RESEARCH ARTICLE

Saccharomyces eubayanus and *Saccharomyces uvarum* associated with the fermentation of *Araucaria araucana* seeds in Patagonia

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Abstract

Mudai is a traditional fermented beverage, made from the seeds of the *Araucaria araucana* tree by Mapuche communities. The main goal of the present study was to identify and characterize the yeast microbiota responsible of *Mudai* fermentation as well as from *A. araucana* seeds and bark from different locations in Northern Patagonia. Only *Hanseniaspora uvarum* and a commercial bakery strain of *Saccharomyces cerevisiae* were isolated from *Mudai* and all *Saccharomyces* isolates recovered from *A. araucana* seed and bark samples belonged to the cryotolerant species *Saccharomyces eubayanus* and *Saccharomyces uvarum*. These two species were already reported in *Nothofagus* trees from Patagonia; however, this is the first time that they were isolated from *A. araucana*, which extends their ecological distribution. The presence of these species in *A. araucana* seeds and bark samples, led us to postulate a potential role for them as the original yeasts responsible for the elaboration of *Mudai* before the introduction of commercial *S. cerevisiae* cultures. The molecular and genetic characterization of the *S. uvarum* and *S. eubayanus* isolates and their comparison with European *S. uvarum* strains and *S. eubayanus* hybrids (*S. bayanus* and *S. pastorianus*), allowed their ecology and evolution us to be examined.

Introduction

Aboriginal communities in Andean Patagonia (Argentina and Chile) used to prepare fermented beverages from several raw sources, including cereals and fruits. The Mapuche community, also known as Araucanians, was the most important aboriginal group inhabiting the temperate forests in Andean Patagonia (de Mösbach, 1992; Donoso & Lara, 1996). This typical gatherer community used several available wild fruits, such as beach strawberries (*Fragaria chiloensis*), '*maqui*' or Chilean wineberry (*Aristotelia chilensis*), '*calafate*' or Magellan barberries (*Berberis* spp.), and others, to produce fermented beverages (Pardo & Pizarro, 2005).

One of the most interesting cases for study is a traditional fermented beverage, called *Mudai*, generally used in religious ceremonies by Mapuche communities. This soft beverage is made from the seeds, *ngülliw* in the Mapuche language, of the *Araucaria araucana* tree, called *Pehuen*, which is a gymnosperm endemic of the lower slopes of the Chilean and Argentinian south-central Andes, typically above 1000 m of altitude. In Argentina, it occupies a narrow strip on the Patagonian Andes ranging from 37°50′ to 39°20′S in Neuquén province. Pehuen seeds

have also constituted an important source of carbohydrates for the Mapuche peoples from this area, who are in fact called Pehuenche (Pehuen people). Pehuen seeds are eaten raw, boiled or roasted and often ground into flour to be used as an ingredient in soups and to make bread and *Mudai* (Herrmann, 2005).

No literature on the microbial biota present during *Mudai* fermentation is available, probably due to the difficulties of obtaining samples from the fermentation performed by Mapuche communities. However, it is well known that yeasts belonging to *Saccharomyces* species, particularly *Saccharomyces cerevisiae*, are related to diverse processes including baking, brewing, distilling, winemaking, cider production, and are used in different traditional fermented beverages and foods around the world (Nout, 2003). In Patagonia, the species *S. cerevisiae* has been associated with winemaking environments (Lopes *et al.*, 2002; Sáez *et al.*, 2011) and fruit surfaces during postharvest cold storage (Robiglio *et al.*, 2011); however, other species of the genus, such as *Saccharomyces bayanus* var. *uvarum* (or *Saccharomyces uvarum*) and the newly described species *Saccharomyces eubayanus,* were isolated from Patagonian natural habitats in association with *Nothofagus* trees (Libkind *et al.*, 2011). Two recent studies (Bing *et al.*, 2014; Peris *et al.*, 2014) extended the geographic range in which *S. eubayanus* has been isolated. These authors reported for the first time the presence *S. eubayanus* strains from different oaks in the Tibetan Plateau in Far East Asia and from *Fagus* and *Acer* trees in Wisconsin, USA, respectively.

Nowadays, 10 species are included in the *Saccharomyces* genus: *Saccharomyces arboricolus, S. bayanus, Saccharomyces cariocanus, S. cerevisiae, S. eubayanus, Saccharomyces kudriavzevii, Saccharomyces mikatae, Saccharomyces paradoxus, Saccharomyces pastorianus* and *S. uvarum*. However, the discovery of *S. eubayanus* reignited discussion in the scientific community about the taxonomic position of *S. bayanus* and *S. pastorianus*. Libkind *et al.* (2011) demonstrated that *S. bayanus* is a taxon composed by heterogeneous hybrid strains between *S. uvarum* and *S. eubayanus* with minor contributions from *S. cerevisiae* in some cases, and *S. pastorianus* is an hybrid between *S. cerevisiae* and *S. eubayanus*. In a recent work by González *et al.* (2006), and modified by Pérez-Través *et al.* (2014), a rapid method was proposed to differentiate both '*uvarum*' and '*eubayanus*' alleles based in the gene sequences obtained from the fully sequenced strains CBS 7001 (also known as MCYC 623, considered the reference strain of *S. uvarum*) and *S. pastorianus* Weihenstephan 34/70, as well as from sequences obtained for *S. bayanus* reference strain NBRC 1948. Additional techniques to differentiate these two species were proposed by Nguyen *et al.* (2011) and Pengelly & Wheals (2013).

The aim of the present study was to identify and characterize fermentative yeasts present during fermentation performed with *A. araucana* seeds, according to the traditional elaboration procedures, in different locations in Northern Patagonia. Additionally, the fermentative yeast biota present in seed and bark samples from the *A. araucana* tree, from which Mapuche communities obtain the seeds used in *Mudai* elaboration, was also sampled and isolated using selective media.

The genetic characterization of *Saccharomyces* strains was performed by PCR-RFLP (polymerase chain reaction restriction fragment length polymorphism) and sequencing of different nuclear genes, and sequencing of the mitochondrial gene *COX2*. The phylogenetic relationships, at the inter- and intra-specific levels, between native isolates were obtained to determine their origins.

The presence of commercial bakery yeasts in artisanal traditional beverages as well as the presence of natural populations of *S. eubayanus* and *S. uvarum* associated with *A. araucana* trees is described for the first time in this study.

Materials and methods

Sampling areas

Samples from *A. araucana* seed fermentation were obtained from three different areas in Northwestern Patagonia (Neuquén province): Villa Pehuenia (38°54′00″S, 71°19′58″W, altitude: 1200 m), Junín de los Andes (39°57′03″S, 71°04′15″W, altitude: 902 m) and Huechulafquen (39°79′90″S, 71°22′57″W, altitude: 875 m) (Fig. 1). Fermentations were performed from April to May, during the Southern Hemisphere autumn.

Araucaria araucana bark and seed samples were collected from three different sampling areas in the same region: Caviahue (37°52′44″S, 71°03′53″W, altitude: 1600 m), Tromen (39°35′03″S, 71°25′33″W, altitude: 1250 m) and Huechulafquen (Fig. 1). Sampling in these areas was carried out during the summer. Annual average precipitation and temperatures in the different localities are as follows: Caviahue, 600–1000 mm, ≤ 10 °C; Tromen, 350 mm, 13 °C; Huechulafquen, ≥ 800 mm, ≤ 10 °C.

Isolation of fermentative yeasts

Sampling from *Mudai* fermentation

Musts were obtained by trituration of *A. araucana* seeds, boiling and addition of commercial sucrose according to traditional methodologies. Musts were transported to the laboratory and fermented at 20 °C. Yeast isolates were

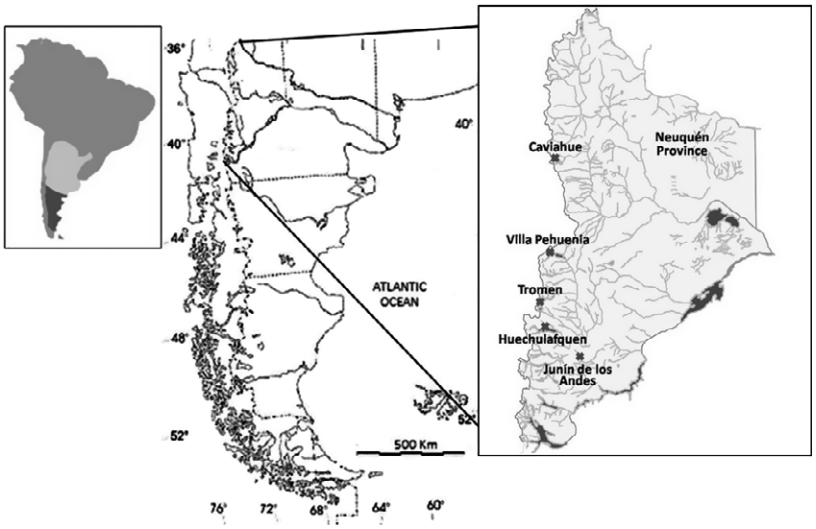

Fig. 1. Location of the sampling areas – Caviahue, Tromen, Huechulafquen, Villa Pehuenia and Junín de los Andes – in Northwestern Patagonia (Neuquén province). Left top corner, from light to dark gray: South America, Argentina and Patagonia.

obtained from different fermentation stages (initial, middle and end). Additionally, samples of musts prepared and fermented totally (end stages) following traditional procedures in the place of origin were also analyzed. Aliquots of appropriate dilutions (0.1 mL each) were spread onto GPY agar (w/v: 2% glucose, 0.5% peptone, 0.5% yeast extract, 2% agar) supplemented with chloramphenicol (50 mg L^{-1}). After incubation at 20 °C for 2–3 days, 20 colonies from each fermentation stage were isolated according to their macroscopic features and frequencies and preserved at −20 °C in a glycerol solution (20% v/v) and conserved in the NPCC (North Patagonian Culture Collection) in Neuquén, Argentina. The fermentations were carried out in duplicate and their evolution was daily followed by weight loss until the same weight was recorded in two consecutive measures.

