Saccharomyces uvarum is responsible for the traditional fermentation of apple chicha in Patagonia

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One sentence summary: Phylogenetic data on Saccharomyces uvarum strains, isolated for the first time from Patagonian apple chicha suggest that these strains might have been introduced in Patagonia during the domestication of apples by Mapuche communities.

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ABSTRACT

Apple chicha is a fresh low alcoholic beverage elaborated by aboriginal communities of Andean Patagonia (Argentina and Chile). In the present work, we identified the yeast microbiota associated with this fermentation, and characterized genetically those belonging to the genus Saccharomyces. Both Saccharomyces cerevisiae and S. uvarum were found in the analyzed fermentations. Phylogenetic and population structure analyses based on genes sequence analysis were carried out for both S. cerevisiae and S. uvarum strains obtained in this study and a set of additional strains from diverse origins. The results demonstrate that S. cerevisiae strains from apple chicha belong to the big group of wine/European strains of this species, while S. uvarum strains were included in the Holartic population of this species. Additionally, some S. uvarum strains from chichas evidenced as an admixture of both pure Holartic and pure South American populations. Our results suggest that Holartic strains could have been introduced in South America together with the domestication of apple trees by Mapuche communities. This Holartic population suffered admixis with the naturally present South American population of this species, originating strains bearing genetic features from the two populations, detectable in both chichas and natural habitats.

Keywords: Holartic; South America; admixture; Mapuche; yeast diversity

INTRODUCTION

A large variety of fermented foods and beverages with traditional and cultural values have been described in the world (Nout 2003). The diversity of such fermented products derives from the heterogeneity of traditions, cultural preference, different geographical areas where they are produced and the staple and/or by-products used for fermentation. In many instances, it
is highly likely that the methods of production were unknown and came about by chance, and were passed down by cultural and traditional values to subsequent generations. This is the case with fermented food elaborated by Mapuche people, aboriginal communities in Andean Patagonia (Argentina and Chile) (de Mösbach 1992; Donoso and Lara 1996). Apples, together with other fruits such as grapes, pears and citric fruits, were introduced to America by the first Spanish settlers, including those that colonized the Chilean region south of the Bio-Bio river. This region was later abandoned by the Spaniards during the Araucanian wars, and kept under Mapuche domination until the 19th century (Cruz 2010). Mapuche people exploited the fruits of these feral apple trees (manshanás-aliven, in Mapuche language) as food but also to produce apple chicha, a fresh low alcoholic beverage.

Although the microflora responsible for most indigenous fermented beverages around the world remains undiscovered, the fermented beverages produced in Patagonia are unusual—based on current knowledge—in that they are generally produced at low temperatures (lower than 20 °C). In a recent study carried out in our laboratory, we studied the diversity of yeasts associated with ‘Mudai’ fermentations, obtained from Araucaria araucana seeds, demonstrating the total dominance of commercial Saccharomyces cerevisiae bakery yeasts in these fermentations. However, the detection of the cryotolerant yeast species S. eubayanus or S. uvarum on A. araucana seeds and barks samples led us to consider the role of these species in the ancient Mudai fermentations, prior to the introduction of commercial yeasts for bakery (Rodríguez et al. 2014). This observation was quite surprising due to the fact that the mesophilic species S. cerevisiae has traditionally not been associated with the elaboration of diverse commercial fermentations around the world (baking, brewing, distilling, winemaking, cider production, etc.), but also with different traditional fermented beverages and foods (Nout 2003).

At present, the genus Saccharomyces comprises a total of seven accepted species—S. arboricolus, S. cerevisiae, S. eubayanus, S. kudriavzevii, S. mikatae, S. paradoxus and S. uvarum (Almeida et al. 2014; Boynton and Greig 2014)—as well as natural interspecific hybrids, including the lager yeasts S. pastorianus (S. cerevisiae × S. uvarum × S. eubayanus) and S. bayanus (S. uvarum × S. eubayanus) (Libkind et al. 2011; Pérez-Través et al. 2014); wine, cider and brewing S. cerevisiae × S. kudriavzevii; S. cerevisiae × S. uvarum, and S. cerevisiae × S. kudriavzevii × S. uvarum hybrids (Peris et al. 2012; Pérez-Torrado et al. 2015). From this complex diversity of species and hybrids, only S. kudriavzevii, S. eubayanus and S. uvarum, and most of their hybrids, have been related to cold temperatures.

The aim of this study was to identify and characterize fermentative yeasts present during apple chicha fermentation. Additionally, the genetic characterization of the Saccharomyces strains responsible for these fermentations was carried out in order to confirm the relevance of cryotolerant Saccharomyces species or hybrids in these fermentations typically carried out at low temperatures.

