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ARTICLE

Ecology of cultivable yeasts in pristine forests in northern Patagonia (Argentina) influenced by different environmental factors

María Cecilia Mestre, Sonia Fontenla, and Carlos A. Rosa

Abstract: Environmental factors influencing the occurrence and community structure of soil yeasts in forests are not well studied. There are few studies dedicated to Southern Hemisphere soil yeasts populations and even fewer focused on temperate forests influenced by volcanic activity. The present work aimed to study the ecology of soil yeast communities from pristine forests influenced by different environmental factors (precipitation, physicochemical properties of soil, tree species, soil region, and season). The survey was performed in 4 northern Patagonian forests: 2 dominated by *Nothofagus pumilio* and 2 by *Nothofagus antarctica*. Yeast communities were described with ecological indices and species accumulation curves, and their association with environmental characteristics was assessed using multivariate analysis. Each forest site showed a particular arrangement of species as a result of environmental characteristics, such as dominant plant species, nutrient availability, and climatic characteristics. *Cryptococcus podzolicus* was most frequently isolated in nutrient-rich soils, *Trichosporon porosum* dominated cold mountain forests with low nutrient and water availability in soil, and capsulated yeasts such as *Cryptococcus phenolicus* dominated forest sites with low precipitation. The present work suggests that environmental factors affecting yeast communities may not be the current soil characteristics but the result of complex interactions of factors including natural disturbances like volcanic activity.

Key words: soil ecology, yeast diversity, community yeast structure, Patagonian forests soil.

Résumé : Les facteurs environnementaux qui ont une incidence que la prévalence et la structure communautaire des levures tericoles sylvestres sont mal étudiées. On trouve peu d'études consacrées aux populations tericoles de l'hémisphère sud, et rares sont celles s'attardant à l'influence de l'activité volcanique sur les forêts tempérées. Le présent ouvrage s'est voué à étudier l'écologie des communautés de levures tericoles de forêts intactes subissant l'influence de divers facteurs environnementaux (précipitations, caractéristiques physico-chimiques du sol, espèces arboricoles, régions de sols et saisons). L'étude a été réalisée dans quatre forêts de la Patagonie du Nord, à savoir deux dominées par *Nothofagus pumilio* et deux par *Nothofagus antarctica*. Les communautés de levures ont été décrites au moyen d'indices écologiques et de courbes d'accumulation des espèces, et l'on a évalué leur association aux caractéristiques environnementales à l'aide d'une analyse multivariée. Chaque site forestier présentait un agencement particulier d'espèces en raison des caractéristiques environnementales telles que les espèces végétales dominantes, la disponibilité des nutriments et les caractéristiques climatiques. *Cryptococcus podzolicus* fut isolé le souvent dans les sols riches en nutriments; *Trichosporon porosum* a dominé les forêts montagneuses froides aux sols pauvres en nutriments et en eau; les levures capsulées comme *Cryptococcus phenolicus* ont dominé les sites forestiers recevant peu de précipitations. Le présent ouvrage tend à démontrer que les facteurs environnementaux affectant les communautés de levures ne se résumeraient pas aux caractéristiques actuelles du sol, mais tiendraient d'interactions complexes de facteurs comprenant entre autres les perturbations naturelles telles que l'activité volcanique. [Traduit par la Rédaction]

Mots-clés : écologie des sols, diversité des levures, structure communautaire des levures, sol des forêts patagoniennes.

Introduction

Yeast distribution and abundance in soils has been described as heterogeneous (Botha 2011). Variation in yeast distribution in soils has been attributed to environmental features, such as precipitation (Vishniac 2006), soil texture (di Menna 1965; Vreulink et al. 2007), management regimes (Yurkov et al. 2012), and seasonal fluctuations (Slaviková and Vadkertiová 2000, 2003). These environmental conditions modify and modulate plant growth patterns and influence yeast populations (di Menna 1965). Soil biota is essential for ecosystem function (Kardol and Wardle 2010), but the relevance of yeasts to soil function is not yet fully understood. It is known, however, that yeasts influence soil aggregation, contribute to nutrient cycles, and interact with vegetation (El-Tarabily 2004; Cloete et al. 2009; Botha 2011; Mestre et al. 2011).

Northern Patagonian forests are characterized by low anthropogenic impact and minimal atmospheric pollution (Satti et al. 2007); most of these forests grow on soils with a low degree of development (Satti et al. 2007), derived from recent volcanic activity in the area. The dominant tree species in Andean Patagonian forests belong to the genus *Nothofagus* (Nothofagaceae), which comprises approximately 35 tree species that inhabit New Zealand, Australia, Tasmania, and cold-temperate woods in South America (Manos 1997). *Nothofagus* forests account for 4% of Argentinean native forest (Gonzalez et al. 2006) and large parts of this

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forest are included in protected areas (Laclau 1997). *Nothofagus pumilio* and *Nothofagus antarctica* are 2 native deciduous species that are widely distributed within natural Andean Patagonian forestlands. *Nothofagus pumilio* is restricted to high mountains and low temperature environments, such as the timberline. This species produces high quality timber, which is generally harvest in naturally occurring forests. Timber exploitation of *N. antarctica* is difficult owing to its morphologic variation (from erect to bush-like individuals) and the harsh environmental conditions where it grows (Donoso Zegers 2006). Forests from both *Nothofagus* tree species are under pressure because of cattle grazing and wood collection by local people.

In a previous survey performed on *N. pumilio* forest soil in northern Patagonia, the authors found that different soil regions (bulk soil (BS), rhizosphere (R), and ectomycorrhizosphere) had a distinct yeast assemblage. The participation of yeast in the carbon (C) cycle, related to hemicellulose degradation, and in the nitrogen (N) and phosphorus (P) cycles was also suggested (Mestre et al. 2011). Considering these previous findings, the aim of the present survey was to study the ecology of soil yeast communities from pristine forests influenced by different environmental factors in an area affected by volcanic activity.

Materials and methods

Study sites

Nothofagus pumilio is the main species in the high altitude treeline forests of the Andes from 35°35′S to 55°31′S (Souza et al. 2000), and its appearance varies from tall trees (about 30 m) to a bush-like form at its highest limit (1000–2000 m above sea level) of distribution in Argentina. In the northwestern Argentinean region of the Andes, *N. pumilio* develops at low temperatures (mean annual temperature 10 °C, with the absolute minimum reaching –29 °C), where precipitation is mainly in the form of snow in winter, with a rainy season from April (autumn) to September (late winter) and a dry season in summer, and the annual rainfall ranging from 800 to 3000 mm (Souza et al. 2000; Gonzalez et al. 2006). *Nothofagus antarctica* occupies a wider geographical range than *N. pumilio*, including high mountain forests (below *N. pumilio*) and extending into the steppe. This species is adapted to extreme environmental conditions, which vary greatly throughout its wide distribution, enduring temperatures of –20 °C (high mountain environment) and low water availability (near the steppe). The present yeast survey was performed on *N. pumilio* and *N. antarctica* forests within the Nahuel Huapi National Park, near San Carlos de Bariloche city (Río Negro, Argentina), at 4 pristine forest sites selected for their differences in precipitation values (mean annual temperature was similar in all forest sites).

The Challhuaco site, located at the base of Cerro Challhuaco, was selected as a low precipitation forest (1500 mm·year^{-1}). This site presents an *N. pumilio* forest with tall trees and a highly developed understory that includes various herbaceous plants.

The Puyehue site, located near the Chilean border in the Puyehue region, was selected as a high precipitation forest (3000 mm·year^{-1}). This is a high mountain *N. pumilio* forest, close to *Nothofagus dombeyi* forests, with a poorly developed understory.

