FEMS Yeast Research, 17, 2017, fow109

doi: 10.1093/femsyr/fow109
Advance Access Publication Date: 23 December 2016
Research Article

RESEARCH ARTICLE

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.

Editor: Isak Pretorius

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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

Received: 24 August 2016; **Accepted:** 20 December 2016
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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 Bío-Bío river. This region was later abandoned by the Spaniards during the Araucarian wars, and kept under Mapuche domination until the 19th century (Cruz 2010). Mapuche people exploited the fruits of these feral apple trees (manshanás-aliwen, in Mapuche language) as food but also to produce apple *chicha*, a fresh low alcoholic beverage.

Although the microbiota 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 only 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: Tirúa (38°20′14.40″S, 73°29′46.66″O), Villarrica (39°16′47.44″S, 72°13′50.81″O) and Pucón (39°16′05.33″S, 71°58′42.94″O) (Fig. 1).

Figure 1. Origin of the *chichas* evaluated in this work.

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 GPY 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

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(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 ProgeneThermocycler (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* 700I, *Cfo I*, *Dde*I, *Eco* RI, *Hae* III, *Hind* III, *Hinf* I, *Msp* I,*Pst* I, *Rsa* I, *Sac* I, *Scr* 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* strains, eight nuclear gene regions including *BRE5*, *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 (Rodríguez *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 KR024782 to KR024801.

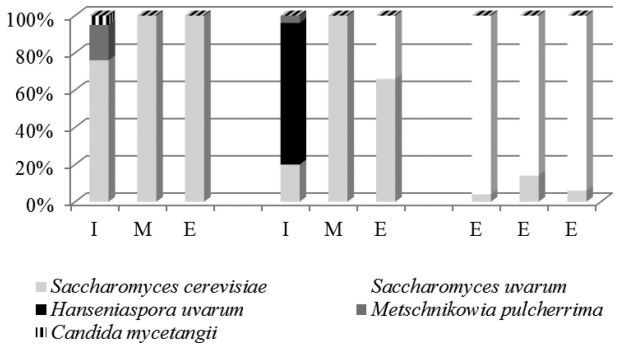

Figure 2. Distribution of yeasts species detected in the analyzed fermentations. Capital letters on the bottom of the bars indicate the fermentation stage: I, initial; M, middle; e, end. Capital letters on top of the bars indicate the corresponding *chicha*: A and B, *chichas* obtained with musts that were allowed to ferment in the laboratory; C, D, E: *chichas* obtained from traditionally fermented musts.

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 ($2 \leq K \leq 8$), 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

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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, evidencing 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 *BRE5* 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.

STRAIN			CHICHA A			CHICHA B			CHICHA C	CHICHA D	CHICHA E
N°	mtDNA-RFLP pattern	NPCC[a]	I	M	E	I	M	E	E	E	E
1	C-I	1303	11 (68)	16 (84)	9 (65)	2 (17)	–	–	–	–	–
2	C-II	1304	1 (8)	–	2 (14)	–	–	–	–	–	–
3	C-III	1305	1 (8)	2 (11)	1 (7)	–	–	–	–	–	–
4	C-IV	1306	–	1 (5)	–	–	–	–	–	–	–
5	C-V	1325	–	–	1 (7)	–	–	–	–	–	–
6	C-VI	1308	–	–	1 (7)	–	–	–	–	–	1 (6)
7	C-VII	1310	2 (16)	–	–	–	–	–	–	–	–
8	C-VIII	1307	–	–	–	10 (83)	11 (100)	8 (66)	–	–	–
9	C-IX	1312	–	–	–	–	–	–	1 (4)	–	–
10	C-X	1313	–	–	–	–	–	–	–	1 (14)	–
11	**U-I**	**1309**	–	–	–	–	–	**4 (34)**	**12 (50)**	**4 (58)**	**1 (6)**
12	**U-II**	**1311**	–	–	–	–	–	–	**5 (21)**	–	–
13	**U-III**	**1323**	–	–	–	–	–	–	**1 (4)**	–	–
14	**U-IV**	**1324**	–	–	–	–	–	–	**2 (8)**	–	–
15	**U-V**	**1328**	–	–	–	–	–	–	**1 (4)**	–	–
16	**U-VI**	**1329**	–	–	–	–	–	–	**1 (4)**	–	–
17	**U-VII**	**1330**	–	–	–	–	–	–	**1 (4)**	–	–
18	**U-VIII**	**1314**	–	–	–	–	–	–	–	**1 (14)**	–
19	**U-IX**	**1317**	–	–	–	–	–	–	–	**1 (14)**	–
20	**U-X**	**1315**	–	–	–	–	–	–	–	–	**3 (19)**
21	**U-XI**	**1316**	–	–	–	–	–	–	–	–	**1 (6)**
22	**U-XII**	**1318**	–	–	–	–	–	–	–	–	**2 (13)**
23	**U-XIII**	**1319**	–	–	–	–	–	–	–	–	**3 (19)**
24	**U-XIV**	**1320**	–	–	–	–	–	–	–	–	**1 (6)**
25	**U-XV**	**1321**	–	–	–	–	–	–	–	–	**1 (6)**
26	**U-XVI**	**1322**	–	–	–	–	–	–	–	–	**3 (19)**
	Total		**13 (100)**	**19 (100)**	**14 (100)**	**12 (100)**	**11 (100)**	**12 (100)**	**24 (100)**	**7 (100)**	**16 100)**