**MATERIALS AND METHODS**

**Sampling areas**

Wild apple musts were obtained from three different areas of Chile: Tiriúa (38°20’14.40”S, 73°29’46.66”W), Villarrica (39°16’47.44”S, 72°13’50.81”W) and Pucón (39°16’05.33”S, 71°58’42.94”W) (Fig. 1).

**Isolation of fermentative yeasts**

Musts were obtained by trituration of fruits of feral apple trees (Malus domestica Borkh.), according to traditional procedures. Musts were transferred to the laboratory and fermented at 20 °C. The fermentations were carried out in duplicate and their evolution was daily followed by weight loss until constant weight during two consecutive measures. Yeast isolates were obtained from different fermentation stages (initial, middle and end). Aliquots of appropriate dilutions (0.1 mL of each one) were spread onto GYP agar (w/v: 2% glucose, 0.5% peptone, 0.5% yeast extract, 2% agar) supplemented with chloramphenicol (50 mg/L). After incubation at 20 °C for 2–3 days, 20 colonies from each fermentation stage were randomly isolated and stored at −20 °C in glycerol solution (20% v/v). Additionally, samples of musts that were prepared totally (end stages) according to traditional procedures in their original areas (Fig. 1) were analyzed following the same methodology explained above.

**Yeasts identification**

Yeasts were identified by restriction analysis PCR-RFLP of the region encompassing the ITS1, 5.8SrRNA and ITS2 (5.8S-ITS). For this purpose, yeast DNA was isolated according to standard procedures (Querol et al. 1992). PCR reactions were performed in 50 μL final volume containing 5 μL of 10x Taq polymerase buffer, 100 μM deoxynucleotides, 1 μM of each primer, 1 U of Taq DNA polymerase (Promega, USA) and 3 μL of DNA diluted to 50 ng/μL. PCR amplifications were performed 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. Simple digestions with one endonuclease were performed using 10 μL of amplified DNA in a final volume of 20 μL. Acc I, Cfo I, Hae III, Hinf I and Scr FI (Promega Corp., WI, USA) restriction endonucleases were used according to the supplier’s instructions. PCR products and restriction fragments were separated on 1.5% w/v and 3% w/v agarose gels in 1 × TAE buffer (Genbiotech, Argentina), respectively. A 50-bp DNA ladder marker

**Figure 1. Origin of the chichas evaluated in this work.**
(Fermentas, Lituania) served as size standard. After electrophoresis, gels were stained with a solution of GelRed (Biotium, USA) and visualized under UV light. PCR-RFLP patterns obtained for each isolate were compared with those of reference strains available at the yeast-id database (www.yeast-id.org). Yeast identity was confirmed by sequencing both the 5.8S-ITS region and the D1/D2 domain of the 26S rRNA gene (Kurtzman and Robnett 1998).

**Mitochondrial DNA restriction analysis**

Mitochondrial DNA (mtDNA)-RFLP patterns were analyzed for all isolates identified as belonging to Saccharomyces genus. Total DNA extraction was performed according to Querol et al. (1992). Total yeast DNA was subsequently digested with Hinf I restriction enzyme (Roche Diagnostics, Mannheim, Germany) according to the supplier’s instructions and the fragments separated in TAE-containing 1% w/v agarose gels.

**PCR-RFLP analysis of nuclear genes**

The detection of the different Saccharomyces uvarum alleles was performed by PCR amplification and subsequent restriction analysis of 33 protein-encoding nuclear genes (Pérez-Través et al. 2014). PCR amplifications were performed in a Progene Thermocycler (Techne) 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 ATF1, DAL1, EGT2, KIN82, MNT2, MRC1, RRI2 and UBP7 genes, annealing was performed at 50°C. Agarose gel preparation and staining were carried out as it was mentioned above. Acc I, Asp I, Asp 7000, Cfo I, Dde I, Eco RI, Hae III, Hind III, Hinf I, Msp I, Pst I, Rsa I, Sac I, Sce FI, Taq I and Xba I restriction endonucleases (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).

**Sequencing and phylogenetic analyses**

For selected S. uvarum, eight nuclear gene regions including BRES, CAT8, CYC3, CYR1, EGT2, GAL4, MET6 and MNL1 as well as the COX2 mitochondrial gene were amplified and sequenced for subsequent phylogenetic and population studies. For S. cerevisiae strains, only CAT8 and GAL4 genes, which resulted useful in previous studies (Peris et al. 2012), were sequenced. Nuclear genes were amplified by PCR as described above and the COX2 gene was amplified using primers and conditions described elsewhere (Belloch et al. 2000). PCR products were cleaned using the AccuPrep PCR purification kit (Bioneer, Inc., Alameda, USA) 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 (Applied Biosystems).