The Ñirihuau site, located in the Ñirihuau river valley, was selected as a low precipitation forest (900 mm·year^{-1}). At this site, the *N. antarctica* trees have a bush-like appearance and represent an interphase region between forest (principally *Austrocedrus chilensis* and conifers) and steppe.

The Tronador site at the base of Mount Tronador was selected as a high precipitation forest (1750 mm·year^{-1}). This is a mountain forest, where *N. antarctica* adult trees are surrounded by *N. dombeyi* and other native tree species, distant from the steppe.

Sampling

At each site, 5 adult trees were randomly selected at a distance of at least 15 m from each other. Three soil cores (5 cm in diameter by 30 cm in depth) from each tree were aseptically collected at a 60 cm distance from the trunk. The litter layer had previously been removed. The soil cores, considered as subsamples for each tree, were individually transported to the laboratory in plastic bags and maintained at 4 °C until processed within 72 h. Sampling was performed in spring (October–December) and autumn (April–June).

Soil characteristics

Soil physicochemical characteristics of the 4 forest sites were determined as follows. Gravimetric water content for each tree was determined as mass difference before and after drying at 90 °C, in spring and autumn samples. A composite soil sample of each site and season was air-dried at room temperature for conservation and later determinations. The following determinations were done from spring samples: soil texture was determined by the Bouyoucos method (Lopez Ritas and Lopez Melida 1990), soil pH was determined in a soil–water (1:5) suspension, organic C was determined by Walkley–Black digestion, total N content was determined by the Kjeldahl method, and ammonium (NH_4^+) and nitrates (NO_3^-) were determined by the distillation method (Keeney and Bremner 1966). Results were expressed as milligram per kilogram of soil dry mass. In addition, the percentage of total N was also calculated from these data. P was extracted with 0.5 mol·L^{-1} $NaHCO_3$ using the molybdate ascorbic acid method (Kuos 1996) in both spring and autumn samples.

Yeast isolation and population count

In each season, the 3 soil subsamples from each tree were pooled together aseptically in the laboratory. Each composite sample was sieved using a 2 mm mesh to separate soil from the roots. Soil without roots was considered the BS. Root fragments, with strongly adhered soil, were then shaken gently to recover the rhizospheric soil (R). The BS and R, termed "soil regions" throughout this work, were suspended at 1:25 (*m*/*v*) in 0.9% NaCl solution (40 g of soil at natural humidity per litre) and shaken at 250 r·min^{-1} for 30 min; 100 μL of the suspension was distributed on the surface of solid MYP medium (0.7% (*m*/*v*) malt extract, 0.05% (*m*/*v*) yeast extract, 0.25% (*m*/*v*) peptone, 1.5% (*m*/*v*) agar) supplemented with Rose Bengal (25 μg·mL^{-1}) and chloramphenicol (200 μg·mL^{-1}). Each sample was plated in triplicate. Incubation of all plates was performed at 20 °C, and colonies were counted after 72 h of incubation, to avoid the interference of mould growth in the yeast count procedure (Mestre et al. 2009). Yeast counts were expressed as the mean colony-forming units per gram of dry soil region (CFU·g^{-1}) per site. After 120 h of incubation, all yeast colonies grown were differentiated into macromorphological types (color, aspect, margin) using a dissection microscope. Representative colonies of each morphotype (up to a maximum of 3) from each count plate were selected and purified. Pure cultures were cryo-preserved (–80 °C) using MYP liquid medium with 12% glycerol.

Statistical analysis

Kruskal–Wallis nonparametric analysis was performed to identify differences in yeast count values between *Nothofagus* tree species, considered to be statistically significant at the level $p < 0.05$.

Yeast count data from the 4 sites were analyzed together with a 3-way ANOVA considering 3 factors: (*i*) site — with 4 modalities: Challhuaco, Puyehue, Ñirihuau, and Tronador; (*ii*) soil region — with 2 modalities: bulk soil and rhizosphere; and (*iii*) season — with 2 modalities: spring and autumn. Yeast count values as CFU per gram were $\log_{10}$ transformed to fit the assumption for the statistical analysis. Statistical analysis was performed with software Statistica 7. Effects were considered to be statistically signif-

icant at the level $p < 0.05$. Tukey's post-hoc test was used to form homogenous groups.

Yeast identification

Yeast cultures were identified using a polyphasic approach that combines morphological, physiological, and molecular features. Pure yeast cultures were grouped according to macro- and micromorphology and characterized at the physiological level according to the keys and descriptions in literature (Kurtzman et al. 2011).

DNA extraction was performed as follows. One loopful of a 72 h pure culture was suspended in 100 μL of lysis buffer (50 mmol·L^{-1} Tris, 1 mol·L^{-1} NaCl, 0.1 mmol·L^{-1} EDTA, 0.5% SDS), incubated for 30 min at −80 °C, and then placed in a 99 °C bath for 15 min. Samples were then centrifuged for 15 min at 21 400*g*, and the supernatant containing genomic DNA was kept. DNA precipitation was performed twice with 100 μL of ethanol and subsequent centrifugation at 21 400*g* for 15 min. Finally, the DNA was resuspended in 100 μL of TE buffer (0 mmol·L^{-1} Tris, 1 mmol·L^{-1} EDTA).

PCR fingerprinting for each group was analyzed with a mini- and microsatellite primed PCR technique using primers $(GTG)_5$ and (or) M13 as described by Libkind et al. (2004). Fragment amplification and size were conferred by 1.5% agarose gel electrophoresis and stained with GelRed (5 μg·mL^{-1}). At least 2 representative isolates from each group were identified using rDNA sequencing.

For DNA sequence analysis, the D1–D2 domain at the 5′ end of large subunit (LSU) rRNA gene was amplified using primers NL-1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL-4 (5′-GGTCCGTGTTTCAAGACGG-3′). Amplification was performed for 35 PCR cycles with denaturation at 94 °C for 15 s, annealing at 54 °C for 1 min, and extension at 72 °C for 20 s. Fragment amplification and size were conferred by 1.5% agarose gel electrophoresis and stained with GelRed (5 μg·mL^{-1}). Sequencing of amplicons was carried out by MACROGEN Inc. using primer NL-1. Comparisons with sequences from GenBank database (http://www.ncbi.nlm.nih.gov/) were carried out using BLASTn search algorithm. Sequence alignment and neighbor-joining tree construction (based on K2P distance method and 1000 bootstrap iterations) was performed with Mega version 4 (Tamura et al. 2007). D1–D2 domain sequences were submitted to the GenBank database.

Community analysis

Species diversity for each community was described using the Shannon–Weaver (*H*) index, and species composition similarity between sites was estimated using the Jaccard classical index (*J*).

Nonparametric classical Chao 2 index and species accumulation curves (S_{est}) were used to estimate species richness. All ecological indices were calculated with EstimateS software package (version 8.2; http://viceroy.eeb.uconn.edu/EstimateS/EstimateSPages/EstSUsersGuide/EstimateSUsersGuide.htm). Species accumulation curves were calculated with the same software using 50 randomizations, sampling without replacement, and default settings for upper incidence limit for infrequent species (Colwell 2006). Observed richness values (S_{obs}) were fitted using Origin 6.1 software with a first order exponential decay model, $y = y_0 + A \times e^{\left(-\frac{x}{t_1}\right)}$, where y_0 is the asymptote, A is the amplitude, x is the number of samples, and t_1 is the decay constant. The curve asymptote corresponds to the estimated richness (S_{est}).