[a]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.

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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*.

		NPCC *S. uvarum* strains (mtDNA pattern)						
S. uvarum Chr.	Gene	**1309 (U-I)**	**1317 (U-IX)**	**1315 (U-X)**	**1316 (U-XI)**	**1318 (U-XII)**	**1319 (U-XIII)**	**1320 (U-XIV) 1323 (U-III) 1329 (U-VI) 1322 (U-XVI) 1314 (U-VIII) 1330 (U-VII) 1311 (U-II) 1324 (U-IV) 1328 (U-V) 1321 (U-XV)**
I	*BUD14*	U1	U1	U1	U1	U1	U1	U1
	CYC3	U1	U1	U1	U1	U1	U1	U1
III	*KIN82*	U1	U1	U1	U1	U1	U1	U1
	MRC1	U1	U1	U1	U1	U1	U1	U1
V	*MET6*	U1	U1	U1	U1	U1	U1	U1
	NPR2	U1	U1	U1	U1	U1	U1	U1
VII	*KEL2*	U1	U1	U1	U1	U1	U1	U1
	MNT2	U1	**U2**	**U2**	**U2**	**U2**	**U2**	U1
IX	*DAL1*	U1	U1	U1	U1	U1	U1	U1
	UBP7	U1	U1	U1	U1	U1	U1	U1
XI	*BAS1*	U1	U1	U1	U1	U1	U1	U1
	CBT1	U1	U1	U1	U1	U1	U1	U1
XII	*MAG2*	U1	U1	U1	U1	U1	U1	U1
	PPR1	U1	U1	U1	U1	U1	U1	U1
XIII	*CAT8*	U1	U1	U1	U1	U1	U1	U1
	ORC1	U1	U1	U1	U1	U1	U1	U1
XVI	*GAL4*	U1	U1	U1	U1	U1	U1	U1
	JIP5	U1	U1	U1	U1	U1	U1	U1
VIIItXV	*CBP2*	U1	U1	U1	U1	U1	U1	U1
	ATF1	U1	U1	U1	U1	U1	U1	U1
XVtVIII	*RRI2*	**U2**	**U2**	**U2**	U1	**U2**	**U2**	U1
VItX	*EPL1*	U1	U1	U1	U1	U1	U1	U1
	GSY1	U1	**U2**	U1	U1	U1	**U2**	**U2**
	PEX2	-	-	-	-	-	-	-
XtVI	*CYR1*	U1	U1	U1	U1	U1	U1	U1
XIVtIItIV	*EUG1*	U1	U1	U1	U1	U1	U1	U1
	BRE5	U1	U1	U1	U1	**U2**	**U2**	U1
IVtIItII	*PKC1*	U1	U1	U1	U1	U1	U1	U1
	RPN4	U1	U1	U1	U1	U1	U1	U1
	UGA3	U1	U1	U1	U1	U1	U1	U1
IItIItXIV	*APM3*	U1	U1	U1	U1	U1	U1	U1
	OPY1	U1	U1	U1	U1	U1	U1	U1
	EGT2	U1	U1	U1	U1	U1	U1	U1

U1: PCR-RFLP pattern detected in the reference strain *S. uvarum* MCYC 623.
U2 without gray shading: alternative PCR-RFLP patterns not reported in the reference strain *S. uvarum* MCYC 623, but detected in other *S. uvarum* strains (Pérez-Través *et al.* 2014).
U2 with gray shading: alternative allele reported for the first time in this work.
–: unamplified.