Sampling from *A. araucana* trees

Yeasts were isolated from both bark and seeds of *A. araucana* trees following the methodology proposed by Sampaio & Gonçalves (2008). *Araucaria araucana* bark samples (2 g) and seeds (12 g) were collected aseptically and introduced into 20-mL sterile flasks containing 10 mL of selective enrichment medium consisting in YNB (yeast nitrogen base; Difco) supplemented with 1% (w/v) raffinose and 8% (v/v) ethanol and incubated at 30 °C or 10 °C without agitation. Samples exhibiting yeast growth (checked microscopically) were plated onto GPY agar and incubated at the same temperature as the bark or seed samples (10 °C or 30 °C). A representative number of yeast colonies were selected according to their frequency and morphology, and were preserved at −20 °C in glycerol solution (20% v/v) in the NPCC.

Yeast identification

Yeasts were identified by PCR-RFLP of the region encompassing the ITS1, 5.8S rRNA and ITS2 (5.8S-ITS region) as described in Lopes *et al.* (2010). PCR-RFLP patterns obtained for each isolate were compared with those of reference strains available in the www.yeast-id.org database. Yeast identifications were confirmed by sequencing both the 5.8S-ITS region and the D1/D2 domain of the 26S rRNA gene (Kurtzman & Robnett, 2003).

Sporulation and spore viability analyses

Sporulation was induced by incubating cells on sodium acetate medium (w/v: 1% sodium acetate, 0.1% glucose, 0.125% yeast extract and 2% agar) for 5–7 days at 26 °C. Following preliminary digestion of the ascus walls with zymoliase (Seikagaku Corporation, Japan) adjusted to 2 mg mL^{-1}, spores were dissected using a Singer MSM Manual micromanipulator in GPY agar plates. After incubation at 26 °C during 3–5 days, the spore viability analysis was performed and the developed colonies were transferred to the same sporulation medium in order to determine the homo/heterothallism of the monosporic cultures.

Mitochondrial DNA restriction analysis

mtDNA-RFLP patterns were analyzed for all isolates identified as belonging to *Saccharomyces*. Total DNA extraction was performed according to Querol *et al.* (1992). Total yeast DNA was subsequently digested with HinfI restriction enzyme (Roche Diagnostics, Mannhein, Germany) according to the supplier's instructions and the

fragments separated in 1% w/v agarose gels containing TAE (Tris-acetate-EDTA).

PCR-RFLP analysis of nuclear genes

The different '*uvarum*' and '*eubayanus*' alleles was detected by PCR amplification and subsequent restriction analysis of 33 protein-encoding nuclear genes according to González *et al.* (2006) and Pérez-Través *et al.* (2014). PCR amplifications were carried out in a Progene Thermocycler (Techne, Cambridge, UK) as follows: initial denaturing at 95 °C for 5 min, then 40 PCR cycles with the following steps: denaturing at 95 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min; and a final extension at 72 °C for 10 min. In the case of genes *ATF1*, *DAL1*, *EGT2*, *KIN82*, *MNT2*, *MRC1*, *RRI2* and *UBP7*, annealing was performed at 50 °C. Agarose gel preparation and staining were carried out as mentioned above. Restriction endonucleases AccI, AspI, Asp700I, CfoI, DdeI, EcoRI, HaeIII, HindIII, HinfI, MspI, PstI, RsaI, SacI, ScrFI, TaqI and XbaI (Fermentas, Lituania) were used according to the supplier's instructions. The PCR-RFLP profiles were compared with those reported by Pérez-Través *et al.* (2014; summarized in their Supporting Information Tables S2 and S3).

When new profiles were detected, their PCR amplifications were sequenced to confirm that they corresponded to new alleles. These PCR products were cleaned using the AccuPrep PCR purification kit (Bioneer, Inc.) and both strands of the DNA were directly sequenced using the BigDyeTM Terminator v3.0 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK), following the manufacturer's instructions, in an Applied Biosystems automatic DNA sequencer Model ABI 3730.

Restriction site maximum parsimony trees

From the restriction site gains and losses required to explain the RFLP patterns present in native *S. eubayanus* and *S. uvarum*, two binary matrices were constructed to codify the presence/absence of restriction sites in the native *S. eubayanus* and *S. uvarum* strains, respectively (Tables S1 and S2). These matrices were used to construct most-parsimonious trees that minimize the number of steps required to connect all the *S. eubayanus* strains and all *S. uvarum* strains. These parsimony trees were obtained with the MIX program included in the PHYLIP 3.695 package (Felsenstein, 2005) by considering restriction site changes as reversible events (Wagner criterion). Trees were rooted by including genotypes from the reference strain *S. uvarum* CBS 7001 and hybrid *S. pastorianus* W34/70 (*eubayanus* subgenome).

Sequencing and phylogenetic analysis

Four nuclear gene regions – *BRE5* and *EGT2*, the D1/D2 domain of the 26S gene and ITS1-5.8s-ITS2 – as well as the mitochondrial gene *COX2* were amplified and sequenced for phylogenetic study.

Nuclear genes were amplified by PCR as described above and the gene *COX2* was amplified using primers and conditions described in Belloch *et al.* (2000). PCR products purification and sequencing were also performed as described above. These sequences were submitted to the GenBank database under accession numbers KJ187251 – KJ187304.

Sequences from all different '*uvarum*' and '*eubayanus*' alleles from the fully sequenced strains *S. uvarum* CBS 7001, *S. pastorianus* Weihenstephan 34/70 (Nakao *et al.*, 2009) and *S. cerevisiae* S288c were used for comparative purposes. Each set of homologous sequences was aligned with the CLUSTAL method (Thompson *et al.*, 1994) available in the program MEGA5 (Tamura *et al.*, 2011). The sequence evolution model that fits our sequence data best was optimized using the maximum-likelihood Bayesian information criterion (BIC) for model comparison, also implemented in MEGA5. The BIC measures the relative support that sequence data give to different models of evolution and can be used to compare nested and non-nested models. It is defined as follows: $BIC_i = Cdels._eL_i + N_i\log_e n$, where n is the sample size (sequence length), N_i is the number of free parameters in the evolution model, and L_i is the maximum likelihood value of the data in the model. The smaller the BIC, the better the fit of the model to the data (Posada & Crandall, 2001).

The best fitting models were the Tamura & Nei (1993) model for *BRE5* sequences, the Tamura (1992) three-parameter model for *EGT2* sequences, and the Tamura 3-parameter model, with a gamma distribution of substitution rates with a shape parameter $\alpha = 0.07$, for *COX2* gene sequences. Nucleotide distances were corrected according to the corresponding models, estimated in the previous analysis, and were used to obtain phylogenetic trees with the neighbor-joining method (Saitou & Nei, 1987). Tree reliability was assessed using non-parametric bootstrap re-sampling of 1000 replicates. All these phylogenetic and molecular evolutionary analyses were also conducted using MEGA5 (Tamura *et al.*, 2011).

In the case of *COX2* sequences, due to evidence of recombination obtained from sequence comparisons, neighbor-net network analyses were also performed using the program SPLITSTREE4 (Huson & Bryant, 2006). Neighbor-net network reliability was also assessed using non-parametric bootstrap analysis based on 1000 replicates.

Tree topologies obtained with the 5′ and 3′ regions of *COX2* were compared with the nonparametric Shimodaira & Hasegawa (1999) test based on maximum likelihood, implemented in the program PAML 4.4 (Yang, 2007). This test is used to simultaneously compare sets of alternative phylogenetic topologies with the same sequence dataset.

Results

The must obtained from mixing ground *A. araucana* seeds, water and sugar was prepared in the traditional way in Villa Pehuenia (North-Patagonia, Argentina) but was fermented in our laboratory to assess the fermentation kinetics, the biomass production and the sampling of the yeast biota associated with this fermentation. Three independent fermentations were carried out from musts prepared in the place of origin. The fermentations were complete in 20 days and the maximum yeast population densities were 1.5×10^8 CFU mL^{-1}.