In the case of the S. cerevisiae intraspecific variability study, sequences from CAT8 and GAL4 genes described in Peris et al. (2012) were included in the subsequent analyses. In the case of S. uvarum, the eight partial gene sequences from strains included in Pérez-Través et al. (2014), as well as from those isolated from Patagonian Araucaria araucana samples (Rodriguez et al. 2014) were also included in the analyses. The final sequences obtained in this study were submitted to Genbank under accession numbers KP874957 to KP875229 and K024782 to K024801.

Additionally, homologous sequences from S. uvarum CBS 7001, S. pastorianus Weihenstephan 34/70, S. bayanus NBRC 1948 and S. cerevisiae S288c strains were used for comparative purposes or as references.

Each set of homologous sequences was aligned with the ClustalW program (Thompson, Higgins and Gibson 1994). Individual gene (MNL1 and COX2 partial genes) trees were obtained using the neighbor-joining method, according to the number of differences. Tree reliability was assessed using nonparametric bootstrap resampling of 1000 replicates.

Partial nuclear gene sequence alignments obtained for each species were concatenated to perform neighbor-net network analyses, which consider reticulate evolution, with the program SPLITSTREE4 (Huson and Bryant 2006). Due to the presence of introgressions from S. cerevisiae to several S. uvarum in MNL1, this gene was removed from the analysis.

**Saccharomyces uvarum and Saccharomyces cerevisiae population structure analyses**

We used the individual-based Bayesian clustering methods implemented in STRUCTURE 2.3.4 (Pritchard, Stephens and Donnelly 2000) to investigate population subdivisions with admixture in S. uvarum and in S. cerevisiae, with the concatenated sequence alignments. STRUCTURE is based on the use of Markov chain Monte Carlo (MCMC) simulations to infer the assignment of genotypes into K distinct clusters (populations). For both species, 20 independent analyses were carried out for each number of clusters K (2 ≤ K ≤ 8), with 500 000 MCMC iterations after a burn-in of 50 000 steps. We determined the amount of additional information explained by increasing K using the ΔK statistic (Evanno, Regnaut and Goudet 2005) with the program STRUCTURE HARVESTER version 0.6.94 (Earl and vonHoldt 2012). For both species, K = 2 was the most suitable according to this ΔK statistic.

**RESULTS**

**General composition of yeast biota in chichas**

A low diversity of yeast species was found in the traditional apple fermentations evaluated in this work (Fig. 2). Two fermentations were allowed to ferment in the laboratory from musts obtained in situ (Pucon and Villarrica regions, Chile). Yeast samples from initial, middle and end stages were obtained for
further yeast isolation and identification by PCR-RFLP and sequencing analyses. Main differences in the yeast biota present in these two chichas were observed at the initial stages of fermentation; while chicha A was dominated by Saccharomyces cerevisiae, in chicha B the most frequent species was Hanseniaspora uvarum (Fig. 2). Middle and end stages of the two fermentations were dominated completely by S. cerevisiae. Interestingly, the fermentative cryotolerant species S. uvarum was recovered from the end stage of fermentation in chicha B (34% of the isolates) together with S. cerevisiae (66%). An additional yeast diversity analysis was carried out from three different chichas (chichas C, D and E) traditionally fermented in the regions of Pucón, Villarrica and Tirúa (Chile). In these cases, we only analyzed the end stages of fermentations, establishing an almost absolute dominance of S. uvarum (Fig. 2). This coexistence of S. cerevisiae and S. uvarum in this traditional fermented product led us to investigate the intraspecific diversity among our isolates.

All S. cerevisiae (82) and S. uvarum (48) isolates obtained from the five analyzed fermentations were subjected to an mtDNA-RFLP analysis in order to rapidly evaluate their molecular variability. Ten and 16 different mtDNA-RFLP patterns were detected among the S. cerevisiae (patterns C-I to C-X) and S. uvarum (patterns U-I to U-XVI), respectively (Table 1). Among S. cerevisiae, pattern C-I was dominant during the complete fermentation of chicha A and it was also recovered as a minority pattern in the initial stage of chicha B. The dominant pattern among the S. cerevisiae isolates in chicha B was designated as C-VIII. This pattern was the same as exhibited by a commercial baker (CB) yeast strain used as control (data not shown).