770 pb 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 phylogenic 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

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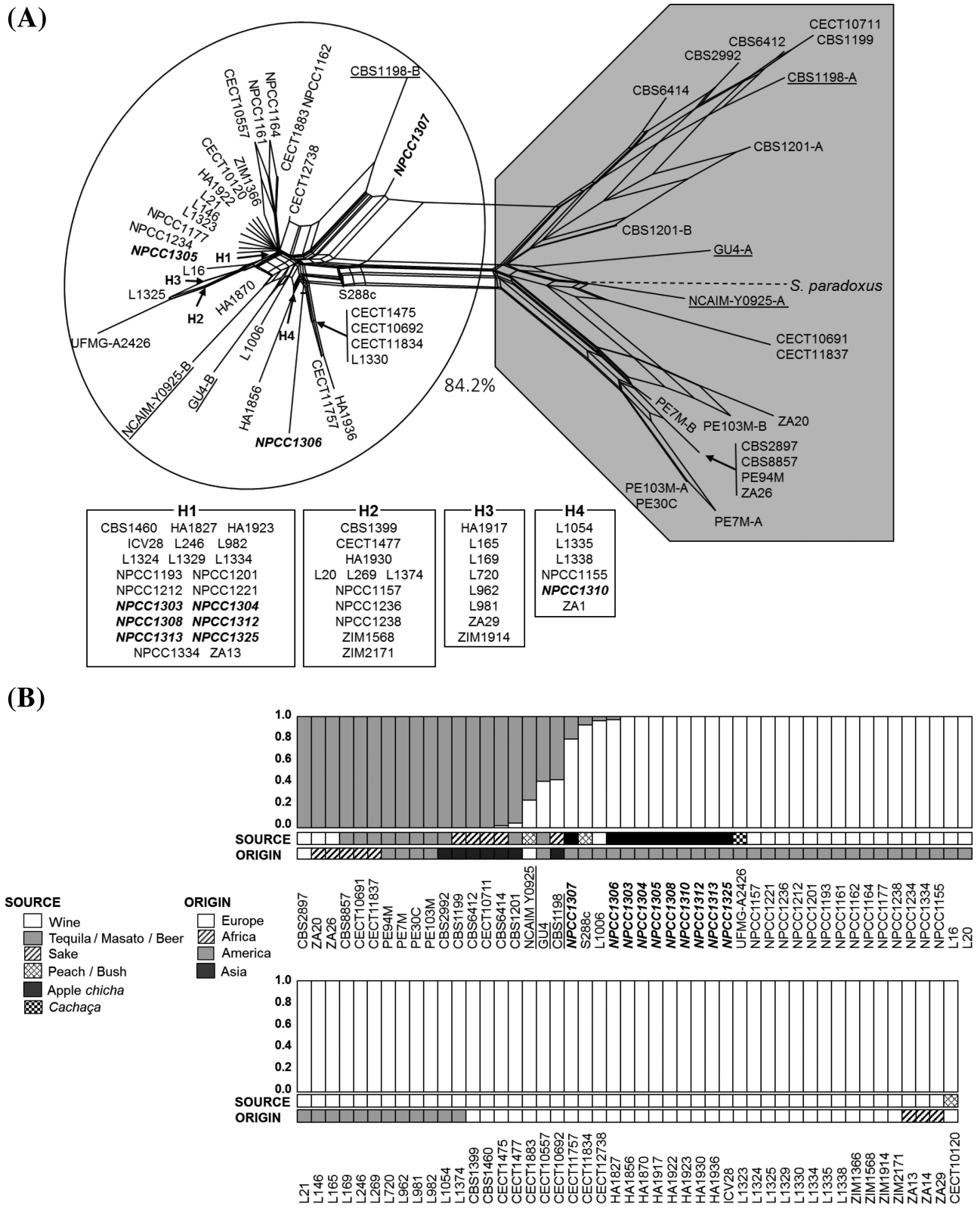

Figure 3. (**A**) Phylogenetic neighbor-net network and (**B**) population structure analysis of *S. cerevisiae* strains used in this work, reconstructed from concatenated partial sequences of *CAT8* and *GAL4* nuclear genes. In population structure analysis, the most consistent number of genetic clusters/populations 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.