A very low morphological diversity was observed among the yeast colonies isolated at the beginning of fermentation. The molecular identification, by PCR-RFLP analysis of the 5.8S-ITS region PCR-RFLP and 26S rRNA D1/D2 domain sequencing, of representative yeasts confirmed the presence of a low species diversity; only two species, *Hanseniaspora uvarum* and *Saccharomyces cerevisiae*, were present in 80% and 20% of the total biomass, respectively. In subsequent stages of fermentation, the yeast biota corresponded exclusively to *S. cerevisiae* in the three analyzed fermentations. Musts that had already been fermented were then obtained from two additional locations from North Patagonia, Junín de los Andes and Huechulafquen, to get a more complete picture of the possible yeast biota responsible for the fermentation of this beverage. In these cases, all isolates obtained were identified as *S. cerevisiae*. The intraspecific analysis of all *S. cerevisiae* isolates from the five kinds of fermentation by means of mtDNA-RFLP showed a unique restriction pattern (Fig. 2). Given this unexpected result and considering a possible cross-contamination with commercial yeasts used for bread elaboration, the mitochondrial DNA of commercial bakery yeast was analyzed. The mtDNA-RFLP pattern obtained for the commercial baker yeast was identical to that detected in our *S. cerevisiae* isolates (Fig. 2). This result led us to search for fermentative yeast populations in the natural environment from where the raw material for the elaboration of this beverage comes from. Seeds and bark samples of *A. araucana* trees were collected aseptically from three different sampling areas: Caviahue, Huechulafquen and Tromen (Fig. 1). Following the methodology for fermentative yeast isolation proposed by Sampaio & Gonçalves (2008), we evaluated 120

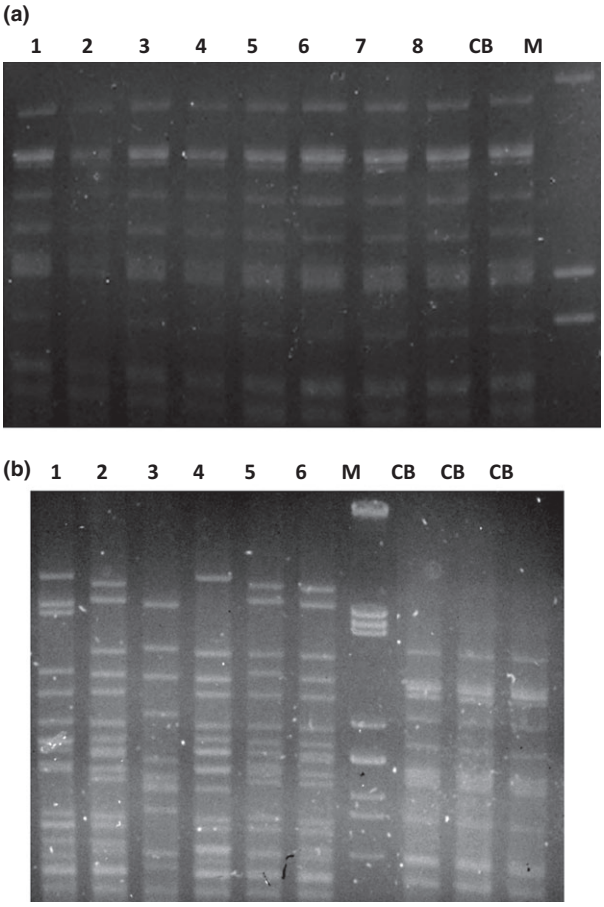

Fig. 2. (a) mtDNARFLP patterns of some *Saccharomyces cerevisiae* isolates from *Mudai* fermentation (lines 1–8) and the commercial bakery yeast (CB). (b) Different isolates of *S. cerevisiae* from fermented apple juice (lines 1–6) used as control to demonstrate the variability obtained with mtDNA-RFLP method and the commercial bakery yeast (CB). M, DNA size marker corresponding to lambda DNA digested with HindIII.

samples from two different substrates (60 samples from seeds and 60 from bark). Yeasts were obtained in 20% and 26.6% of the seed samples incubated at 10 and 30 °C, respectively (Table 1). Lower percentages of yeast recovery were obtained for bark samples at the two temperatures, 16.6% at 10 °C and 10% at 30 °C. According to the yeast macroscopic morphology and its frequency in GPY agar plates, a representative number of colonies were selected and identified using 5.8S-ITS PCR-RFLP and confirmed by sequencing the D1/D2 domain of the 26S rRNA gene. All the isolates were identified as *S. eubayanus* and *S. uvarum*, except for those obtained from the seed sample from Huechulafquen incubated at 30 °C (Table 1), which corresponded to the species *Kazachstania servazzii*. Both *S. eubayanus* and *S. uvarum* were detected in samples from *Tromen*, whereas only one of

Table 1. Number of bark and seed samples showing yeast growth at 10 and 30 °C and yeast species detected

		Samples with yeast (%)*		Yeast species	
Sampling area	Substrate	10 °C	30 °C	10 °C	30 °C
Caviahue	Bark	2 (20)	0	*Saccharomyces eubayanus*	–
	Seed	2 (20)	4 (40)	*Saccharomyces eubayanus*	*Saccharomyces eubayanus*
Huechulafquen	Bark	1 (10)	2 (20)	*Saccharomyces uvarum*	*Saccharomyces uvarum*
	Seed	0	1 (10)	–	*Kazachstania servazzii*
Tromen	Bark	2 (20)	1 (10)	*Saccharomyces uvarum/Saccharomyces eubayanus*	*S. eubayanus*
	Seed	4 (40)	3 (30)	*Saccharomyces eubayanus*	*S. uvarum/S. eubayanus*

*Number and percent of the 10 samples analyzed at 10 and 30 °C of each substrate and sample area (120 samples in total).

these two species was detected in the two other locations (*S. eubayanus* in Caviahue and *S. uvarum* in Huechulafquen) (Table 1). The two yeast species were obtained at both isolation temperatures and in bark and seed substrates (Table 1).

All the *Saccharomyces* isolates identified were subsequently subjected to mtDNA restriction analysis to evaluate the existence of one or more different molecular patterns, i.e. different strains, in the natural populations of *S. eubayanus* and *S. uvarum*. A total of five (U1m–U5m) and 13 (E1m–E13m) mtDNA profiles were detected among the *S. uvarum* and *S. eubayanus* isolates, respectively (Table 2). Each sampling area exhibited unique profiles; however, a shared profile was detected in several samples from the same area, e.g. the E2m profile was detected in different seed and bark samples from Caviahue but was not found in Huechulafquen or in Tromen (Table 2). The greatest profile diversity was observed in Tromen area, showing three and seven different profiles for *S. uvarum* and S. *eubayanus*, respectively. Only one species, *S. eubayanus* or *S. uvarum*, was found in each separate seed and bark sample, although in some cases more than one mitochondrial profile was observed among the isolates obtained from the same sample (Table 2).

To evaluate the pure nature of the *S. uvarum* and *S. eubayanus* yeast isolates, as well as the potential presence of natural hybrids between these two sympatric species, isolates representative of each mtDNA restriction profile were subjected to PCR amplification and subsequent restriction analysis of 33 nuclear gene regions located on different chromosomes. This methodology permits the differentiation of '*uvarum*' and '*eubayanus*' alleles along the genome based on the restriction patterns deduced from the complete genome sequences of the reference strains *S. uvarum* CBS 7001 and *S. pastorianus* (*S. eubayanus* × *S. cerevisiae* hybrid) Weihenstephan 34/70 (Pérez-Través *et al.*, 2014). In most cases, the RFLP patterns found in native isolates for the 33 gene regions were identical to those found in the non-*cerevisiae* (i.e. *S. eubayanus*) subgenome of *S. pastorianus* Weihenstephan 34/70 or in *S. uvarum* CBS 7001. These alleles were indicated as E1 or U1, respectively, in Table 3. However, new patterns (corresponding to new alleles) differing in one restriction site gain or loss were also found for some particular genes (Table 3). These new alleles, named E2 and E3, were detected in nine gene regions of native *S. eubayanus* isolates: *MET6*, *GSY1*, *PEX2*, *CBP2*, *DAL1*, *UBP7*, *CBT1*, *PPR1* and *ORC1* (Table 3). The nuclear genes *GAL4* and *KIN82* were successfully amplified and digested from all

Table 2. mtDNA-RFLP genetic characterization of *Saccharomyces eubayanus* and *Saccharomyces uvarum* native isolates obtained from bark and seed samples of *Araucaria araucana* from three different areas

		mtDNA-RFLP profile*					
		Bark samples		Seed samples			
Sampling area	T (°C)	1	2	1	2	3	4
Caviahue	10	$E1_m$ $E2_m$	$E3_m$	$E4_m$ $E2_m$ $E5_m$	$E2_m$ $E6_m$	–	–
	30	–	–	$E2_m$	$E2_m$	$E2_m$	$E2_m$ $E3_m$
Huechulafquen	10	$U1_m$		–	–	–	–
	30	$U1_m$	$U2_m$	–	–	–	–
Tromen	10	$E7_m$	$U3_m$	$E9_m$	$E10_m$ $E11_m$	$E12_m$	$E10_m$ $E12_m$
	30	$E8_m$	–	$U4_m$ $U5_m$	$E13_m$	$E12_m$ $E8_m$ $E9_m$	–

**Saccharomyces eubayanus* and *S. uvarum* mtDNA profiles are indicated as E_m and U_m, respectively. The associated numbers indicate the different profiles.