The dominant S. uvarum pattern in chichas B, C and D was the pattern U-I (Table 1). This pattern was also detected in a minor proportion in chicha E. This phenomenon is relevant due to the fact that three out of four fermentations came from different sampling areas (Fig. 1). Particularly, chicha E showed a codominance of different mtDNA-RFLP patterns instead of a unique predominant pattern.

As a part of the S. uvarum strain characterization, we carried out a PCR-RFLP analysis of the 33 gene regions proposed by Pérez-Través et al. (2014) to evaluate the presence of introgressions or chimeric chromosomes in the S. uvarum strains obtained in this work. In this case, strains representatives of each different mtDNA-RFLP pattern were analyzed by this methodology and the obtained patterns for each gene were compared to those obtained by Pérez-Través et al. (2014) for the complete panel of S. uvarum and some hybrid strains and to those obtained by Rodríguez et al. (2014) for the S. uvarum from Araucaria araucana. A high homogeneity was observed among the analyzed strains, being the allele U1 (present in the reference strain CBS 7001) the allele most frequently found in our strains independently from the analyzed gene region (Table 2). Alternative U2 alleles were only found for MNT2, RRI2, GSY1 and BRES genes, but only one of them (GSY1; 770 pb with Hae III and

### Table 1. Number (%) of isolates of S. cerevisiae and S. uvarum belonging to each different mtDNA-RFLP pattern (strain) detected in the analyzed beverages.

<table>
<thead>
<tr>
<th>STRAIN CHICHA A</th>
<th>CHICHA B</th>
<th>CHICHA C</th>
<th>CHICHA D</th>
<th>CHICHA E</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>mtDNA-RFLP pattern</strong></td>
<td><strong>NPCC</strong></td>
<td><strong>I</strong></td>
<td><strong>M</strong></td>
<td><strong>E</strong></td>
</tr>
<tr>
<td>C-I 1303</td>
<td>I 11 (68)</td>
<td>M 16 (84)</td>
<td>E 9 (65)</td>
<td>I 2 (17)</td>
</tr>
<tr>
<td>C-II 1304</td>
<td>I 1 (8)</td>
<td>M -</td>
<td>E 2 (14)</td>
<td>-</td>
</tr>
<tr>
<td>C-III 1305</td>
<td>I 1 (8)</td>
<td>M 2 (11)</td>
<td>E 1 (7)</td>
<td>-</td>
</tr>
<tr>
<td>C-IV 1306</td>
<td>-</td>
<td>M 1 (5)</td>
<td>E -</td>
<td>-</td>
</tr>
<tr>
<td>C-V 1325</td>
<td>-</td>
<td>M -</td>
<td>E 1 (7)</td>
<td>-</td>
</tr>
<tr>
<td>C-VI 1308</td>
<td>-</td>
<td>M -</td>
<td>E 1 (7)</td>
<td>-</td>
</tr>
<tr>
<td>C-VII 1310</td>
<td>I 2 (16)</td>
<td>M -</td>
<td>E -</td>
<td>-</td>
</tr>
<tr>
<td>C-VIII 1307</td>
<td>-</td>
<td>M -</td>
<td>E 10 (83)</td>
<td>I 11 (100)</td>
</tr>
<tr>
<td>C-IX 1312</td>
<td>-</td>
<td>M -</td>
<td>E -</td>
<td>-</td>
</tr>
<tr>
<td>C-X 1313</td>
<td>-</td>
<td>M -</td>
<td>E -</td>
<td>-</td>
</tr>
<tr>
<td>U-I 1309</td>
<td>-</td>
<td>M -</td>
<td>E 4 (34)</td>
<td>I 12 (50)</td>
</tr>
<tr>
<td>U-II 1311</td>
<td>-</td>
<td>M -</td>
<td>E 5 (21)</td>
<td>-</td>
</tr>
<tr>
<td>U-III 1323</td>
<td>-</td>
<td>M -</td>
<td>E 1 (4)</td>
<td>-</td>
</tr>
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<td>M -</td>
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<td>-</td>
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<td>U-V 1328</td>
<td>-</td>
<td>M -</td>
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</tr>
<tr>
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<td>-</td>
<td>M -</td>
<td>E -</td>
<td>-</td>
</tr>
<tr>
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<td>-</td>
<td>M -</td>
<td>E -</td>
<td>-</td>
</tr>
<tr>
<td>U-X 1315</td>
<td>-</td>
<td>M -</td>
<td>E -</td>
<td>-</td>
</tr>
<tr>
<td>U-XI 1316</td>
<td>-</td>
<td>M -</td>
<td>E -</td>
<td>-</td>
</tr>
<tr>
<td>U-XII 1318</td>
<td>-</td>
<td>M -</td>
<td>E -</td>
<td>-</td>
</tr>
<tr>
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<td>-</td>
<td>M -</td>
<td>E -</td>
<td>-</td>
</tr>
<tr>
<td>U-XIV 1320</td>
<td>-</td>
<td>M -</td>
<td>E -</td>
<td>-</td>
</tr>
<tr>
<td>U-XV 1321</td>
<td>-</td>
<td>M -</td>
<td>E -</td>
<td>-</td>
</tr>
<tr>
<td>U-XVI 1322</td>
<td>-</td>
<td>M -</td>
<td>E -</td>
<td>-</td>
</tr>
</tbody>
</table>