Multivariate analysis

To study the association between environmental characteristics and yeast community attributes at each site, we performed a normed principal component analysis using SPAD version 5.5 software package. This analysis included the soil chemical data from spring samples of each site (NO_3^-, NH_4^+, N, C, C/N, P content, gravimetric humidity, pH, % silt contents); and yeast count (CFU·g^{-1}), Shannon's diversity index (*H*), and the estimated richness (S_{est}), which were obtained using the data from spring samples.

Results

Yeast colonies were observed in every sample, and nonsignificant differences (Kruskal–Wallis; $p = 0.799$) were found between tree species.

Figure 1 shows the number of yeast CFU per gram of soil per site in each season and soil region sampled. The mean yeast count number per site varied from 7.33×10^3 CFU·(g soil)$^{-1}$ in Challhuaco to intermediate values in Ñirihuau (3.99×10^3 CFU·(g soil)$^{-1}$) to the lowest values in Tronador and Puyehue (3.16×10^3 and 3.10×10^3 CFU·(g soil)$^{-1}$, respectively) (Fig. 1*a*). The values for Challhuaco and Puyehue (both are *N. pumilio* forests) present higher numbers of yeasts in spring samples while in Ñirihuau (*N. antarctica* forests) values seemed to be higher in autumn (Fig. 1*b*). Rhizospheric soil samples from Challhuaco, Puyehue, and Tronador tended to have higher numbers of yeast than the BS region (Fig. 1*c*), whereas in Ñirihuau an opposite tendency was observed. The statistical analysis, based on $\log_{10}$ transformed data, showed significant differences between sites (ANOVA, $F_{[3]} = 7.19$; $p = 0.0003$), with Challhuaco forest presenting a higher yeast count than Puyehue and Tronador (Fig. 1*a*). Significant interacting effects were also observed between site and season (ANOVA, $F_{[3]} = 3.89$; $p = 0.013$), with the highest abundance of yeasts in Challhuaco spring samples (Fig. 1*b*), and between site and soil region (ANOVA, $F_{[3]} = 2.89$; $p = 0.043$), with a higher abundance of yeast in Challhuaco rhizospheric region than Puyehue and Tronador BS region (Fig. 1*c*).

Yeast inventory

A total of 123 isolates were obtained in the present work, representing 28 yeast species (Table 1). The phylogenetic placement of model strains from the species recovered in the present work is shown in Figs. 2 and 3. *Cryptococcus* sp. 1, represented by 4 isolates, was related to *Cryptococcus aerius* CBS 155^T (GenBank sequence AF075486) and had 16–18 nt differences in the D1–D2 region. *Cryptococcus* sp. 2, represented by 1 isolate, was related to *Cryptococcus saitoi* CBS 1975^T (GenBank sequence AF181540) and had 29 nt differences in the D1–D2 region. The ascomycetous species *Wickerhamomyces* sp., represented by 4 isolates, was related to *Candida quercuum* NRRL Y-12942 (GenBank sequence EF550292), from the *Wickerhamomyces* clade, and had more than 30 nt differences in the D1–D2 region. Further studies need to be performed to confirm the identity of 4 other species: *Trichosporon* sp. (10 nt difference with *Trichosporon middelhovenii* JCM 12592^T), *Rhodothorula* sp. (8 nt difference with *Rhodothorula fujisanensis* CBS 4551^T), *Ambrosiozyma* sp. (6 nt differences with *Ambrosiozyma angophorae* NRRL Y-7118^T), and *Cryptococcus* cf. *aerius* (4 nt differences with *Cryptococcus aerius* CBS 155^T).

A high number of basidiomycetous species were observed (20, including 2 possible new species), which correspond to 76% of isolates, with the order Filobasidiales represented by the largest number of species (10 species), followed by Trichosporonales and Cystofilobasidiales (3 species each). All ascomycetous species (8, including possible new *Wickerhamomyces* species) belong to the order Saccharomycetales.

Trichosporon porosum was the most frequently recovered species, representing 26% of the isolates, while the other species each represented less than 10% of the total isolates. Five species showed a wide distribution, as they were recovered in 3 of the 4 sites sampled: *Trichosporon porosum*, *Candida sake*, *Holtermanniella wattica*, *Saccharomyces eubayanus*, and *Cryptococcus* sp. 1, which are found in forests of both *Nothofagus* species. Eleven species were represented by only 1 isolate, all of which were isolated from spring samples except *Rhodotorula* sp. Six species were recovered solely from autumn samples, and 13 species were recovered solely from spring samples. Nine species were found during both seasons, including 4 of the most widely distributed species, plus *Cryptococcus podzolicus*,

Fig. 1. Yeast count per site in each sampled soil region and season. Whiskers correspond to standard error, and middle points to mean. Letters indicate different homogenous groups calculated by Tukey's post-hoc test after $\log_{10}$ transformation of yeast count value data. A, autumn; S, spring; BS, bulk soil; R, rhizosphere.

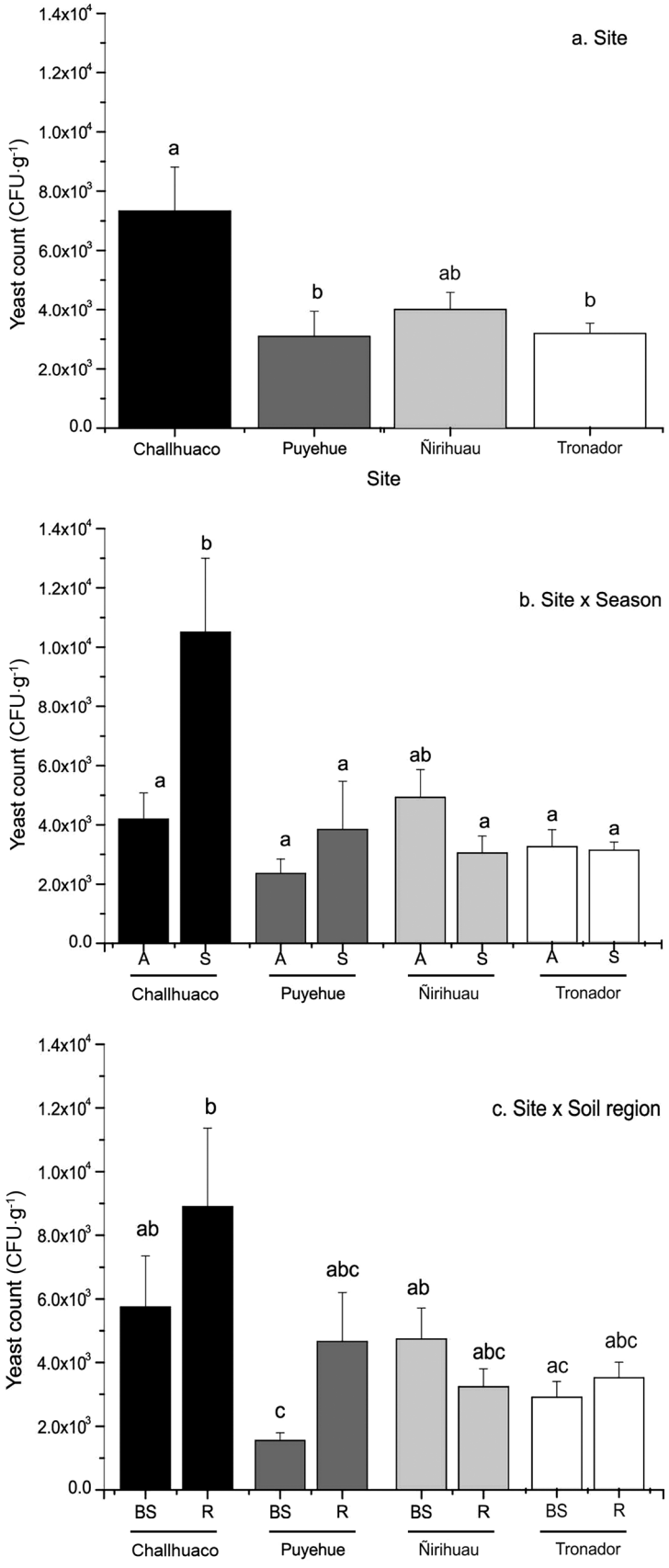


Table 1. Inventory and abundance of yeast taxa isolated at 4 *Nothofagus* forest sites, from bulk soil (BS) and rhizosphere (R), during 2 sampling seasons: autumn (A) and spring (S).