Table 3. Restriction patterns detected among *Saccharomyces eubayanus* and *Saccharomyces uvarum* indigenous yeast. Chromosome order is based on that described for *S. uvarum* CBS 7001

		S. eubayanus strains (mtDNA pattern)								*S. uvarum* strains (mtDNA pattern)			
S. uvarum Chr.	Gene	NPCC 1283 ($E2_m$) 1284 ($E5_m$) 1285 ($E6_m$) 1287 ($E3_m$)	NPCC 1294 ($E13_m$) 1297 ($E9_m$) 1302 ($E11_m$)	NPCC 1282 ($E4_m$)	NPCC 1286 ($E1_m$)	NPCC 1291 ($E12_m$)	NPCC 1292 ($E8_m$)	NPCC 1296 ($E7_m$)	NPCC 1301 ($E10_m$)	NPCC 1288 ($U5_m$)	NPCC 1289 ($U1_m$)	NPCC 1290 ($U4_m$) 1298 ($U3_m$)	NPCC 1293 ($U2_m$)
I	*CYC3*	E1	E1	E1	E1	E1	E1	E1	E1	U1	U1	U1	U1
III	*KIN82*	E1*	E1	E1	E1	E1	E1	E1	E1	U1	U1	U1	U1
	MRC1	E1	E1	E1	E1	E1	E1	E1	E1	U1	U1	U1	U1
V	*MET6*	E1	E1	E1	E2	E1	E1	E1	E1	U1	U1	U1	U1
	NPR2	E1	E1	E1	E1	E1	E1	E1	E1	U1	U1	U1	U1
VII	*KEL2*	E1	E1	E1	E1	E1	E1	E1	E1	U1	U1	U1	U1
	MNT2	E1	E1	E1	E1	E1	E1	E1	E1	U2	U2	U1	U2
IX	*DAL1*†	E3	E1	E3	E3	E1	E1	E1	E1	U1	U2	U1	U2
	UBP7	E2	E2	E2	E2	E2	E3	E2	E2	U1	U2	U1	U1
XI	*BAS1*	E1	E1	E1	E1	E1	E1	E1	E1	U1	U2	U1	U1
	CBT1	E1	E1	E1	E1	E1	E2	E1	E1	U1	U1	U1	U1
XII	*MAG2*	E1	E1	E1	E1	E1	E1	E1	E1	U1	U1	U1	U1
	PPR1	E2	E2	E2	E2	E2	E2	E2	E2	U1	U1	U1	U1
XIII	*CAT8*	E1	E1	E1	E1	E1	E1	E1	E1	U1	U1	U1	U1
	ORC1	E1	E2	E1	E1	E1	E1/E2	E1	E1	U1	U1	U1	U1
XVI	*GAL4*	E1*	E1	E1	E1	E1	E1	E1	E1	U1	U1	U1	U1
	JIP5	E1	E1	E1	E1	E1	E1	E1	E1	U1	U1	U1	U1
VIIItXV	*CBP2*	E1	E2	E1	E1	E1	E2	E1	E2	U1	U1	U1	U1
	ATF1	E1	E1	E1	E1	E1	E1	E1	E1	U1	U1	U1	U1
XVtVIII	*RRI2*	E1	E1	E1	E1	E1	E1	E1	E1	U1	U1	U1	U1
VItX	*EPL1*	E1	E1	E1	E1	E1	E1	E1	E1	U1	U1	U1	U1
	GSY1	E2	E1	E2	E2	E2	E3	E2	E1	U1	U1	U1	U1
	PEX2	E2	E3	E2	E2	E2	E3	E3	E3	U2	U2	U2	U2
XtVI	*CYR1*	E1	E1	E1	E1	E1	E1	E1	E1	U1	U1	U1	U1
XIVtIItIV	*EUG1*	E1	E1	E1	E1	E1	E1	E1	E1	U1	U1	U1	U1
IVtIItII	*PKC1*	E1	E1	E1	E1	E1	E1	E1	E1	U1	U1	U1	U1
	RPN4	E1	E1	E1	E1	E1	E1	E1	E1	U1	U1	U1	U1
	UGA3	E1	E1	E1	E1	E1	E1	E1	E1	U1	U1	U1	U1
IItIItXIV	*APM3*	E1	E1	E1	E1	E1	E1	E1	E1	U1	U1	U1	U1
	OPY1	E1	E1	E1	E1	E1	E1	E1	E1	U1	U1	U1	U1
	EGT2	E1	E1	E1	E1	E1	E1	E1	E1	U1	U1	U1	U1
XIVtIItIV	*BRE5*	E2	E2	E2	E2	E2	E2	E2	E2	U2	U2	U2	U2

E1 and U1 correspond to RFLP patterns exhibited by the reference strains *S. pastorianus* W34/70 and *S. uvarum* CBS 7001, respectively.
E1*refers to *Saccharomyces eubayanus* alleles lost in the reference hybrid strain *Saccharomyces pastorianus* Weihenstephan 34/70, but described for European *S. eubayanus* × *S. uvarum* hybrids (Pérez-Través *et al.*, 2014).
E2, E3 and U2 (with black background) correspond to new allele patterns reported in the present study.
E2 and U2 (with white background) are alternative PCR-RFLP patterns not reported in the reference strains but described in other *S. pastorianus* and European *S. uvarum* strains, respectively (Pérez-Través *et al.*, 2014).
†In the case of *DAL1* there is an E2 pattern described in a European *S. eubayanus* × *S. uvarum* hybrid (Pérez-Través *et al.*, 2014), therefore the new pattern exhibited by the Patagonian *S. eubayanus* strains is called E3.
NPCC, North Patagonian Culture Collection, Neuquén, Argentina.

the native isolates of *S. eubayanus*; however, these genes are lost in the non-*cerevisiae* subgenome of *S. pastorianus* W34/70. The comparison between the sequences of *GAL4* and *KIN82* of *S. eubayanus* native isolates and those present in *S. uvarum* CBS 7001 showed a low nucleotide similarity, 90.99% for *GAL4* and 92.30% for *KIN82* (Table 4), of the same level as other *S. eubayanus* gene regions, which confirms the *S. eubayanus* origin of the *GAL4* and *KIN82* genes present in these isolates.

Among native *S. uvarum* strains, restriction patterns different from those present in the reference strain *S. uvarum* CBS 7001 were found for six gene regions: *PEX2*, *MNT2*, *DAL1*, *UBP7*, *BAS1* and *BRE5* (Table 3). From them, only the new alleles for *DAL1* and *PEX2* were

Table 4. Restriction patterns and nucleotide similarities of new alleles found in native isolates *Saccharomyces eubayanus* (E2 and E3) and *Saccharomyces uvarum* (U2). Restriction fragment lengths are given in base pairs. Reference patterns correspond to those of the alleles present in the reference strain *S. uvarum* CBS 7001 and in the '*eubayanus*' genome fraction of the *Saccharomyces pastorianus* Weihenstephan 34/70 hybrid

								Nucleotide similarity (%) between new alleles and	
Gene	RE	Reference patterns		Restriction patterns of new alleles				*S. pastorianus* Weihenstephan 34/70	*S. uvarum* CBS 7001
CBP2	CfoI HinfI	605 180 370 255 120 35	E1	785 595 150 35	E2			96.56	85.05
CBT1	HaeIII MspI	360 120 430 50	E1	360 120 240 190 50	E2			98.11	92.65
DAL1	HaeIII HaeIII	470 210 80 345 170 100 75 65 5	E1 U1	335 210 135 80 345 235 100 75	E3* U2			99.85 93.58	93.12 98.78
GSY1	EcoRI HaeIII	450 315 765	E1	315 240 210 540 225	E2	450 315 540 225	E3	99.06 (E2) 99.19 (E3)	95.42 (E2) 95.29 (E3)
MET6	AspI Asp700 I	340 340 680	E1	340 340 440 240	E2			99.35	96.52
ORC1	HaeIII TaqI	430 350 100 295 240 155 75 60 55	E1	780 100 295 240 155 75 60 55	E2			98.99	87.47
PEX2	HaeIII	270 190 150 60 45	E1	340 230 60 45 40	E2	340 270 60 45	E3	98.15 (E2) 99.15 (E3)	93.45 (E2) 92.59 (E3)
	HaeIII	230 210 115 100 45 10	U1	330 210 115 45 10	U2			92.59	99.72
PPR1	TaqI	260 240 140 70	E1	260 180 140 70 60	E2			99.03	94.96
	XbaI	710		710					
UBP7	HaeIII HinfI	460† 440 95 405 400 171† 20	E1	909† 95 405 320 180† 80 20	E2	891† 95 405 320 162† 80 20	E3	94.83 (E2) 95.06 (E3)	90.25 (E2) 92.24 (E3)

*In the case of *DAL1*, there is an E2 pattern described in a European *S. eubayanus* × *S. uvarum* hybrid (Pérez-Través *et al.*, 2014), therefore the new pattern exhibited by the Patagonian *S. eubayanus* strains is called E3.

†These *UBP7* fragments show 3-bp microsatellite variations corresponding to CAA/CAG codons for Gln.

not previously reported by Pérez-Través *et al.* (2014) for *S. uvarum* strains. The new alternative alleles were also confirmed by sequence analysis.