| Total | 13 (100) | 19 (100) | 14 (100) | 12 (100) | 11 (100) | 12 (100) | 24 (100) | 7 (100) | 16 (100) |

- NPCC: North Patagonian Culture Collection, Neuquén, Argentina; strain selected as a representative of the mtDNA-RFLP pattern. Strains identified as S. cerevisiae are indicated in regular letters and strains identified as S. uvarum are indicated in **bold** letters. I: initial stage of fermentation. M: middle stage of fermentation. E: end stage of fermentation.
Table 2. Alleles composition of S. uvarum indigenous strains based in PCR-RFLP restriction patterns. Chromosome order is based on the S. uvarum genome considering the translocations with regard to S. cerevisiae.

<table>
<thead>
<tr>
<th>S. uvarum Chr.</th>
<th>Gene</th>
<th>NPCC S. uvarum strains (mtDNA pattern)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1309 (U-I)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1317 (U-IX)</td>
</tr>
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<td></td>
<td></td>
<td>1315 (U-X)</td>
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<td></td>
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<td></td>
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<td></td>
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<td></td>
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<td>1324 (U-IV)</td>
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<td></td>
<td></td>
<td>1328 (U-V)</td>
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<tr>
<td></td>
<td></td>
<td>1321 (U-XV)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

|               |        | 770pb with Eco RI) had been detected neither in European (Pérez-Través et al. 2014) nor in South American (Rodríguez et al. 2014) strains of this species. In particular, the U2 allele found in RRI2 gene in most strains from chichas was detected only in European strains.

Phylogeny analysis and population structure of Saccharomyces cerevisiae

In order to evaluate the origin of the S. cerevisiae isolates obtained from the analyzed chichas, the partial sequences of CAT8 and GAL4 nuclear genes were obtained for an isolate representative of each mtDNA-RFLP pattern (10 strains in total). Both CAT8 and GAL4 genes sequences have been previously demonstrated to be useful for S. cerevisiae phylogeny studies (Peris et al. 2012). The sequences obtained were compared with those reported by Peris et al. (2012) for 84 yeast strains belonging to S. cerevisiae species isolated from multiple substrates and countries around the world, including strains from wines elaborated in Argentina and Chile (Supplementary Material Table S1). The neighbor-net phylogenetic analysis carried out with the concatenated sequences of the two genes analyzed clearly differentiated two clusters of strains. One cluster grouped a high number of strains including all wine/European isolates (cluster in white in Fig. 3A), and it contains all S. cerevisiae strains from chichas, and the other cluster corresponded to no-wine strains (cluster
Figure 3. (A) Phylogenetic neighbor-net network and (B) population structure analysis of *S. cerevisiae* strains used in this work, reconstructed from concatenated partial sequences of *CAT8* and *GAL4* nuclear genes. In population structure analysis, the most consistent number of genetic clusters/populations was $K = 2$. Each bar represents a particular strain. Colors in the groups in A and colors in the bars in B represent the population membership: wine/European (white) or non-wine (gray). The presence of several colors in the same bar (strain) in B suggests admixture.
in gray in Fig. 3A). These results demonstrated that S. cerevisiae strains from chichas were related to S. cerevisiae wine strains isolated from different origins including Chilean and Argentinean winemaking environments (Fig. 3A). In fact, the alleles most frequently detected in chichas for the two genes were also the alleles most frequently found among wine strains (Fig. 3A, cluster H1). The NPCC 1307 strain (pattern C-VIII), which showed the same mtDNA-RFLP pattern detected in the CB yeast, conforms an independent group, showing no relationship to the remaining S. cerevisiae strains isolated from chichas (Fig. 3A).