		Nothofagus pumilio				*Nothofagus antarctica*			
		Challhuaco		Puyehue		Ñirihuau		Tronador	
Yeast taxa	Total no. of isolates[a]	BS	R	BS	R	BS	R	BS	R
Basidiomycota									
Filobasidiales									
Cryptococcus bhutanensis	2 (1.62)						2, S–A		
Cryptococcus filicatus	1 (0.81)							1, S	
Cryptococcus gastricus	1 (0.81)						1, S		
Cryptococcus phenolicus	10 (8.13)					5, S	5, S		
Cryptococcus saitoi/friedmannii	4 (3.25)	1, A	2, A				1, S		
Cryptococcus terreus	1 (0.81)					1, S			
Cryptococcus terricola	1 (0.81)		1, S						
Cryptococcus sp. 1	4 (3.25)	1, S				1, S	1, S	1, S	
Cryptococcus sp. 2	1 (0.81)						1, S		
Cryptococcus cf. *aerius*	2 (1.62)					1, S	1, S		
Holtermanniales									
Holtermanniella wattica	9 (7.32)		2, S–A			2, S	1, S	3, S–A	1, A
Tremelalles									
Cryptococcus podzolicus	9 (7.32)	2, S	5, S–A	2, S					
Microbotryomycetes									
Rhodotorula sp.	1 (0.81)		1, A						
Trichosporonales									
Trichosporon sp.	1 (0.81)								1, S
Trichosporon moniliiforme	1 (0.81)							1, S	
Trichosporon porosum	32 (26.02)	1, S		2, S	5, S			12, S–A	12, S–A
Cystofilobasidiales									
Cystofilobasidium infirmominiatum	1 (0.81)	1, S							
Cystofilobasidium capitatum	5 (4.07)								5, A
Guehomyces pullulans	4 (3.25)		2, S			1, A	1, A		
Sporidiobolales									
Rhodotorula colostri	3 (2.43)					2, A		1, A	
Ascomycota									
Saccharomycetales									
Ambrosiozyma sp.	1 (0.81)								1, S
Candida maritima	2 (1.62)	2, S–A							
Candida railenensis	2 (1.62)					2, A			
Candida saitoana	1 (0.81)							1, S	
Candida sake	9 (7.32)				1, A		1, A	5, S–A	2, S–A
Lachancea nothofagi	5 (4.07)	3, A	1, A		1, A				
Saccharomyces eubayanus	6 (4.88)			1, S	2, S		1, A		2, S–A
Wickerhamomyces sp.	4 (3.25)	2, A	1, A						1, A
Total	**123**	**13**	**15**	**5**	**9**	**15**	**17**	**25**	**25**

[a]Occurrence frequency percentages are shown in parentheses.

Cryptococcus saitoi/friedmannii, *Guehomyces pullulans*, *Cryptococcus bhutanensis*, and *Candida maritima*. Three red-pigmented species were recovered: *Cystofilobasidium infirmominiatum*, *Cystofilobasidium capitatum*, and *Rhodotorula colostri*. Most of the species isolated exclusively in one soil region (8 in each soil region) were represented by a single isolate, while the other 12 species were found in both soil regions.

Community analysis

Challhuaco and Ñirihuau forests showed similar species diversity values (2.24 and 2.32, respectively), Tronador had intermediate values (1.85), and Puyehue was the least diverse community (1.46). The highest species similarity index was observed for Tronador and Ñirihuau communities (J = 0.23), both *N. antarctica* forests, and for Challhuaco and Puyehue communities (J = 0.21), both *N. pumilio* forests. Ñirihuau (900 mm·year^{-1}) and Puyehue (3000 mm·year^{-1}) were the least similar communities (J = 0.12). These forest sites are at opposite extremes of the selected precipitation gradient. In the 2 *N. pumilio* forests, the number of species observed was different: 12 species in Challhuaco and 5 in Puyehue, while the number of species in the *N. antarctica* forests was similar: 14 in Ñirihuau and 13 in Tronador (Table 2). Richness estimation for every site is higher than the number of species observed (S_{obs}), with the exception of Puyehue forest where richness estimator values and S_{obs} were similar. Chao 2 richness estimator ranged from 5 in Puyehue to 29 in Tronador (Table 2), while estimated richness from rarefaction curves (S_{est}) ranged from 5.14 in Puyehue to 21.10 in Ñirihuau (Fig. 4). The S_{est} values for Tronador and Challhuaco were similar: 18.87 and 18.71, respectively, and richness estimator Chao 2 was higher than S_{est} in both forest soils (Table 2).

The R soil region presented higher diversity values than the BS region in Puyehue, Ñirihuau, and Tronador, while in Challhuaco the R community was less diverse than the BS community (Table 2). S_{obs} is similar in BS and R communities, with a higher number in the R community from Ñirihuau (8 in BS vs. 11 in R). Nevertheless, the richness estimation for BS and R communities using Chao 2 estimator differed greatly from S_{obs} (except in Puyehue), and the Chao 2 values for the R community were higher than for the BS community for all 4 forest sites.

Fig. 2. Phylogenetic placement of the soil yeast species from *Nothofagus* forests within Phylum Basidiomycota obtained by neighbor-joining (distance K2P method) of the LSU rRNA gene D1–D2 domains. Names in bold type are strains described in this work. Bar, substitutions accumulated every 100 nt. Bootstrap values higher than 50% are shown (1000 replicates). T, type strain.