Nucleotide sequence similarities between the new alleles reported for the first time in this work and those present in the reference strains are described in Table 4. We found high similarities between the sequences of the new alleles in native *S. eubayanus* isolates (E2 or E3) and the non-*cerevisiae* sequences from *S. pastorianus* W34/70. These similarity percentages were higher than 98% in most cases. Conversely, the nucleotide similarities were significantly lower when these sequences were compared with those present in the reference strain *S. uvarum* CBS 7001, with percentages lower than 95%, indicating that they correspond to *S. eubayanus*. In the same way, the new '*uvarum*' allele sequence was compared with the reference strains, confirming its *S. uvarum* origin (Table 4).

No hybrid strains were found among native isolates of *S. eubayanus* and *S. uvarum*; however, *S. eubayanus* strain NPCC 1292 is a particularly interesting native isolate since it exhibits eight new allelic variants, four of them unique (genes *GSY1*, *UBP7*, *CBT1* and *ORC1*). Moreover, this strain is heterozygous for *ORC1*, the only heterozygosity observed among our isolates (Table 3).

From the allele sequences for each gene, the restriction site gains/losses required to connect the different restriction patterns present in native *S. eubayanus* and *S. uvarum* can be deduced (see Tables S1 and S2, respectively). Each restriction site may be treated as a discrete 'character' or trait that is present or absent in any given strain. Most-parsimonious trees that minimize the number of steps required to connect all the *S. eubayanus* strains and all *S. uvarum* strains can then be constructed (Fig. 3a and b, respectively). Microsatellite variation in *UBP7* was not considered in these analyses. These trees were rooted by including genotypes from the reference strain *S. uvarum* CBS 7001 and hybrid *S. pastorianus* W34/70 (its *eubayanus* subgenome), depicted in Fig. 3 as black circles. These trees show a phylogeographic structure in the native *S. eubayanus* and *S. uvarum* strains. In this way,

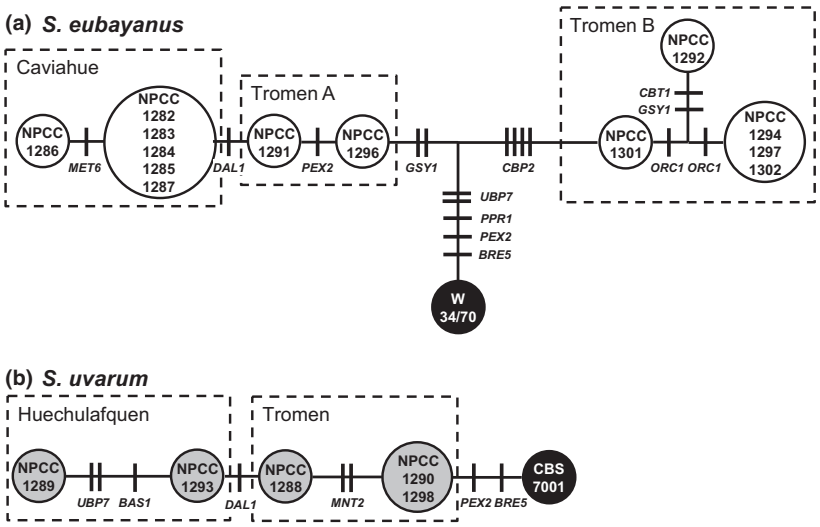

Fig. 3. Minimum number of restriction site changes needed to connect the different genotypes, represented by white circles, exhibited by the Patagonian *Saccharomyces eubayanus* (a) and *Saccharomyces uvarum* strains (b). Genotypes from the reference strains are represented by black circles. Restriction site changes can be reversible (gain/loss) and are represented by short lines. The gene regions involved are also indicated (for a description, see Tables 3 and 4). Microsatellite variation was not considered. Dotted squares show strain groups according to their geographic origins.

S. eubayanus strains from Caviahue and Tromen can be differentiated by their *DAL1* pattern, and Tromen strains can be divided into two subgroups, A and B, by their *CBP2* and *GSY1* patterns. *Saccharomyces uvarum* strains can also be differentiated into two populations, Huechulafquen and Tromen, by their *DAL1* patterns.

We also evaluated the sporulation capability of the complete set of native isolates. All of them produced abundant tetraspore asci after 15 days of incubation in sodium acetate agar medium and, in general, they showed high spore viability, ranging from 75% to 99% (Table 5). The only exception was *S. eubayanus* NPCC 1302, which showed a spore viability of 55%. F1 cultures obtained from viable spores were subsequently seeded in sporulation medium and all the monosporic cultures were able to sporulate, evidence of the homothallic nature of all *S. eubayanus* and *S. uvarum* native strains.

To obtain a more reliable picture of the relations among the native isolates from different Patagonian regions, we performed a phylogenetic analysis of partial sequences of the nuclear genes *BRE5* and *EGT2* and the mitochondrial gene *COX2*. Sequences of '*eubayanus*' and '*uvarum*' alleles from *S. pastorianus* W34/70 and *S. uvarum* CBS 7001, respectively, as well as sequences of the reference *S. cerevisiae* strain S288c were included in the phylogenetic analyses for comparative purposes.

Phylogenetic trees obtained for the nuclear genes clearly confirmed the species assignation of the native strains (Fig. 4). Those identified as belonging to *S. eubayanus* clearly clustered with the sequences of the '*eubayanus*' alleles of *S. pastorianus* W34/70 and those classified as *S. uvarum* grouped with the reference *S. uvarum* CBS 7001, in all cases with high bootstrap values (100%).

In the case of *BRE5* (Fig. 4a), no correlation was found between nucleotide sequences exhibited by native strains and their origin (Caviahue, Tromen and Huechulafquen). In particular, *S. eubayanus* strains from Caviahue and Tromen grouped together exhibiting four allelic variants, two of them being the most frequent in the two locations studied. Due to the low number of native strains belonging to the species *S. uvarum*, it was difficult to observe a geographical segregation within this species for this or the other genes. Four different allelic variants were found for this gene among *S. uvarum* strains; one of them (NPCC 1289) showed an identical allele to that found in the reference strain CBS 7001, whereas the remaining corresponded to three new alleles.

Table 5. Spore viability of *Saccharomyces eubayanus* and *Saccharomyces uvarum* indigenous strains

Yeast species	Sampling area	Strains NPCC (mtDNA-RFLP pattern)	Spore viability (%)
S. eubayanus	Caviahue	1282 ($E4_m$)	86
		1283 ($E2_m$)	86
		1284 ($E5_m$)	95
		1285 ($E6_m$)	86
		1286 ($E1_m$)	87
		1287 ($E3_m$)	80
	Tromen	1291 ($E12_m$)	81
		1292 ($E8_m$)	98
		1294 ($E13_m$)	98
		1296 ($E7_m$)	75
		1297 ($E9_m$)	90
		1301 ($E10_m$)	80
		1302 ($E11_m$)	55
S. uvarum	Huechulafquen	1293 ($U2_m$)	75
		1289 ($U1_m$)	98
	Tromen	1298 ($U3_m$)	89
		1288 ($U5_m$)	89
		1290 ($U4_m$)	99

NPCC, North Patagonian Culture Collection, Neuquén, Argentina.