Population structure inference carried out in STRUCTURE also demonstrated the presence of two clusters with different genetic background (K = 2) in our sampling, with all strains from chichas included in the same wine/European cluster (Fig. 3B). Although more related to the wine/European group than to the non-wine group, particular strains from chichas named NPCC 1306 and NPCC 1307 (associated with the CB yeast strain) are an evidence of admixture between the two populations according to this analysis (bars in two colors in Fig. 3B).

**Phylogeny analysis and population structure of Saccharomyces uvarum**

Six S. uvarum strains isolated from chichas elaborated in different regions were selected to carry out a phylogenetic analysis: NPCC 1309 and 1323 strains from Villarrica, NPCC 1317 and 1314 strains from Pucón and NPCC 1322 and 1321 strains from Tirua (strains showing different mtDNA-RFLP pattern were selected from a same region). The objective of this analysis was to evaluate the potential origin (Holartic or South American) of the strains present in this beverage. For this purpose, partial sequences of eight nuclear genes (BRE5, CAT8, CYC3, CYR1, EG2, GAL4, MET6 and MNL1) resulting in a total of 3.75 Kbp and the COX2 mitochondrial gene were obtained for each strain and were compared. We also obtained sequences for the same nine genes from the complete panel of 26 S. uvarum strains studied by Pérez-Través et al. (2014), most of them isolated from wine or other fermentative sources in Europe, and from five S. uvarum strains isolated from A. araucana trees in a previous work carried out in our laboratory (Rodríguez et al. 2014).

Phylogenetic analysis carried out with MNL1 partial gene sequences evidenced a low diversity among S. uvarum strains (Fig. 4A). All S. uvarum strains from chichas showed a certain degree of relationship with European strains of this species, although three well different situations were observed: (i) NPCC 1314 strain evidenced the same allele found in the reference S. uvarum CBS 7001; (ii) NPCC 1317, 1321, 1322 and 1323 strains shared a same allele variant also present in another set of wine/European strains; and (iii) the NPCC 1309 strain evidenced a S. cerevisiae allele (introgression) for MNL1 partial gene and grouped with the S4, S10 and S14 strains isolated from Irish ciders (Fig. 4A). All strains from A. araucana showed differential alleles for this gene, different from those exhibited by strains isolated from both chichas and wine/European origin (Fig. 4A).

COX2 mitochondrial gene phylogenetic analysis evidenced a similar phenomenon observed for MNL1 (Fig. 4B). NPCC 1317, 1321, 1322 and 1323 strains were phylogenetically related to the S14, S10, S4 and CBS 2986 strains obtained from Ireland and Switzerland, and NPCC 1314 strain showed again the same allele evidenced in the reference CBS 7001 (Fig. 4B). NPCC 1309 strain from chicha as well as most strains from A. araucana showed unique and differential alleles (Fig. 4B).

Finally, a neighbor-net analysis was carried out with the concatenated sequences of all nuclear genes evaluated. MNL1 partial gene sequences were not included in this study because of the presence of S. cerevisiae introgressions observed in different strains (Fig. 4A). This analysis clearly evidences that S. uvarum NPCC 1288, 1298, 1290 and 1293 strains from A. araucana are separated from the rest of strains (Fig. 5A). The remaining NPCC 1289 strain from natural habitats and all strains from chichas were located together in the same group of European strains of this species (Fig. 5A). As it was evidenced for S. cerevisiae, population structure analysis carried out with all S. uvarum strains confirmed the presence of the two well-differentiated populations (K = 2) as well as evidence of admixture in two strains from chichas (NPCC 1314 and 1317 strains) and one strain from A. araucana (NPCC 1289) (Fig. 5B).

**DISCUSSION**

The well-known yeast species Saccharomyces cerevisiae has been largely associated with human history. Due to its importance for the elaboration of bread, beer and wine, a high number of strains of this species have been isolated and deposited in diverse culture collections around the world (Liti et al. 2009). A significantly lower amount of strains has been recovered from non-human-related environments; in fact, the natural niche of S. cerevisiae is still a matter of contention (Wang et al. 2012; Eberlein, Leducq and Landry 2015). In a recent study carried out by Almeida et al. (2015), which enlarged the collection of natural strains of this species, the authors proposed that oaks from the Mediterranean region of Europe harbor the wild genetic stock of domesticated wine yeasts.