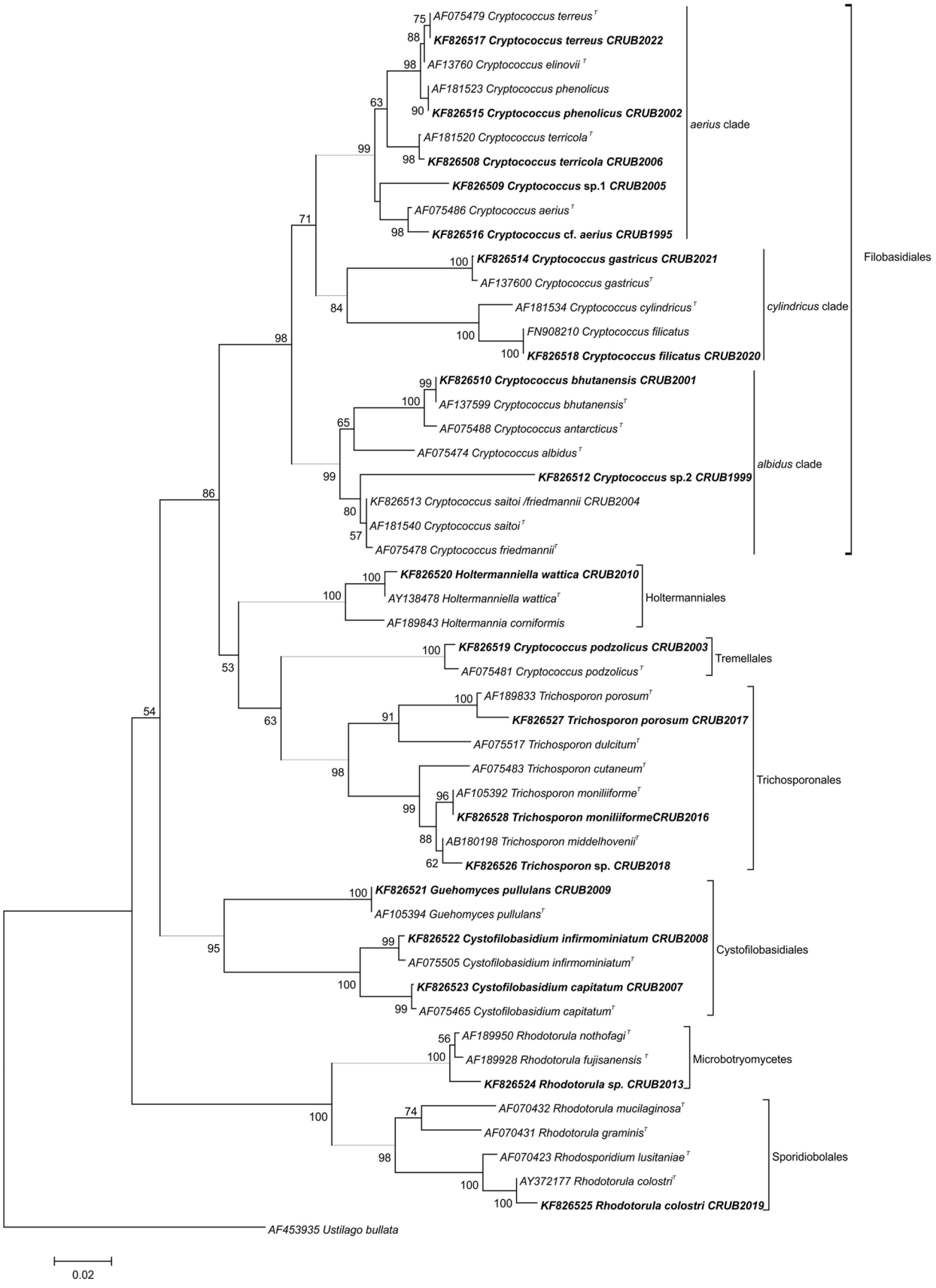
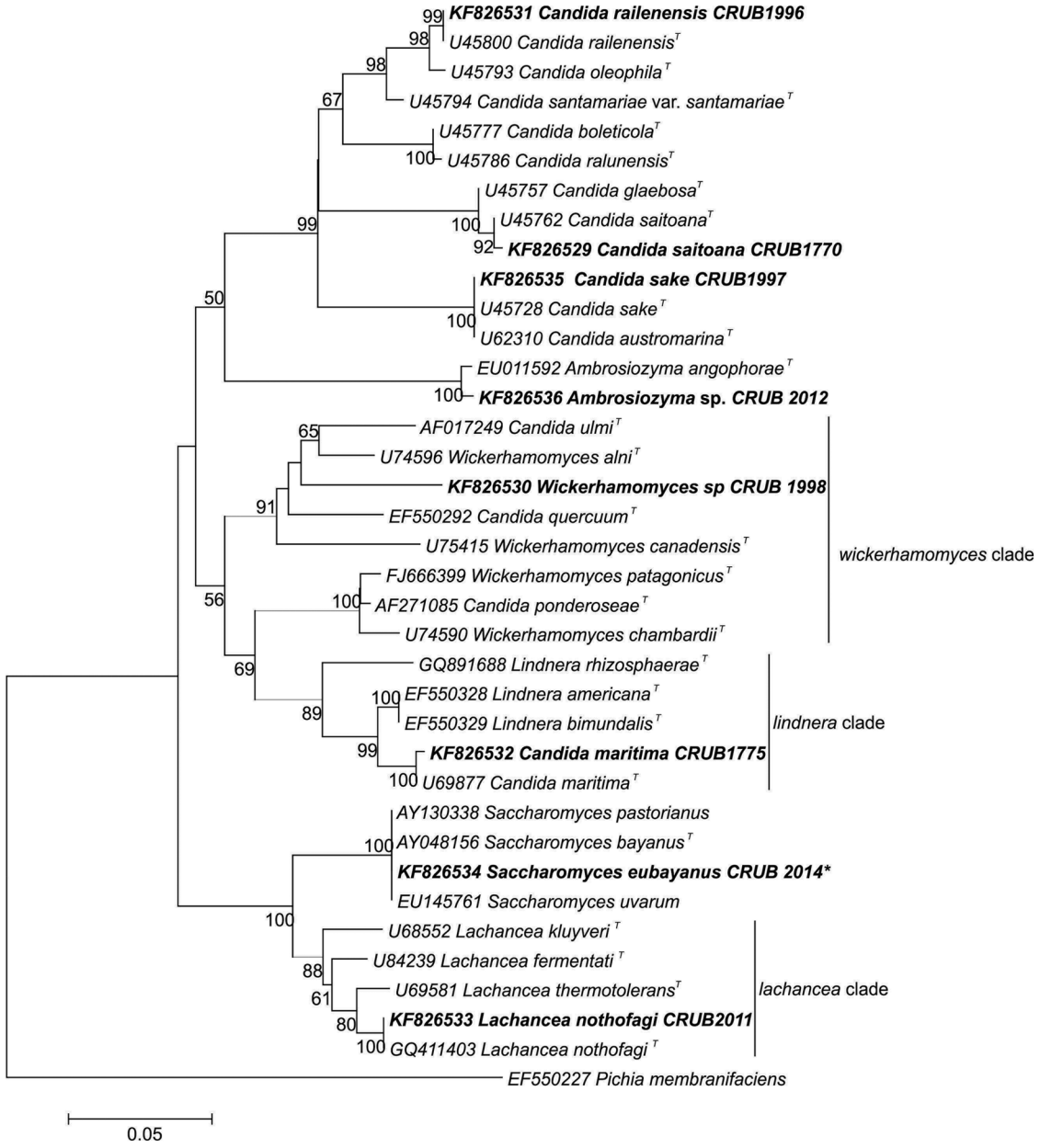


Fig. 3. Phylogenetic placement of the soil yeast species from *Nothofagus* forests within Phylum Ascomycota obtained by neighbor-joining (distance K2P method) of the LSU rRNA gene D1–D2 domains. Names in bold type are strains described in this work. Bar, substitutions accumulated every 100 nt. Bootstrap values higher than 50% are shown (1000 replicates). An asterisk (*) indicates the species was identified based on PCR fingerprinting, as suggested by Libkind et al (2011).



Soil characterization

The soils of Tronador and Challhuaco forest sites was sandy loam while that of Ñirihuau and Puyehue was loamy sand (Table 3). All 4 soils had the same clay content (4%). All forest soils were slightly acidic, but Ñirihuau and Tronador (*N. antarctica* forest sites) had higher pH values (6.27 and 6.22, respectively). The soils from Tronador and Ñirihuau (both *N. antarctica* forests) had similar nutrient contents for NH_4^+, NO_3^-, N, C, and P, which are relatively lower than that of *N. pumilio* forest soils. Challhuaco forest soil had the highest values for NO_3^- (19.34 mg·kg^{-1}), N (0.58%), and C (8%), while Puyehue had the highest values for NH_4^+ (13.54 mg·kg^{-1}). The highest C-to-N ratio was observed in Puyehue (C/N = 23.86). P and NH_4^+ values were up to 6 and 3 times higher, respectively, in Puyehue than in any of the other forest soils. In all forest soils, P content increased in autumn, but levels were much higher at the Puyehue site than at any of the others.