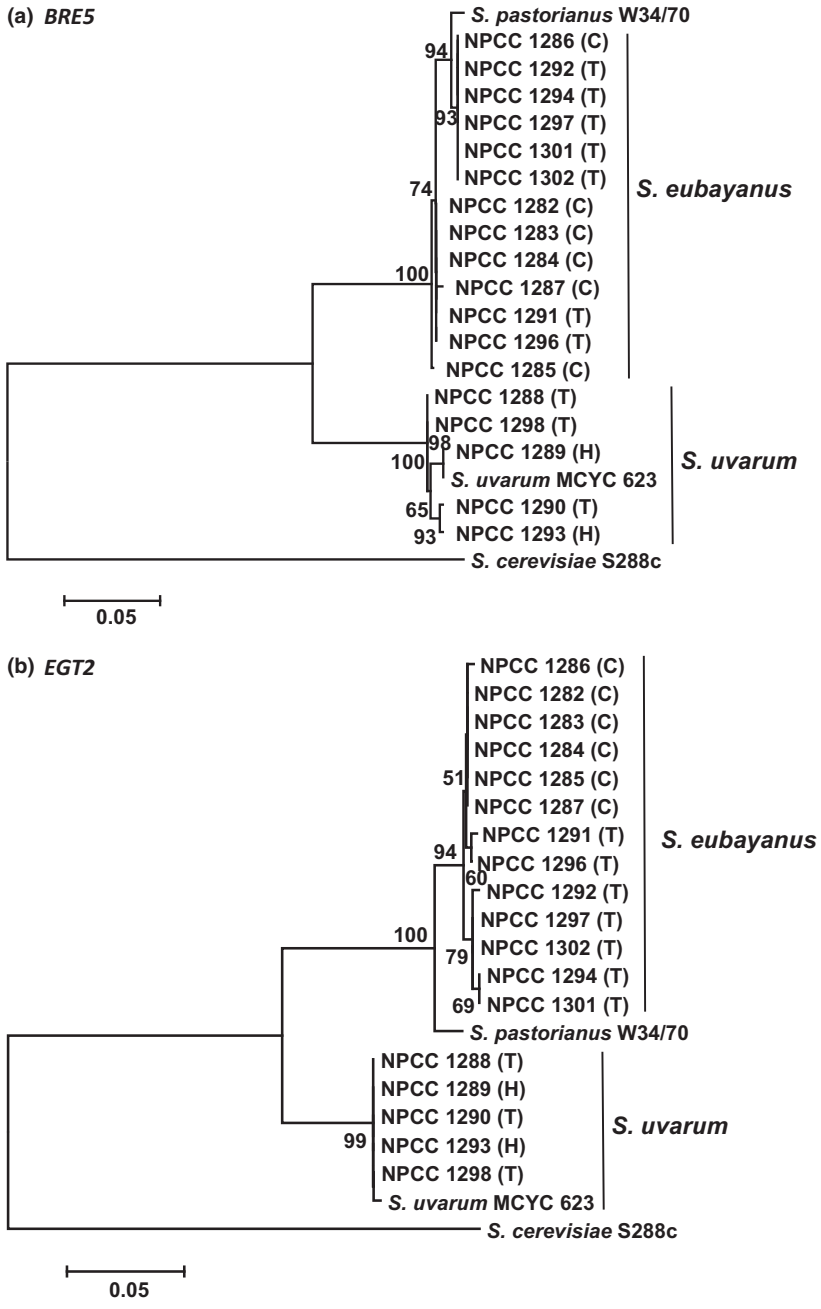

Fig. 4. Neighbor-joining trees obtained with partial sequences of the genes *BRE*5 (a) and *ETG*2 (b) from *Saccharomyces eubayanus* and *Saccharomyces uvarum* native isolates and reference strains of *Saccharomyces*. Nucleotide distances were corrected with the best fitting models according to the maximum-likelihood Bayesian information criterion for model comparison. The best models were the Tamura & Nei's (1993) for *BRE5* and Tamura's (1992) three-parameter model for *EGT2*. All these analyses were performed with the program MEGA5 (Tamura *et al.*, 2011). Numbers at the nodes correspond to bootstrap values based on 1000 pseudo-replicates. The scale is given in nucleotide substitutions per site. The geographic origin of the strains is indicated by: (C) Caviahue, (H) Huechulafquen and (T) Tromen.

The phylogenetic analysis of gene *EGT2* evidenced a higher genetic variability among *S. eubayanus* native isolates (seven different alleles) (Fig. 4b). In this case, there was a possible correlation between '*eubayanus*' allele sequences and the origin of the isolates (Caviahue or Tromen), because no common alleles were detected in the two areas, with the same groupings observed in the maximum parsimony tree based on restriction site variation (Fig. 3a). In contrast, no genetic variability was observed among native *S. uvarum;* all isolates showed the same allelic variant, highly similar to that found in the reference strain *S. uvarum* CBS 7001.

Finally, the analysis of the mitochondrial gene *COX2* showed six different alleles among *S. eubayanus* and five among *S. uvarum* native strains. In these phylogenetic analyses, we included reference strains representative of the *COX2* haplotypes described in a previous study for *S. uvarum, S. bayanus and S. pastorianus* (Pérez-Través *et al.*, 2014). In the *COX2* neighbor-joining tree (not shown), the '*eubayanus*' haplotype cluster appeared to be

a 'paraphyletic' ancestral group from which the *S. pastorianus COX2* and the monophyletic '*uvarum*' haplotype cluster derived. In this tree the non-*cerevisiae COX2* haplotype P1, present in five *S. pastorianus* strains, was located in an intermediate position between the '*eubayanus*' and '*uvarum*' haplotype clusters. This intermediate position may be due to the presence of a recombinant *COX2* haplotype in this strain. A detailed analysis of the *COX2* sequence alignment suggested the possibility of reticulate evolution due to recombination (Table S3). This way, the *S. pastorianus COX2* haplotype P1 (called E-I in Pérez-Través *et al.*, 2014) appears to be a chimerical sequence with a 5′ region (nucleotides 1–369) very similar to that of Patagonian *S. eubayanus COX2* haplotypes, showing only three to four differences, but 11–13 compared with *S. uvarum*; and a 3′ region (nucleotides 370–553) highly similar to that of Patagonian *S. uvarum COX2* haplotypes U6 and U7, and European haplotype U4 (UrE for Pérez-Través *et al.*, 2014), with only one difference, but 12–16 differences with respect to *S. eubayanus*.

As the presence of recombinant *COX2* haplotypes in *Saccharomyces* hybrids was already described in a previous study (Peris *et al.*, 2012), we performed a neighbor-net network phylogenetic analyses of the whole *COX2* gene and the 5′ and 3′ end regions separately (Fig. 5). In the complete *COX2* network, the *S. pastorianus COX2* haplotype again occupies an intermediate position; however, in the 5′ region, network clusters with the *S. eubayanus* sequences and in the 5′ region, phylogeny appears within the *S. uvarum* group, confirming the chimerical nature of the *S. pastorianus COX2* haplotype. Tree topologies depicted in Fig. 5b and c were compared with the non-parametric Shimodaira & Hasegawa (1999) test based on maximum likelihood, and proved to be significantly different ($P = 0.003$ for the *COX2* 5′ region sequences, and $P = 0.000$ for the *COX2* 3′ region sequences).

Therefore, this chimerical sequence could be the result of a recombination in the *COX2* gene during a hybridization event between a *S. uvarum* and a *S. eubayanus* strains bearing haplotypes not sampled yet, or could correspond to a chimerical sequence derived from an ancestral recombinant form after nucleotide substitution fixations.

Discussion

Loss of yeast diversity in traditional fermentation

Mudai is a traditional beverage elaborated from *A. araucana* seeds by indigenous Mapuche communities who have inhabited the cold regions in southwestern Argentina since the 18th century. Although there are historical records about its preparation and cultural connotations within the native communities, nothing is known about the microbiota associated to this fermentation process. However, only two species, *H. uvarum* and *S. cerevisiae*, were isolated from different *Mudai* fermentation stages in this work. Both species have been widely described in different fermentative processes. The apiculate yeast species *H. uvarum* and other related species of the same genus *Hanseniaspora* have long been associated with the fermentation of different sugar-rich raw materials including grapes (Barata *et al.*, 2008; Sáez *et al.*, 2011), apples (Morrissey *et al.*, 2004; Suárez-Valles *et al.*, 2007), oranges (Las Heras-Vazquez *et al.*, 2003) and cocoa beans (Nielsen *et al.*, 2007), particularly in the initial stages. Later, these apiculate yeasts are replaced by *S. cerevisiae*, which is generally the dominant species during the middle and final stages of most fermentation processes (Romano *et al.*, 2006).

The molecular characterization of the *S. cerevisiae* isolates obtained in this work from fermentation elaborated in different regions, exhibited a genetic homogeneity frequently observed in inoculated fermentation, in which the selected yeast starter dominates the fermentation process, but not in non-inoculated processes (Lopes *et al.*, 2007).

Traditional natural production of *Mudai* does not involve the use of commercial yeasts; however, the use of commercial bakery yeasts in breadmaking by people from the Mapuche communities has been reported (Pardo & Pizarro, 2005). Therefore, the environment in which this homemade product is nowadays elaborated, is in constant contact with commercial yeast cultures used in baking. MtDNA-RFLP analysis of a commercial bakery strain showed the same molecular pattern detected in our fermentation, evidencing a clear cross-contamination in this traditional fermented product.

The use of commercial strains in the industrial production of traditional beverages from Latin America is a common practice to ensure fast and reproducible fermentation. The use of wine or bakery yeasts has been reported in the fermentation of agave juice to produce Tequila (Aguilar-Uscanga *et al.*, 2007) and in the fermentation of sugarcane juice to produce Cachaça (Marini *et al.*, 2009). The use of commercial bakery or wine yeasts in the industrial production of traditional beverages results not only in lower quality products properties with less desirable sensory attributes (Marini *et al.*, 2009), but also in a modification of the yeast microbiota by means of a replacement of the native *Saccharomyces* strains or the formation of intraspecific hybrids between native and wine yeasts (Badotti *et al.*, 2014).