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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*, *EGT2*, *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 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 linage 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 *Mudai*, another traditional fermented beverage elaborated by Mapuche communities from *Araucaria araucana* seeds, the presence of a CB yeast strain

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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 Goncalves 2008; Almeida *et al.* 2014), South America (Libkind *et al.* 2011; Almeida *et al.* 2014; Rodríguez *et al.* 2014) and Australasia (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

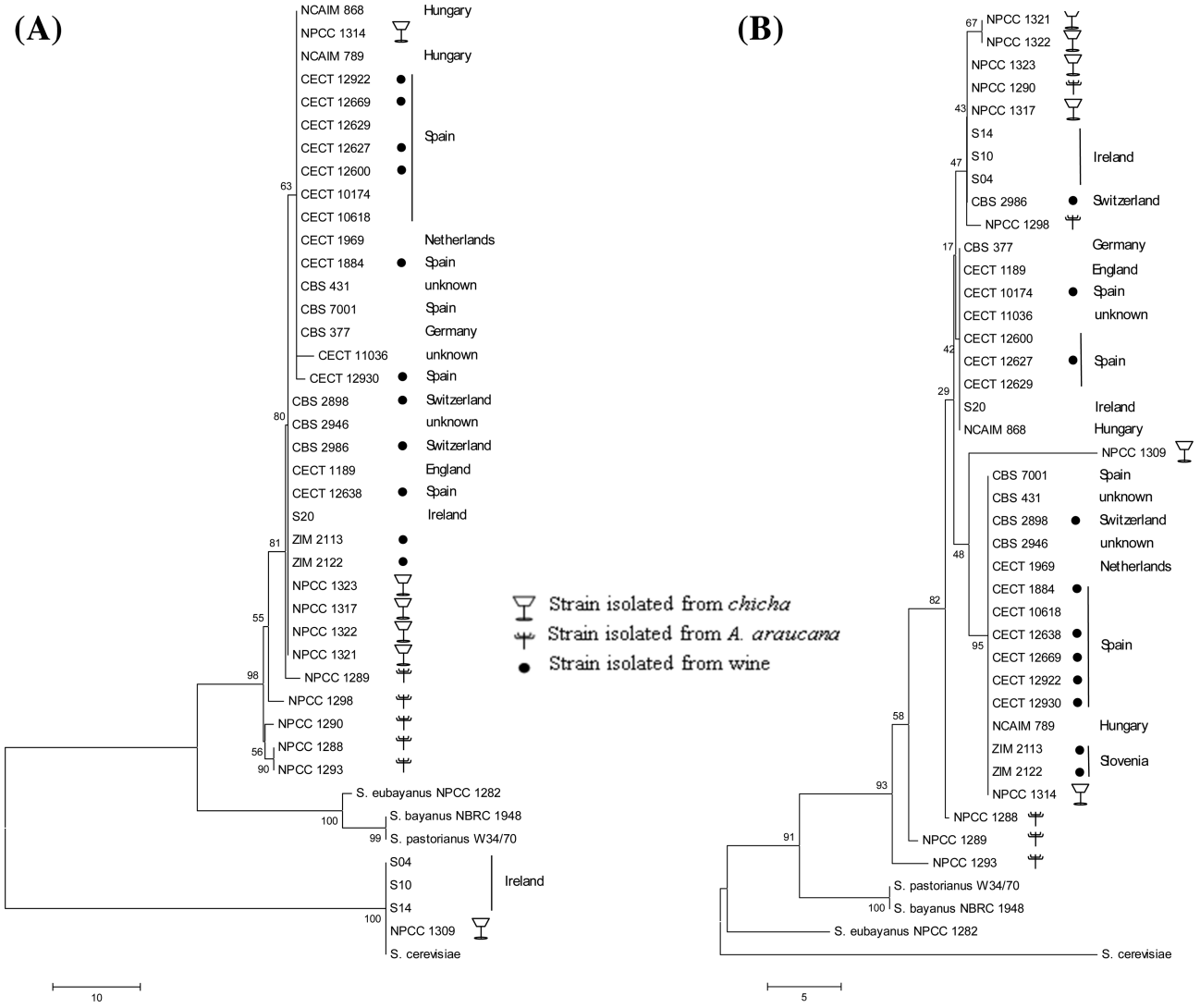

Figure 4. Neighbor-joining trees obtained with partial sequences of the genes MNL1 (**A**) and COX2 (**B**) from all *S. uvarum* strains included in this study. Numbers at the nodes correspond to bootstrap values based on 1000 pseudoreplicates. The scale is given in nucleotide substitutions per site.

