and the washing process for breaking seed dormancy) alter microbial communities present on the fruit, and this information is relevant to domestication programs. If native microbiota present on fresh fruits benefits germination or seedling stand, then fruits should not be preserved at 4 °C for long periods of time.

Adaptations to the phyllosphere environment

Resident phyllosphere microorganisms are presumably endowed with suitable niche-specific traits for survival and growth on their particular surface habitats. Some

of these attributes include high growth rates, the ability to compete for nutrients and to withstand periods of drought, and varying osmotic and temperature conditions. Some of the specific adaptive properties that epiphytic yeasts possess are a capacity for substrate adherence (mycelia, capsules) and pigmentation (carotenes, melanin) (Andrews & Harris, 2000; Lindow & Brandl, 2003; Kowalchuk *et al.*, 2010).

It has been demonstrated in *Candida* spp. that adherence is largely influenced by mycelium development, associated with high attachment capacity for tissue colonization (Trochin *et al.*, 1991). Most of the species described in this study, except for those included in the *Cryptococcus* and *Rhodotorula* genera, are capable of forming conspicuous mycelia, suggesting that this structure is a widespread attachment strategy among the yeast and yeast-like fungi present on *N. nervosa* fruits. Another common trait described for phylloplane yeasts is the production of capsules, mainly in *Cryptococcus* and *Rhodotorula* species, which lack mycelium (Golubev, 1991; Glushakova & Chernov, 2004). Experimental evidence suggests that this structure increases yeast fitness and survival when subject to stress and protects them from drying out during low water activity (Golubev, 1991; Bhadra *et al.*, 2008). Capsules also play an important adhesion role (Deak, 2006) and seem to explain, at least in part, the observed differences between resident and transient microorganisms in the phyllosphere (Fonseca & Inácio, 2006). The species described in this work, then, have various adhesion strategies that allow them to survive in this extreme environment, exposed to rain and wind, and remain attached to the substrate even after the washing treatment.

Most phyllosphere microorganisms are capable of withstanding high UV light levels, mainly because of two mechanisms: pigmentation and DNA repair (Kowalchuk *et al.*, 2010). Yeasts can be damaged by UV wavelengths of sunlight, so pigmented species are abundant on plant surfaces, mostly corresponding to the genera *Rhodothorula* and *Sporobolomyces* (Fonseca & Inácio, 2006). These organisms contain red, orange, and pink carotenoid pigments, which provide indirect protection for the cells by quenching the reactive oxygen species produced by radiation (Young, 1991). In this work, *R. colostri* was the only species belonging to this group. However, a high proportion of species (53%) were found to be darkly pigmented as a consequence of their melanin content (Table 1). Melanins are not only responsible for the dark-green, brown, and black color of the fungi but also for a number of properties helping them to survive under conditions of environmental stress, such as temperature and osmotic extremes, UV radiation, and desiccation (Sterflinger, 2006).

Mycosporines are hydrosoluble molecules with UV absorption at wavelengths mainly around 310 nm (Bandaranayake, 1998). Several yeast species are able to synthesize and accumulate UV-radiation-absorbing mycosporine metabolites, such as the mycosporine–glutaminol–glucoside (MGG). This molecule has been shown to play an important role as a UVB photoprotective metabolite in yeasts by protecting them against direct DNA damage (Moliné *et al.*, 2011). This idea is supported by the fact that most of the species isolated from *N. nervosa* fruits (71%), which in nature are generally exposed to high levels of solar radiation, synthesize mycosporines. Another interesting observation is that mycosporinogenesis seems to be a feature related to certain phylogenetic groups (taxon-specific), as was suggested by Libkind *et al.* (2005). Our findings are in agreement with these authors, because species included in the orders *Dothideales* and *Chaetothyriales* constitutively synthesize mycosporines, while those belonging to the orders *Coniochaetales* and *Sporidiobolales* are not able to do so. The order *Tremellales* is highly heterogeneous and polyphyletic, so it is not surprising that the ability to produce mycosporines varied among the isolated species included in it (Fig. 1).