However, the present work is the first evidence from an ecological-molecular point of view of the impact of commercial yeast in very traditional fermentation, resulting in

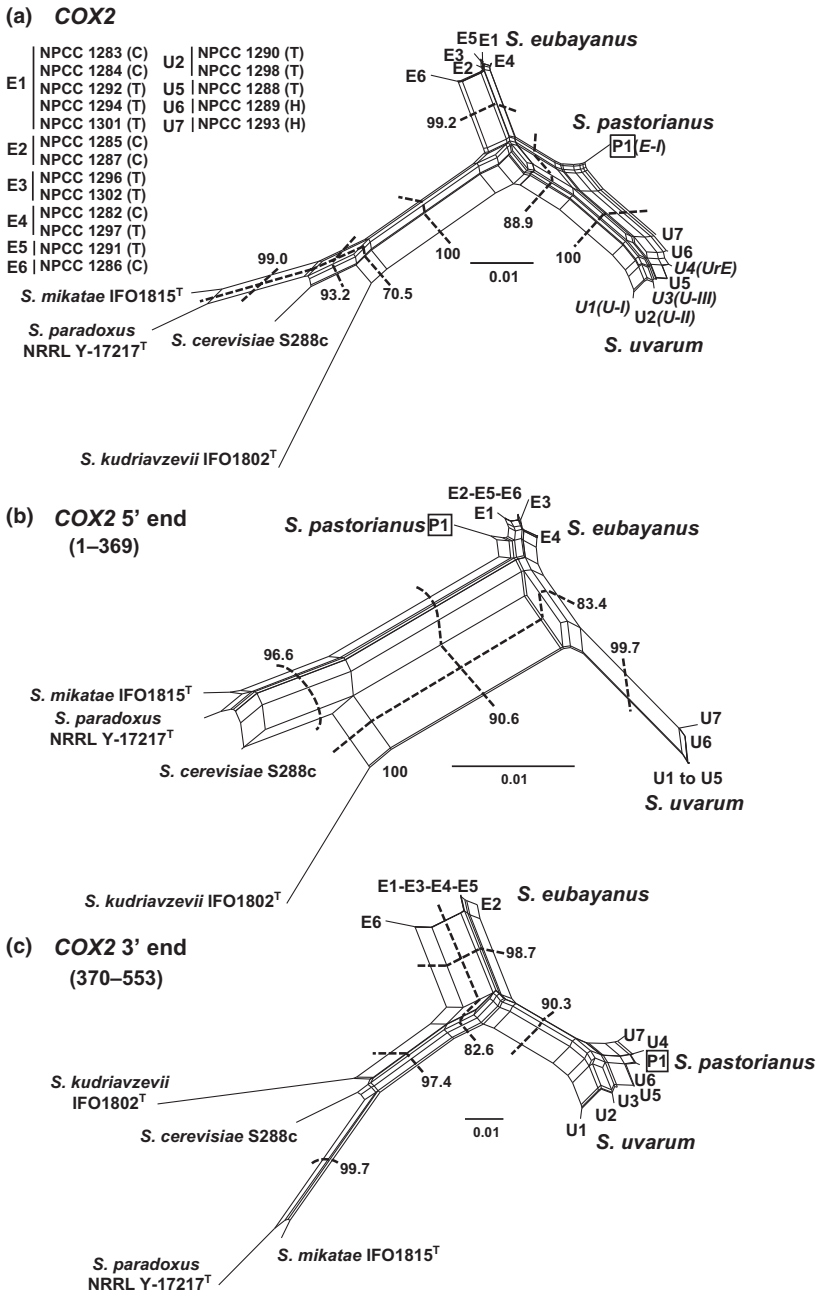

Fig. 5. Phylogenetic neighbor-net networks obtained with complete (a) and partial 5′-end (b) and 3′-end (c) sequences of the mitochondrial *COX2* gene from *Saccharomyces eubayanus* and *Saccharomyces uvarum* native isolates and reference strains of *Saccharomyces*. The different *COX2* sequence haplotypes are named by the initial of the species name of the closest parental (U for *S. uvarum,* E for *S. eubayanus,* P for *Saccharomyces pastorianus*) followed by a number. The *COX2* haplotype references used by Pérez-Través *et al.* (2014) are given in parentheses. Strains sharing the same haplotype are given at the upper left corner. The *S. pastorianus* haplotype P1 is highlighted by a square to indicate its changing positions in the phylogenetic networks due to its chimerical nature; its 5′ and 3′ ends are closely related to *S. eubayanus* and *S. uvarum COX2* haplotypes, respectively. Numbers located on the branches correspond to bootstrap values based on 1000 replicates for those branches crossed by the dashed lines.

a radical substitution of the natural yeast diversity. In fact, no indigenous *Saccharomyces* isolates were present in the *Mudai* samples.

These results show the difficulties faced in studying traditional fermentation, and led us to reformulate our methodological approach. We decided to isolate native fermentative yeasts from *A. araucana* seeds, the raw material from which *Mudai* is prepared. To our great surprise, all *Saccharomyces* isolates recovered from *A. araucana* seed and bark samples belonged to the closely related species *S. eubayanus* and *S. uvarum*. There is no previous report on the occurrence of *S. uvarum* or *S. eubayanus* in traditional fermentation from Latin America, but their presence in *A. araucana* seeds allows us to postulate a potential role for these strains in the production of *Mudai*. *Saccharomyces eubayanus*, a recently described taxon (Libkind *et al.*, 2011), has only been isolated so far from natural environments in Patagonia, but strains of this species, as well as of *S. uvarum*, can be used to make *Mudai* and carry out other traditional fermentation under

laboratory conditions with promising results (Rodríguez ME, Barbagelata RJ, Origone AC and Lopes CA manuscript in preparation). In the case of *S. uvarum*, this species is a better candidate because it is frequently found in low-temperature fermentation processes from European regions of oceanic or continental climates, in which it coexists and even replaces *S. cerevisiae* as the main yeast responsible for wine (Naumov *et al.*, 2000b, 2002; Rementería *et al.*, 2003; Demuyter *et al.*, 2004; Lopandic *et al.*, 2008; Csoma *et al.*, 2010) and cider (Naumov *et al.*, 2001; Coton *et al.*, 2005; Suárez-Valles *et al.*, 2007) fermentation.

Nonetheless, we cannot discard that *S. uvarum* or *S. eubayanus* could be replaced by native *S. cerevisiae* during *Mudai* fermentation, in the same way that a commercial bakery *S. cerevisiae* strain has recently done. However, the absence of native *S. cerevisiae* strains in *A. araucana* seeds and bark samples, as well as the absence of hybrids between commercial *S. cerevisiae* and native *S. eubayanus* or *S. uvarum* strains may be indicative of a recent substitution of native *S. uvarum* or *S. eubayanus* strains by commercial *S. cerevisiae* strains for *Mudai* fermentation.

Ecology and distribution of *S. eubayanus* and *S. uvarum* in Northern Patagonia

Information on the natural occurrence of *S. uvarum* and *S. eubayanus* is really scarce. As mentioned, *S. uvarum* has mainly been found associated with low-temperature fermentation processes in regions of oceanic or continental climates. Only a few isolates have been recovered from insects (*Mesophylax adopersus* and *Drosophila* spp.), bark from *Quercus*, *Arbutus* and *Prunus* trees, and exudates from *Ulmus*, *Carpinus* and *Nothofagus* trees and mushrooms (Sampaio & Gonçalves, 2008; Libkind *et al.*, 2011; Naumov *et al.*, 2011).

Saccharomyces eubayanus is a new taxon described from isolates obtained from *Nothofagus* trees, including *Nothofagus pumilio* and *Nothofagus antarctica*, as well as from stromata of their parasitic fungi *Cyttaria* in Northern Patagonia (Libkind *et al.*, 2011). The areas from which we obtained our isolates are characterized by mixed woodlands containing different species of *Nothofagus*, as well as *A. araucana* trees. Very recently, new populations of *S. eubayanus* were discovered from *Fagus* and *Acer* trees in Wisconsin, USA (Peris *et al.*, 2014) as well as from oak bark samples in Tibetan Plateau in the Far East Asia (Bing *et al.*, 2014). Both the present study and these new reports are evidence that *S. eubayanus* is not host-specific, as proposed by Libkind *et al.* (2011).

The *Saccharomyces* genus contains both cryotolerant and non-cryotolerant species. *Saccharomyces kudriavzevii*, *S. uvarum* and *S. eubayanus* species are considered cryotolerant and have been successfully isolated from bark samples by means of selective isolation methods at low temperature (Sampaio & Gonçalves, 2008; Lopes *et al.*, 2010; Libkind *et al.*, 2011). In spite of this, all *Saccharomyces* isolates obtained in this work at both 10 and 30 °C temperatures corresponded to the cryophilic species *S. uvarum* and *S. eubayanus*. This result agrees with those reported by Libkind *et al.* (2011) and supports the idea that the cold forests from Patagonia may be an unfavorable ecosystem for the non-cryotolerant *Saccharomyces* species such as *S. cerevisiae* or *S. paradoxus*, which is easily isolated in other regions using the same methodology (Sampaio & Gonçalves, 2008; Lopes *et al.*, 2010; Naumov *et al.*, 2013). The climatic conditions of the sampling areas in this study, characterized by temperatures between −4 and 11 °C, are highly selective and make Northwestern Patagonia a region suitable only for the cryotolerant species *S. eubayanus* and *S. uvarum*. Interestingly, *S. kudriavzevii*, the other cryotolerant species of the genus isolated from European and Asian areas of similar climatic conditions (Sampaio & Gonçalves, 2008; Lopes *et al.*, 2010; Naumov *et al.*, 2013), was not isolated in this study or in the previous study by Libkind *et al.* (2011), perhaps due to competitive exclusion, as suggested by Sampaio & Gonçalves (2008).

Of the three areas under study, only Tromen showed the sympatric distribution of both species; however, only a single species was isolated from each seed and bark sample. These results suggest that the two species could be unable to coexist in the same microhabitat. The fact that we only obtained isolates belonging to one species in samples from Caviahue and Huechulafquen (*S. eubayanus* and *S. uvarum*, respectively) may also suggest that these locations are exclusive habitats for each particular species; however, further sampling in these areas and different hosts would be needed to support this claim.