In a recent phylogenetic study carried out by multilocus sequence analysis of more than 250 S. cerevisiae yeast isolates from wine (Europe, South America and South Africa) and non-wine origins (wild, brewing, cider, sake and traditional beverage fermentations mainly from Latin America, Africa and Asia), we demonstrated the existence of two groups of alleles (Arias 2008; Peris et al. 2012). A group present only in strains isolated from non-wine sources (non-wine alleles), and another group of alleles typically present in wine strains (wine alleles). The last group corresponded to the wine/European lineage observed by Liti et al. (2009) using complete genome sequences, while the non-wine alleles corresponded to the non-wine lineages according to Liti et al. (2009). According to Peris et al. (2012), S. cerevisiae wine yeasts isolated from North Patagonian winemaking environment were included in a wine/European lineage. This discovery, together with historical evidence that both grapes and winemaking were introduced to America during the 16th century during the European colonization (Pretorius, 2000), supports the hypothesis that Patagonian wine S. cerevisiae strains arrived to the New World by means of this human activity. This hypothesis is also supported by the lack of natural strains of this species in Patagonia, where only the cryotolerant species S. eubayanus and S. uvarum were detected (Libkind et al. 2011; Rodríguez et al. 2014).

Regarding the presence of S. cerevisiae in the traditional apple chichas analyzed in this study, both neighbor-net and population structure analyses, together with data obtained from individual genes (both the MNL1 nuclear gene and the COX2 mitochondrial gene) phylogenetic analysis, demonstrated that all strains obtained also belonged to the big group of wine/European strains.

As it was previously observed for Ñudai, another traditional fermented beverage elaborated by Mapuche communities from Araucaria araucana seeds, the presence of a CB yeast strain
among the *S. cerevisiae* populations was also confirmed in one apple chicha in this work. This phenomenon was detected by both mtDNA-RFLP and genes sequences analyses. The same mtDNA-RFLP pattern observed in NPCC 1307 strain (pattern C-VIII), the major pattern in chicha B, was detected in Mudai fermentations and in the CB yeast strain (Rodríguez et al. 2014). Concatenated sequences analysis of CAT8 and GAL4 genes evidenced the divergence between NPCC 1307 strain and the remaining strains from chichas. Although the traditional production of fermented beverages like chichas (made from different raw materials including cereals or fruits) does not involve the use of commercial yeasts, its use in bread making by people from the Mapuche communities (Pardo and Pizarro 2005) is the principal focus for the cross-contamination in this traditional fermented products. Contrarily to that observed in Mudai, where the CB yeasts dominated the fermentations, their presence in chichas was limited to chicha B, evidencing a higher diversity of naturally occurring *S. cerevisiae* strains (nine different mtDNA-RFLP patterns). This observation is consistent with the elaboration procedure of the two beverages: while Mudai is performed using seed must, boiled to eliminate all natural microbiota present in the seeds surfaces, apple chicha is made by natural fermentation of apple juice containing the complete yeast communities present on fruit surfaces.

The putative origin of the wine/European *S. cerevisiae* strains detected in apple chichas in South America could be elucidated by analyzing the characteristics of the other *Saccharomyces* species detected in our fermentations, *S. uvarum*. This species has been isolated from both natural habitats and anthropic environments. It was associated with natural sources in Europe (Naumov et al. 2011), North America (Sampaio and Gonçalves 2008; Almeida et al. 2014), South America (Libkind et al. 2011; Almeida et al. 2014; Rodríguez et al. 2014) and Australia (Almeida et al. 2014). Among man-made environments, *S. uvarum* has been typically associated with some wine and cider fermentations from Europe, usually conducted at low temperatures (Naumov et al. 2000; Sipiczki 2002; Demuyter et al. 2004; Coton et al. 2006; Suárez Valles et al. 2007). While in natural habitats *S. uvarum* coexists with *S. eubayanus* (Libkind et al. 2011; Almeida et al. 2014; Rodríguez et al. 2014), in fermented beverages it generally forms mixed cultures with *S. cerevisiae* (Sipiczki 2002). This mixed culture of *S. cerevisiae* and *S. uvarum* was...
observed in the apple chichas evaluated in this work; chicha A was dominated by *S. cerevisiae*, and chichas B, C, D and E evidenced the presence of *S. uvarum* in different proportions. This cryotolerant species was the dominant yeast in some cases, evidencing the existence of different strains during the fermentations as suggested by mtDNA-RFLP analysis.

This work is the first in South America reporting the presence of *S. uvarum* in a fermented beverage, although the previous finding of this species in natural environments in the same region already suggested its putative participation (Libkind et al. 2011; Rodríguez et al. 2014). The detection of this species in apple chichas elaborated in diverse regions and the presence of different strains of this species in each fermentation (as it was observed in *chichas* C, D and E by means of mtDNA-RFLP analysis) are clear evidence of its relevance in the analyzed fermentations. The origin of the strains of this species detected in this South American traditional fermentation is an interesting matter of study.