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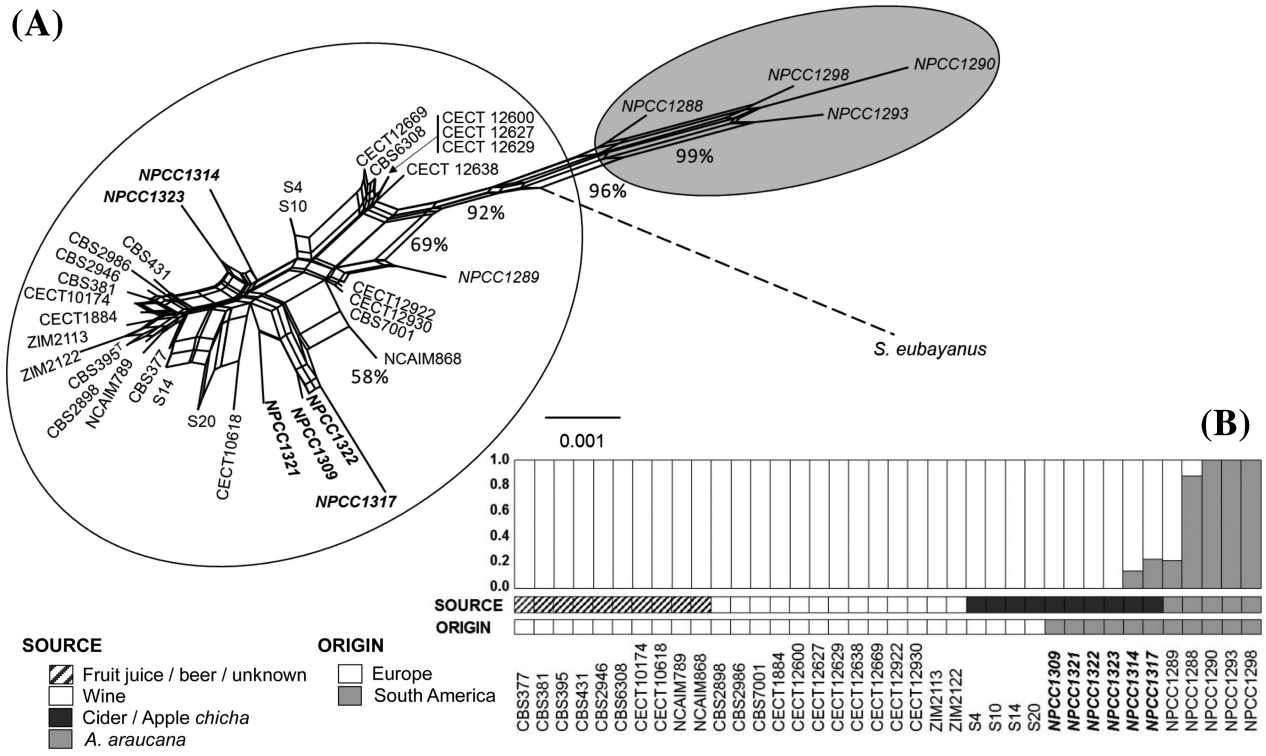

Figure 5. (**A**) Phylogenetic neighbor-net network and (**B**) population structure analysis of *S. uvarum* strains used in this work, reconstructed from concatenated partial sequences of *BRE5*, *CAT8*, *CYC3*, *CYR1*, *EGT2*, *GAL4* and *MET6* nuclear genes. In population structure analysis, the most 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: Holartic (white) or South American (gray). The presence of several colors in the same bar (strain) in B suggests admixture.

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-Través *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 scene, 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

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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 Argentinean 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.

ACKNOWLEDGEMENTS

We thank Olga Apablaza and Jimena Soleño for their help during samples collection. We are also grateful to Mapuche families for kindly providing *chicha* samples and to the Spanish Type Culture Collection (CECT), University of Valencia and CSIC, for the online access to the yeast identification database (http://www.yeast-id.org).

FUNDING

This work was supported by grants PICT 2011-1738 from the National Agency for Scientific and Technical Promotion and PI04-173 from National University of Comahue to C.L., as well as by grants AGL2012-39937-C02-01 and 02 to A.Q. and E.B., respectively, from the Spanish Ministry of Economy and Competitiveness.

Conflict of interest. None declared.

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