Multivariate approach

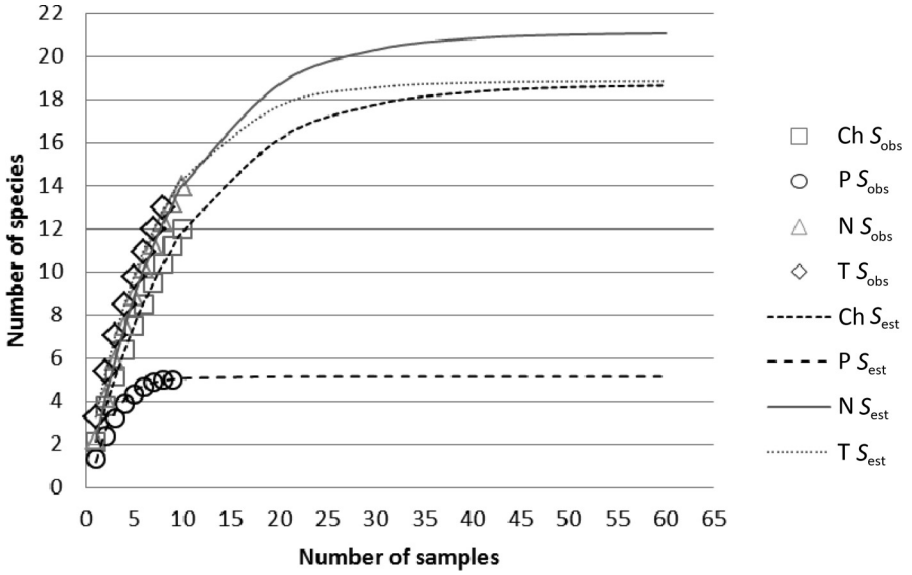
Principal component analysis showed the association of environmental characteristics and yeast community attributes at each site, with axes 1 and 2 explaining 95.88% of the effects of these variables (Fig. 5). The number of yeasts (CFU·g^{-1}) was positively correlated with NO_3^-, N, and C contents. Estimated richness (S_{est}) was negatively correlated with NH_4^+ and P contents. P was also negatively correlated with pH. Puyehue and Challhuaco forest soils (*N. pumilio* forests) were placed at opposite ends of axis 1, while Tronador and Ñirihuau (*N. antarctica* forests) were placed together at the same end of axis 2 (Fig. 5). Challhuaco is associated with higher C, NO_3^-, and N contents and a higher abundance of

Table 2. Species diversity and richness estimators for bulk soil (BS), rhizosphere (R), and site communities (identified as "Total").

Tree species	Forest site	No. of isolates	Diversity (*H*)	Richness		
				S_{obs}	Chao 2	S_{est}
Nothofagus pumilio	Challhuaco					
	Total	28	2.24	12	28.00	18.71
	BS	13	1.99	8	14.25	
	R	15	1.87	8	26.00	
	Puyehue					
	Total	14	1.46	5	5.00	5.14
	BS	5	1.05	3	3.25	
	R	9	1.29	4	4.17	
Nothofagus antarctica	Ñirihuau					
	Total	31	2.32	14	20.40	21.10
	BS	15	1.89	8	26.00	
	R	16	2.18	11	51.50	
	Tronador					
	Total	50	1.85	13	29.00	18.87
	BS	25	1.57	8	8.90	
	R	25	1.63	8	10.67	

Note: *H*, Shannon's diversity index; S_{obs}, number of species recovered; S_{est}, number of species estimated by accumulation curves.

Fig. 4. Soil yeast species accumulation curves for 4 *Nothofagus* forest soils. Observed data (S_{obs}) were fitted with an exponential first order decay model (S_{est}). Ch, Challhuaco (R^2 = 0.999); P, Puyehue (R^2 = 0.979); N, Ñirihuau (R^2 = 1.000); T, Tronador (R^2 = 0.999). S_{obs}, number of species recovered; S_{est}, number of species estimated by accumulation curves.



yeast (CFU·g^{-1}). Puyehue is associated with higher P and NH_4^+ contents. Tronador and Ñirihuau are associated with higher values for diversity (*H*), S_{est}, and pH.

Discussion

The mean yeast count recorded in the present study was 10^3 CFU·g^{-1}, a value that is similar to those found in a previous study on *N. pumilio* forest soils in the region (Mestre et al. 2011) and within the range previously reported for other forest soils worldwide (di Menna 1965; Slaviková and Vadkertiová 2000; Maksimova and Chernov 2004; Botha 2011; Yurkov et al. 2011).

Net primary production in temperate forest ecosystems is considered to be strongly dependent on internal nutrient cycling, with plant litter fall being the main pathway for nutrient return to the soil (Schlesinger 1991); nutrient availability (quality and quantity) shapes the microorganism populations (Buée et al. 2009; Birkhofer et al. 2012). Despite the influence of dominant tree species on nutrient availability, the present work did not find significant differences in soil yeast numbers when comparing the forests of the 2 *Nothofagus* species.

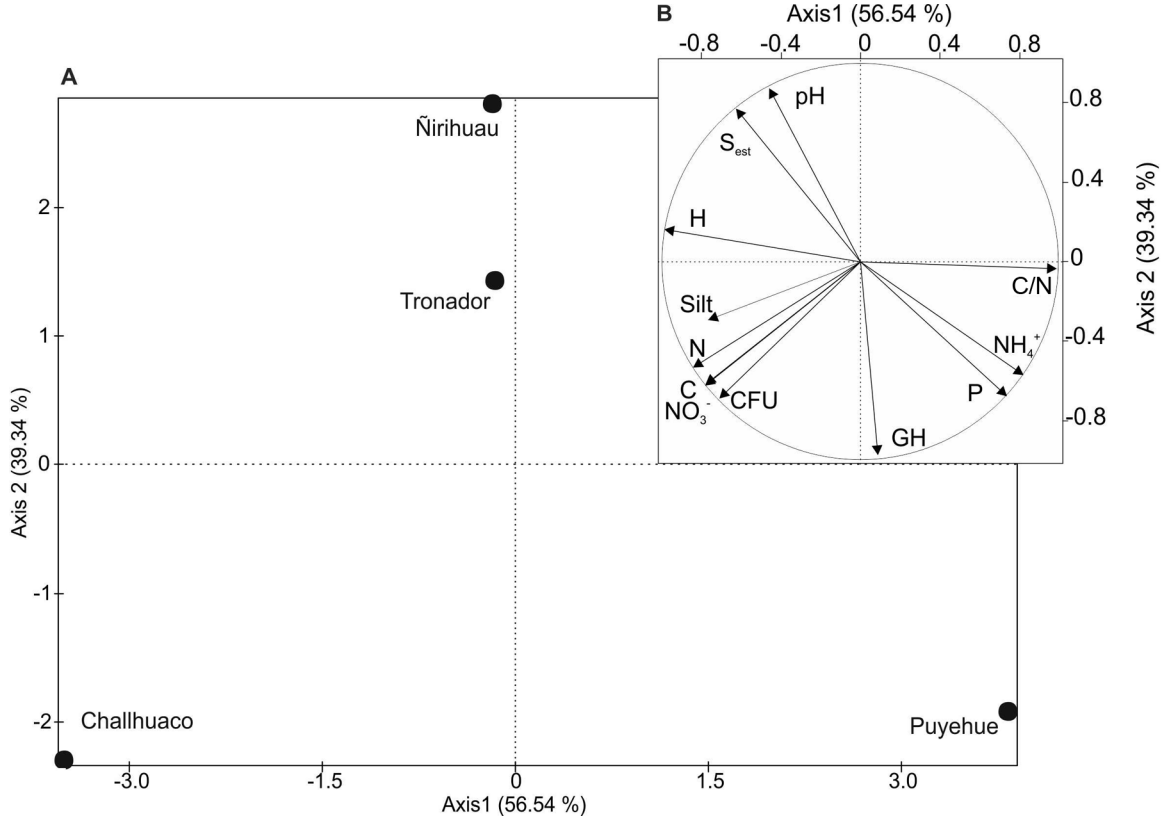
Raw data tendencies and statistical analyses showed differences in yeast count when comparing different forest sites, seasons, and soil regions. Nutrient availability in soils might change with the seasonal input of lignocellulosic material introduced into soil by leaf fall (Bertiller et al. 2006; Diehl et al. 2008), and with rhizodeposition (Buée et al. 2009). The quality and quantity of this nutrient input is closely associated with the aboveground plant community (Maksimova and Chernov 2004; Wardle et al. 2006; Diehl et al. 2008). In general, Challhuaco forest site stands out when compared with the other 3 sites, showing the highest yeast values at site, soil region (R region), and seasonal levels (spring). These results showed that the yeast count from a given site during one season (or soil region) does not necessarily reflect a general pattern that can be extrapolated to other sites.