Therefore, the present study is of utmost significance since this is a second report confirming the sympatric coexistence of the two cryotolerant species in a same region, Northwestern Patagonia, but in areas and on a plant species different from those previously reported (Libkind *et al.*, 2011). In particular, this work extends the habitat of this species northward in the South Hemisphere, from 40°09′5″S reported by Libkind *et al.* (2011) to 37°52′44″S (Caviahue area in this work).

Hybridization and introgression in *S. eubayanus* and *S. uvarum*

The genetic characterization of Northern Patagonian *S. eubayanus* and *S. uvarum* native isolates showed that they are homothallic and homozygous for most analyzed

genes (the only exception is *ORC1* from *S. eubayanus* NPCC 1292), showing high sporulation ability and spore germination. Most *Saccharomyces* strains of enological origin are characterized by being homothallic, with a low level of heterozygosity (Mortimer *et al.*, 1994; Johnston *et al.*, 2000; Pretorius, 2000; Bradbury *et al.*, 2006; Legras *et al.*, 2007). High homozygosity levels but a low ascospore viability was also observed among natural isolates of *S. kudriavzevii* (Lopes *et al.*, 2010). These features are important for the survival of yeasts in nature and may be involved in the removal of deleterious alleles and chromosome rearrangements (Mortimer *et al.*, 1994), and are indicative of the non-hybrid nature of these *S. eubayanus* and *S. uvarum* native isolates. The absence of natural hybrids between *S. uvarum* and *S. eubayanus* in the Patagonian sampling regions was also confirmed by their molecular characterization based on the restriction analysis and sequencing of different gene regions.

The absence of hybrids among native *S. eubayanus* and *S. uvarum* isolates contrasts with the very complex situation found in Europe, where hybridization is very common among strains related to *S. eubayanus* and *S. uvarum* (Rainieri *et al.*, 2006; Nguyen *et al.*, 2011; Pérez-Través *et al.*, 2014). Among them, we found strains belonging to the *S. uvarum* species that contain a single type of genome related to that sequenced for the strain CBS 7001 (also called MCYC 623), although some small introgressed regions from *S. cerevisiae* can be present (Naumova *et al.*, 2005, 2011; Pérez-Través *et al.*, 2014). There is also a panoply of hybrid strains containing different portions of the *S. uvarum* and *S. eubayanus* genomes (included in the *S. bayanus* taxon), *S. cerevisiae* and *S. eubayanus* genomes (included in the *S. pastorianus* taxon), and *S. cerevisiae* and *S. uvarum* genomes (González *et al.*, 2006; Le Jeune *et al.*, 2007; Pérez-Través *et al.*, 2014), as well as hybrids with portions of the genomes from *S. cerevisiae*, *S. uvarum* and a third species, *S. kudriavzevii* (Peris *et al.*, 2012).

COX2 gene sequencing has shown useful to decipher the origin of mitochondria in *Saccharomyces* hybrid strains (González *et al.*, 2008; Peris *et al.*, 2012; Pérez-Través *et al.*, 2014). The presence of a chimeric *COX2* haplotype in the European *S. pastorianus* hybrids may be due to the presence of a recombinant *COX2* haplotype in strains belonging to this species. The presence of recombinant *COX2* haplotypes in *Saccharomyces* hybrids was already described in our previous studies (Peris *et al.*, 2012; Pérez-Través *et al.*, 2014) and may also be the result of the interspecies hybridization during the evolution of these strains. In the recent study by Peris *et al.* (2014), recombination in the mitochondrial *COX2* sequences from *S. eubayanus* and *S. pastorianus* strains has also been found.

Introgressed strains have also been described both in fermentation processes (Naumova *et al.*, 2011; Nguyen *et al.*, 2011; Pérez-Través *et al.*, 2014) and in native wild environments (Liti *et al.*, 2006; Wei *et al.*, 2007; Doniger *et al.*, 2008; Muller & McCusker, 2009; Dunn *et al.*, 2012). However, introgressions are due to unstable hybridization followed by successive backcrossing with one or the other parental species, resulting in introgressed but fertile strains (Naumova *et al.*, 2011).

In conclusion, these observations suggest that even though hybridization may be occurring in nature, stable hybrids seem to be only successful in fermentative environments, under conditions different from those present in the ancestral habitat of the parental species.

Origin of European *S. eubayanus* hybrids

Restriction analysis and sequencing of nuclear genes revealed that Patagonian *S. uvarum* and *S. eubayanus* share alleles with the European *S. uvarum* strains and *S. pastorianus* hybrids, respectively. However, specific allelic variants were exhibited by Patagonian *S. eubayanus* and, to a lesser extent, by Patagonian *S. uvarum*. This new variation allowed us to differentiate Patagonian strains and detect a certain degree of population structure based on the association between their geographic origin and the molecular variation of the native *S. eubayanus* strains.

Libkind *et al.* (2011) suggested that, because *S. eubayanus* has not been found in Europe, *S. pastorianus* hybrids may have appeared in the lager brewery environments of Central Europe by hybridization between ale *S. cerevisiae* strains and immigrant *S. eubayanus* yeasts arriving from America after the advent of trans-Atlantic trade. The major caveat to the validity of this assessment is that although lager brewing is more or less contemporary with the arrival of Europeans to America (Hornsey, 2003), the Patagonian region was not colonized until the late 19th century, during the Chilean occupation of Araucanía and the Argentine Conquest of the Desert, mainly due to the fierce resistance of the Mapuche (Araucanian) peoples (Bengoa, 2000). However, the presence of *S. eubayanus* has been recently reported in other regions of America (Peris *et al.*, 2014) that were colonized earlier, which may explain its presence in Patagonia. In addition, lager brewing may have originally been performed using other cryophilic yeasts also present in brewery environments, e.g. *S. cerevisiae* × *S. kudriavzevii* hybrids (González *et al.*, 2008) and only later have been replaced by the newly generated *S. pastorianus* (*S. cerevisiae* × *S. eubayanus*) hybrids.

In their recent study, Peris *et al.* (2014), reported the presence of two diverse and highly differentiated populations in *Nothofagus* from Patagonia and the rare isolation of *S. eubayanus* strains in Wisconsin, USA, which represent a recent mixture of the two Patagonian populations.

This new Patagonian population (so-called B) seems to be closer to the *S. eubayanus* subgenome of the hybrid lager strains, although the populations reported by Bing *et al.* (2014) in Asia, may be even closer.

The genetic differentiation between our Patagonian *S. eubayanus* population from *A. araucana* and the *S. eubayanus*-genome fraction of the European *S. pastorianus* and *S. bayanus* hybrids suggests that these *A. araucana* strains belong to the *Nothofagus* population first described (so-called population A). Although the Peris *et al.* (2014) results suggest that European hybrids were generated by an ancestor of the Patagonian B population, a possible origin in an unknown European *S. eubayanus* population, not sampled yet, as proposed by Gibson *et al.* (2013), cannot be discarded, in a similar way to the species *S. kudriavzevii*, which was first described from Japanese isolates (Naumov *et al.*, 1995, 2000a) but whose hybrids with *S. cerevisiae* were found in fermentation environments from Europe (González *et al.*, 2006) before any European strain of this species were isolated from nature (Sampaio & Gonçalves, 2008; Lopes *et al.*, 2010). Both possibilities would solve the problem of the origin of the lager yeast *S. pastorianus* mentioned above. Finally, Bing *et al.* (2014) strongly suggest that the new Tibetan population of *S. eubayanus* is the direct donor of the non-*S. cerevisiae* subgenome of lager yeast due to the proximity between Europe and Asia and the trade history between the two continents.

In contrast, nobody has posited colonization events to explain the distribution of *S. uvarum* and the origin of its hybrids. However, as pointed out, European and Patagonian *S. uvarum* populations share more alleles than Patagonian *S. eubayanus* and their European hybrids. This species is considered to have a world-wide distribution because it has recurrently been isolated in Europe, mainly in industrial environments (for a review see Naumov *et al.*, 2011) and from natural environments in Far East Asia (Naumov *et al.*, 2003) and in America (Naumov *et al.*, 1996, 2006; Sampaio & Gonçalves, 2008; Libkind *et al.*, 2011; present study). However, although it seems to be quite frequent in wild environments of Patagonia (Naumov *et al.*, 2006; Libkind *et al.*, 2011; present study), it is very rare in non-fermentative environments of Europe (only three cases, Table 3 in Naumov *et al.*, 2011). It is clear that additional environmental sampling is necessary to understand the geographic distribution and niche occupation of these cryophilic sibling species in relation to other *Saccharomyces* species.

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Supporting Information

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

Table S1. Presence/absence matrix of variable restriction sites (constant sites are not shown for simplification) present in different genes of native *S. eubayanus* strains.
Table S2. Presence/absence matrix of variable restriction sites (constant sites are not shown for simplification) present in different genes of native *S. uvarum* strains.
Table S3. Comparison of *COX2* haplotype sequences from Patagonian *S. eubayanus* and *S. uvarum* strains, and reference strains of *S. uvarum* and *S. pastorianus* hybrids from Pérez-Través *et al.* (2014).