As previously mentioned, both the diversity and domestication of *S. cerevisiae* have been studied in several studies during the last years; however, only a few researches have addressed the diversity of *S. uvarum* and only one reported data about the potential domestication of this species (Almeida et al. 2014). According to a broad phylogenetic study carried out by Almeida et al. (2014) on *S. uvarum* strains, obtained from diverse geographic origins and habitats, three main clades were clearly distinguished. Clade A contained Holartic strains and a few strains from South America (named SA-A), clade B phylogenetically related to clade A and composed of strains from South America (named SA-B) and a distant clade C corresponding to the Australasian population. In order to elucidate the location of the *S. uvarum* strains involved in the apple *chichas* in a particular clade, our strains were genetically compared with strains isolated from natural habitats in Patagonia (Rodríguez et al. 2014) and a complete set of *S. uvarum* strains mainly obtained from European fermentations (Pérez-Traves et al. 2014). Neighbor-net and population structure analyses evidenced two populations among *S. uvarum* strains employed in our study. As a general rule, most strains from *chichas* (NPCC 1309, 1321, 1322 and 1323) are located in the Holartic clade, while most strains from natural habitats from Patagonia (NPCC 1288, 1290, 1293 and 1298 strains) are located in the South American clade, apparently in lineage SA-B. On the basis of our analysis and the population structure proposed by Almeida et al. (2014), one *S. uvarum* strain isolated from *A. araucana* (NPCC 1289) must be part of the lineage SA-A in the Holartic clade. In the same sense, NPCC 1314 and 1317 strains from chicha also evidenced an admixture of both pure Holartic and pure South American (SA-B) populations, and must be included in lineage SA-A.

According to Almeida et al. (2014), SA-A lineage of *S. uvarum* is geographically restricted to the northern part of Patagonia, whereas the lineage SA-B is distributed along the whole Andean region of Patagonia including the Austral island of Tierra del Fuego. The authors also observed admixture between both SA-A and SA-B populations in overlapping areas and suggest a correlation between this fact and the distribution of different *Nothofagus* tree species along the Andes. However, our results suggest an alternative scenario, which could be in part explained by analyzing the history of this traditional beverage, the apple chicha, in Patagonia.

The history of apple domestication has just begun to be unveiled; genetic studies have demonstrated the Central Asian
origin of the wild species Malus sieversii (Cornille et al. 2012). During late Neolithic or early Bronze Age, travelers introduced Asian wild apples through the Danube to Europe. This fact evidenced a large secondary genetic contribution to the European wild species M. sylvestris (Cornille et al. 2012). Colonists introduced apples to America in the 16th to 17th centuries, and this culture was particularly important in USA, Mexico, Chile and Argentina. As previously mentioned, Mapuche people exploited the fruits of apple trees introduced by the Spanish citizens in the Chilean side of the Andes to produce the apple chicha. These aboriginal communities propagated this culture in the Argentine side of the Andes during their travels (Bandieri 2005). The land extension occupied by apple trees in Patagonia was so wide that this region was even called ‘el país de las manzanas’ (the country of apples). The apple trees propagated by the Mapuche communities survived and reproduced naturally in the North region of Andean Patagonia sharing habitat with other native tree species including those in the Nothofagus genus, and the gymnosperm A. araucana. Calvo et al. (2010) evidenced the existence of different feral apple trees populations and a strong human influence in their geographical distribution.

Altogether, the results obtained in our work and previous studies suggest that the lineage SA-A in the South American population of S. uvarum, detected in both natural habitats and apple chichas, could be the result of the genetic flow between the naturally present populations of this species in Patagonia before the introduction of apple cultures by Spanish citizens and Mapuche people. These admixture populations are now found in both natural (Nothofagus spp and A. araucana trees) and antropic (chichas) environments; however, pure South American populations of this species are found only in natural habitats and Holartic strains are found only in chichas. Whether the genetic interchange occurs in natural habitats or it happens during chichas elaboration is still a matter of discussion.

On the other hand, representatives of S. cerevisiae were not able to grow and reproduce in the very extreme climatic conditions of Patagonia, being only detected in chichas but not in natural habitats even when using selective isolation strategies at 30°C (Libkind et al. 2011; Rodríguez et al. 2014; Peris et al. 2014, 2016).

Summarizing, we proposed a first colonization of Holartic S. uvarum strains during the development of the Mapuche communities after first Spanish invasions and a second relatively recent colonization with bakery strains of S. cerevisiae caused by the introduction of commercial dry yeasts.

SUPPLEMENTARY DATA
Supplementary data are available at FEMSyr online.

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