Table 3. Site characteristics determined in this study: altitude, precipitation, and soil physicochemical parameters.

	Nothofagus pumilio		*Nothofagus antarctica*	
	Challhuaco	Puyehue	Ñirihuau	Tronador
Altitude (m above sea level)	1240	1260	990	843
Precipitation (mm·year^{-1})	1500	3000	900	1750
Nitrogen (%)	0.58	0.15	0.19	0.2
NH_4^+ (mg·kg^{-1})	5.38	13.54	4.49	5.36
NO_3^- (mg·kg^{-1})	19.34	3.04	2.94	3.10
Carbon (%)	8	3.5	3.3	3.8
%C/%N	13.72	23.86	17.53	19.16
Phosphorus				
Spring (mg·kg^{-1})	5.90	16.50	2.70	4.10
Autumn (mg·kg^{-1})	8.10	20.30	7.20	8.00
Gravimetric humidity				
% in spring	35.69	36.46	14.1	25.03
% in autumn	34.93	30.54	21.04	28.93
Soil texture	Sandy loam	Loamy sand	Loamy sand	Sandy loam
% Sand	70	80	80	72
% Clay	4	4	4	4
% Silt	26	16	16	24
pH (H_2O)	5.68	5.17	6.27	6.22

Note: NO_3^-, nitrate; NH_4^+, ammonium.

Fig. 5. Principal component analysis using spring data for physicochemical soil characteristics and yeast community attributes from Challhuaco, Puyehue, Tronador, and Ñirihuau forests sites. (A) Relative position of sites along Axes 1 and 2. (B) Variable correlation circle. Soil characteristics: NH_4^+, ammonium; NO_3^-, nitrate; N, nitrogen; C, carbon; P, phosphorus; GH, gravimetric humidity; C/N, carbon to nitrogen ratio; pH. Community attributes: H, yeast diversity; S_{est}, number of species estimated by accumulation curves; CFU, yeast count (CFU·g^{-1}).



Twenty-eight yeast taxa were retrieved in this survey. Five species accounted for 56% of the total isolates (123) while 15 species were represented by 1 or 2 isolates and accounted for 15% of the isolates. This pattern was also observed at individual forest sites. This is in agreement with other authors who found that a few taxa account for most of the species abundance in soils, while the majority of the species are retrieved only rarely (Buée et al. 2009; Yurkov et al. 2012). In all forest sites, the dominance of basidiomycetous yeast species was observed, which has also been reported in different forest soils worldwide (Maksimova and Chernov 2004; Mestre et al. 2011; Yurkov et al. 2012). Most of the basidiomycetous species belonged to the Filobasidiales order, including some known pedobiont species, such as *Cryptococcus aerius*, *Cryptococcus phenolicus*, *Cryptococcus podzolicus*, and *Cryptococcus terreus* (Maksimova

and Chernov 2004; Vishniac 2006). These and other widely distributed species, such as *Trichosporon porosum*, *Holtermanniella wattica*, *Cryptococcus* sp. 1, *Candida sake*, and *Saccharomyces eubayanus*, were also isolated in previous studies performed in Patagonia, including substrates such as *N. pumilio* forest soil (Mestre et al. 2011), water from an acidic river (Russo et al. 2008), and *Nothofagus* bark and a parasitic fungus (Libkind et al. 2011). These species also seem to be frequent inhabitants of forest soil in the Northern Hemisphere (Maksimova and Chernov 2004; Wuczkowski and Prillinger 2004; Yurkov et al. 2012).

Northern Patagonian environments, with their particular ecogeographic characteristics, have a relatively recent history of yeast diversity surveys, and most of these surveys report the isolation of new yeast species. Isolation and identification of new species increases information on yeast diversity and ecology and also provides an opportunity to find new biotechnological resources (de García et al. 2007; Moliné et al. 2009; Libkind et al. 2011). In this work, some new species were recognized. *Cryptococcus* sp. 1 was also isolated in a previous study on *Nothofagus* forest soils (Mestre et al. 2011) and seems to be widespread in forest soils in the northwestern Patagonian region. The closest relative to this species is *Cryptococcus aerius*, which is a cosmopolitan species and has been recovered from white willow, spruce, and birch forests in Europe (Maksimova and Chernov 2004; Wuczkowski and Prillinger 2004) and from *Nothofagus* forest in Patagonia (Mestre et al. 2011). *Cryptococcus* sp. 2 was represented by 1 isolate related to *Cryptococcus saitoi* (also isolated in the present survey). *Cryptococcus saitoi* has also been isolated in the northern Patagonian region from an acidic river (Russo et al. 2008) and high altitude lakes (Libkind et al. 2009). *Wickerhamomyces* sp. strain CRUB 1998 was placed within the *Wickerhamomyces* clade along with a species from the region recently described and identified as *Wickerhamomyces patagonicus* (de García et al. 2010), which is proposed to be closely associated with *N. dombeyi* exudates. The recovery of these yeast species in the forest supports the contention that some of the yeast species found in water bodies in Patagonia may be attributed to surface runoff from surrounding *Nothofagus* forests (Russo et al. 2008; Libkind et al. 2009; de García et al. 2010).

Yeast species occurrence in *Nothofagus* forests seems to follow nutrient and plant seasonal cycles. In deciduous forests, the input of new material occurs as a result of leaf fall, thus increasing the yeast species in the soil; these species were recovered from soil in low numbers and predominantly in autumn. This could be the case for several species in the present survey: *Lachancea nothofagi* is a recently described species isolated from different *Nothofagus*-related substrates, including soil (Mestre et al. 2010); *Candida railenensis* has been recovered from *Quercus robur* fruits, along with *Cystofilobasidium capitatium* (Isaeva et al. 2009); and *Rhodotorula colostri* was isolated from *Nothofagus* dry fruits (Fernández et al. 2012). Three pigmented yeast (reddish-pink) species were recovered in this study: *Cystofilobasidium infirmominiatum* (from spring samples), *Cystofilobasidium capitatium*, and *Rhodotorula colostri* (from autumn samples). The presence of pigment has been associated with epiphytic habitats (Fonseca and Inácio 2006; Fernández et al. 2012), which are exposed to high UV radiation so pigments could act as photoprotective compounds (Libkind et al. 2009; Moliné et al. 2009). *Candida sake* was a frequently isolated species (7.3% of total isolates), predominantly from autumn samples. Isolates of this species recovered from storage apples have been studied as a postharvest biocontrol agent on pome fruits (Viñas et al. 1998). This species could be important in regulating microorganism interaction in soil, and it could also be of value in forest regeneration processes if it acts as a biocontrol agent for microorganisms that could damage seed, preventing it from germinating. These hypotheses should be evaluated further.