As the phyllosphere environment is not homogenous, resident microbial populations present a marked variation in exposure to light, wind, rainfall, and airborne inoculum (Deak, 2006; Fonseca & Inácio, 2006). Although many aerial structures of trees are usually within or beneath the tree canopy, most of the yeasts present in *N. nervosa* fruits are darkly pigmented and mycosporine-producing species. Consequently, it would be highly probable that these molecules (carotenes, melanin, and mycosporines) have another biological function in addition to UV protection. *Nothofagus* fruits are usually exposed to prolonged desiccation periods, and it is known that desiccation is an important factor that causes oxidative stress (Alpert, 2006). Carotenes, melanin, and mycosporines are known to have antioxidant properties, so it is possible that these molecules play an antioxidant role in these microorganisms. This hypothesis is supported by the work carried out by Moliné *et al.* (2011), who have recently demonstrated the ability of the mycosporine-derived glucosides to scavenge or quench reactive oxygen species, indicating that in these fungi, mycosporines might play a role in fighting oxidative stress, in addition to UV protection.

All these findings are in agreement with Muñoz (2010), who found dark pigmented yeast and yeast-like fungi in the phylloplane of *Nothofagus pumilio*, most of which were also capable of forming mycelia and producing mycosporines. According to this information, it seems that in spite of having different community composition, the yeast and yeast-like fungi present on different substrates within *Nothofagus* phyllospheres have the same adaptations to this environment.

Potential biotechnological application of epiphytic yeast and yeast-like fungi

A basic understanding of the organisms, relationships, and driving forces that have evolved in the phyllosphere is crucial to the manipulation of plant-associated microbiota for the development of biocontrol methods that can contribute to more effective and less environmentally damaging methods of plant protection (Droby *et al.*, 1993; Berger *et al.*, 1996; Schoeman *et al.*, 1999; Lindow & Brandl, 2003). In addition, microorganisms present in this environment are important sources of diverse compounds with biotechnological applications (Bull *et al.*, 1992), such as xylanases, cellulases, nitrogenases, lipases, amylases, pectinases, esterases, proteases (Middelhoven, 1997; de García *et al.*, 2007; Bhadra *et al.*, 2008), and antifungal compounds, such as pyrrolnitrin. Pyrrolnitrin is produced by different bacteria, including some *Burkholderia cepacia* isolated from apple leaves (Janisiewicz & Roitman, 1988; Janisiewicz & Yourman, 1991). This bacterial metabolite is currently used in some successful fungicides (e.g. fenpiclonil – Nevill *et al.*, 1988 and fludioxonil – Gehmann *et al.*, 1990), against fruit decays produced by different pathogens, such as *Fusarium graminearum* Schwabe, *Botrytis cinerea* Pers., *Rhizoctonia solani* J.G. Kuhn, and *Penicillium expansum* Link.

The biological control of plant diseases has been focused primarily on the use of bacteria or filamentous fungi (Schoeman *et al.*, 1999). The application of yeast and yeast-like fungi for controlling plant diseases seems to be a new trend in this area (El-Sayed & El-Nady, 2008), despite its potential as biocontrol agents was described a long time ago (Janisiewics, 1987). These fungi have been said to provide a natural buffer against infection by several pathogens (Fokkema *et al.*, 1979; Punja & Utkhede, 2003; El-Tarabily, 2004), and some attributes that make them suitable as biocontrol agents are as follows: rapid colonization of surfaces and survival for long periods under varying conditions; the production of extracellular polysaccharides that enhance their chances of survival and restrict both colonization sites and the flow of germination cues to pathogen propagules; the use of available nutrients for rapid proliferation; and minimal reaction to pesticides (Janisiewicz, 1991). These fungi have shown great potential for reducing foliar diseases, especially those caused by mildew fungi (Urquhart & Punja, 1997; El-Mehalawy, 2004), and for effectively inhibiting the development of postharvest pathogens on various fruits (McLaughlin *et al.*, 1992; Droby *et al.*, 1993; Elad *et al.*, 1994; Janisiewicz *et al.*, 1994; Chand-Goyal & Spotts, 1996a, b). Among the yeasts that have been described to have antagonistic effects on plant pathogens are different *Candida*, *Cryptococcus*, *Kloeckera*, *Pichia*, *Rhodotorula*, *Sporobolomyces*, and *Trichosporon* species (McLaughlin *et al.*, 1992; Droby *et al.*, 1993; Chand-Goyal & Spotts, 1996a, b; Buck, 2004; El-Mehalawy, 2004; Medina *et al.*, 2009; Pusey *et al.*, 2009; Janisiewicz *et al.*, 2010). Moreover, commercial yeast-based formulations have been developed (see Reglinski *et al.*, 2011), and some are available commercially, such as Bionext (*Candida oleophila* Montrocher – Bionext, Belgium and Leasaffe International, France), Shemer (*Metschnikowia fructicola* Kurtzman & Droby – AgroGreen Israel) and Candifrut (*Candida sake* (Saito & M. Ota) Uden & H.R. Buckley ex S.A. Mey. & Ahearn – IRTA, Spain). Special attention has to be paid to *A. pullulans*. This is a ubiquitous yeast-like fungus present on plant surfaces and several other substrates worldwide, and it has been shown to be antagonistic to different plant pathogens (Schena *et al.*, 2002; Janisiewicz *et al.*, 2010; Reglinski *et al.*, 2011). This fungus has been used for the formulation of ecologically harmless products to control postharvest diseases, such as Boniprotect and Blossom-protect (Bio-protect, Germany).

Greenhouse or nursery conditions (temperature, light, and fertilizer regimes) are optimized for maximal plant growth, but they are also favorable for diseases. Pathogens enter this system via air, irrigation water, insects, contaminated shoes, tools, or equipment. Many pathogens are also introduced on seeds. Disinfested soil or soilless substrates (peat or rockwool) lack the microbial diversity responsible for the natural buffering against pathogens, so soilborne pathogens such as *Pythium* and *Rhizoctonia* can grow and seriously affect seeds and seedlings (Paulitz & Bélanger, 2001). The success of biocontrol depends on how the microbial searching and screening process is carried out. For example, finding microorganisms to protect postharvest fruit is likely to require screening for microorganisms that colonize the surface of the fruit, because a biocontrol agent must occupy an ecological niche similar to that of the plant pathogen (Janisiewics, 1987; Paulitz & Bélanger, 2001; Janisiewicz & Korsten, 2002; Fravel, 2005). In the same way, the screening of yeast and yeast-like fungi present on *N. nervosa* fruits to be used in nurseries for seedling propagation would be a good strategy for finding microorganisms with an antagonistic effect against different phytopathogens, including those causing damping-off (Mao *et al.*, 1997; El-Mehalawy *et al.*, 2007; Elwakil *et al.*, 2009). Some of the yeast and yeast-like fungi isolated in this work belong to genera (*Cryptococcus*, *Rhodotorula*, *Trichosporon*) or even species (*A. pullulans*, *R. colostri*) that have been successfully used as biocontrol or growth promoting agents. Thus, future studies will focus on determining the effect of these microorganisms on the control of plant pathogens and on improving germination and seedling stand of *N. nervosa* in domestication programs.

Conclusion

This study describes the occurrence of yeasts and yeast-like fungi present on the indehiscent dry fruits of an ecologically and economically important forestry species. It contributes to the description of microbial populations present in *N. nervosa* phyllosphere as well as to the general knowledge of fungal biodiversity in Patagonia.

The species described in this work have adaptation strategies that allow them to survive in extreme environments where they are exposed to rain, wind, and high levels of UV radiation. Most of them are capable of forming mycelia, which allow them to remain attached to the substrate even after the washing treatment. In addition, the majority of the species analyzed are pigmented and can produce mycosporines, which is not surprising because a high proportion of melanin and carotene-producing microorganisms are associated with environmentally stressed areas, such as hot and cold deserts, alpine regions, and the upper biosphere (Sterflinger, 2006).

This investigation has revealed that both preservation and washing processes alter microbial communities on *N. nervosa* fruits. The former considerably reduces the number of yeast and yeast-like fungi, while the washing process tends to change species composition. The valuable information obtained through this study constitutes the first step toward exploring native yeast and yeast-like fungi for improving seed germination and seedling stand in *N. nervosa* domestication programs. More research is needed, however, to determine the effects of this type of practice on seed germination, plant improvement, and biocontrol strategy development.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Accession numbers, isolation substrates and type of fungi corresponding to the nucleotide sequences obtained in this work (HQ629551–HQ629619) and to the reference strains. Sequences used for constructing the trees presented in Figure 1 are indicated in bold.

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