Identification of species is a critical point for ecological studies and in particular for index calculation for yeast communities (Yurkov et al. 2012). The polyphasic approach used for species identification in the present survey has been widely used by other authors (Libkind et al. 2004; Wuczkowski and Prillinger 2004; de García et al. 2007; Fernández et al. 2012) and makes the reliable identification of yeast species possible. In previous paragraphs, we mentioned differences in yeast abundance and in the occurrence of species from different sites, soil regions, and seasons. The mathematical description of these differences could also be achieved using ecological indices to describe the community richness and structure. When using Shannon's diversity index, it was observed that the rhizosphere community was more diverse than the BS one, and when using the Chao 2 richness estimator, a higher number of estimated species for the R community was also found. These 2 patterns might be the result of higher nutrient availability derived from rhizodeposition (Buée et al. 2009). The species richness in different soil regions seems to be overestimated by the Chao 2 estimator; this estimator bases calculus on the number of rare species, leading to higher richness estimation in communities with a high number of rare species (Moreno 2001). Richness estimation at each site from rarefaction curves results in a more realistic estimation in comparison with other forest soils in the world, where observed and estimated species richness ranges from 4 to 20 (Maksimova and Chernov 2004; Wuczkowski and Prillinger 2004; Mestre et al. 2011; Yurkov et al. 2012).

The soil from the Challhuaco site had the highest values for total C and N and high levels of NO_3, indicating a fertile soil and, indirectly, a high potential primary productivity. *Cryptococcus podzolicus* represented 25% of isolates from Challhuaco. This species belongs to the "*luteolus* clade", which was previously associated with a high rainfall site and high net primary productivity (Vishniac 2006). *Cryptococcus podzolicus* is a common inhabitant of soils (Maksimova and Chernov 2004; Vishniac 2006), and it was previously found to be the dominant soil yeast species in a *N. pumilio* forest nearby (23% of total isolates, Mestre et al. 2011). Interestingly, this species was isolated only from *N. pumilio* forest soils in the present survey.

The Ñirihuau site lies on the interphase between forest and steppe, with low precipitation and semiarid characteristics, where trees of *N. antarctica* have a bush-like morphology. *Cryptococcus phenolicus* was isolated only from Ñirihuau; it represented 30% of isolates from this site and 8.13% of total isolates (it is the second most frequently isolated species in this survey). At this site, the yeast community seems to be largely dominated by capsulated basidiomycetous species from the orders Filobasidiales (*albidus* and *aerius* clades) and Holtermanniales. Vishniac (2006) associated the presence of these groups with a desert site, owing to xerotolerance conferred by the extracellular capsules. The original description of *Cryptococcus phenolicus* was achieved from a strain isolated from decaying wood in Portugal, and it was named after its ability to assimilate phenol (Fonseca et al. 2000); the ability of this species to assimilate phenol and other aromatic compounds was later demonstrated (Middelhoven et al. 2001). Diehl et al. (2008) showed that *N. antarctica* litter contains a higher quantity of lignin than the litter of *N. pumilio* in northern Patagonia. In an environment with dry conditions and low C availability such as Ñirihuau, the ability to assimilate aromatic compounds could be an advantage. The Tronador site has soil characteristics similar to Ñirihuau; nevertheless, at this site there is a virtual absence of capsulated species from the order Filobasidiales but high abundance of Trichosporonales species, mainly *Trichosporon porosum* (48% of isolates). This was also the most frequently isolated species in Puyehue forest (50% of isolates).

Tronador and Puyehue forests are typical cold mountain forests with frequent snow precipitation, where soil might freeze for long periods of time during winter, lowering water availability. *Trichosporon porosum* also has the ability to degrade aromatic compounds (Middelhoven et al. 2001) and forms creamy colonies in pure culture with true mycelia (personal observation; Sugita 2011). The assimilation of alternative nutrient sources, such as aromatic

compounds derived from lignin degradation, and the presence of hyphae that enhance nutrient access might be advantageous to survival in harsh soil environments with low water and nutrient availability.

Puyehue forest soil had the lowest number of both observed and estimated species. At this site we observed 2 main differences from the other forest soils: very high P values and high NH_4^+ values in spring. Puyehue forest soils have originated from volcanic eruption depositions, the most recent volcanic events having occurred in 1960 (Lara et al. 2006) and 2011. In these soils, the formation of allophanes might strongly retain P; however, the high P levels could be attributed to frequent volcanic deposition (Broquen et al. 2004). N content in soil is strongly associated with mineralization, which depends largely on microbial activity. High C/N ratio, acidic pH, and low temperature, as well as anaerobic conditions (which might be produced by too much rain during the rainy season — autumn to winter), lead to the accumulation of NH_4^+ in spring. Differences in mineralization processes determine differences in available nutrients and in the microbial community. Puyehue forest could be seen as a highly (naturally) disturbed site, with low abundance and a species-poor yeast community.

Much of the work published from the Northern Hemisphere has had to deal with the virtual absence of natural, undisturbed forest (Yurkov et al. 2011). The low anthropic impact that characterized most of the *Nothofagus* forest sites make this forest of great value in terms of understanding the functional aspect and yeast community dynamics in naturally occurring forests. The present analysis indicates that a complex set of environmental factors affects yeast community composition and structure, and even within the same geographical region these factors can vary greatly. It was suggested by di Menna (1965) that yeast populations are strongly affected not only by soil moisture but also by soil drainage, related to soil texture and organic matter content. In good moisture and high nutrient availability sites such as Challhuaco forest, we found higher numbers of yeast in soils. In harsh environments, with fewer easily assimilable nutrients, the number of yeasts decreased and the yeast communities have a different species composition. In these cases, species composition seems to be strongly related to soil texture, water availability (frozen water is not really available), the ability of different yeast species to assimilate complex molecules to survive (such as aromatic compounds), and the frequency of natural disturbances such as volcanic eruption.

Final remarks

The present survey described different soil yeast communities in northern Patagonian *Nothofagus* forest soils. There were no differences in yeast numbers when comparing the 2 tree species. Yeast distribution in this study followed a pattern also observed in other forest soils: higher numbers of yeast in high nutrient soils, dominance of basidiomycetous yeasts, and uneven distribution of isolates with few taxa accounting for a high percentage of isolates, while most of the taxa were scarcely represented. Each forest site showed a particular assemblage of species as a result of environmental characteristics, such as dominant plant species, nutrient availability, and climatic characteristics. Within each forest site, yeast distribution varied with soil region and season. Many of the yeast species recovered in the present survey were also found in other habitats in the region (lakes, leaf, glacier), which suggests a close relation between the yeast communities inhabiting them. The environmental factors affecting yeast communities comprise not only current soil characteristics but also a complex interaction of factors that includes previous natural disturbances such as volcanic activity. This survey focused on natural, pristine forests with low anthropic disturbance, and it sets the baseline for further studies regarding either natural or man-related disturbance in the northern Patagonian region.

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