environmental microbiology
environmental microbiology reports

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Journal:	*Environmental Microbiology and Environmental Microbiology Reports*
Manuscript ID	EMI-2018-0850
Journal:	Environmental Microbiology
Manuscript Type:	EMI - Research article
Date Submitted by the Author:	29-May-2018
Complete List of Authors:	Libkind, Diego Eizaguirre, Juan
Keywords:	Yeast, phylogeography, microbe domestication, beer, population genetics

Phylogeography of the wild Lager-brewing ancestor (*Saccharomyces eubayanus*) in Patagonia

Juan I. Eizaguirre[1], David Peris[2,3], María E. Rodríguez[4], Christian A. Lopes[4], Patricio De Los Ríos[5], Chris Todd Hittinger[2], Diego Libkind[1*].

[1]Laboratorio de Microbiología Aplicada, Biotecnología y Bioinformática de Levaduras, Instituto Andino Patagónico de Tecnologías Biológicas y Geoambientales (IPATEC), Universidad Nacional del Comahue, CONICET, CRUB, Quintral 1250 San Carlos de Bariloche, 8400 Río Negro, ARGENTINA

[2]Laboratory of Genetics, Genome Center of Wisconsin, DOE Great Lakes Bioenergy Research Center, Wisconsin Energy Institute, J. F. Crow Institute for the Study of Evolution, University of Wisconsin-Madison, Madison, WI 53706, USA.

[3]Department of Food Biotechnology, Institute of Agrochemistry and Food Technology (IATA), Spanish National Research Council (CSIC), Valencia, Spain

[4]Instituto de Investigación y Desarrollo en Ingeniería de Procesos, Biotecnología y Energías Alternativas (PROBIEN, CONICET-UNCo), Neuquén, ARGENTINA.

[5]Lab. Ecología Aplicada y Biodiversidad, Univ. Católica de Chile, Temuco, CHILE.

*Correspondence: Diego Libkind

Quintral 1250, San Carlos de Bariloche, Río Negro C.P. 8400, Argentina.

Tel: +54 2944 428505 INT.:102

Fax: +54 2944 428505

diego.libkind@gmail.com, libkindfd@comahue-conicet.gob.ar

Running title: *Saccharomyces eubayanus* in Patagonia

Original Significance Statement

The discovery of the missing close relative of Lager-brewing yeasts, *Saccharomyces eubayanus*, solved a mystery that had puzzled scientists and brewers for decades, and has created a unique opportunity for studying microbe domestication processes and for the breeding novel yeasts for the brewing industry. Through the processing of 400 samples and genetic characterization of more than 200 *S. eubayanus* we demostrated that Patagonia harbours a very high abundance and genetic diversity of this species. Five subpopulations were found in Patagonia and their phyleogeographic analyses suggest that ancient geological events helped shaping the observed population structure. The first large screening for fermentation properties in *S. eubayanus* was performed and revealed the most interesting strains for brewing.

Summary

Saccharomyces eubayanus is the close relative of the Lager-brewing yeast and was firstly found in North Patagonia associated with *Nothofagus* trees. In recent years additional strains were found in North America, Asia and New Zealand, and genomic analyses showed the existence of two main populations of this yeast, both of them present in Patagonia. Here we performed the most comprehensive study of *S. eubayanus* in Patagonia natural environments (400 samples) and confirmed that this region has the highest isolation success rate for this species described worldwide (more than 10 fold). The genetic characterization of 200 isolates (*COX2*, *DCR1*, *intFR*) revealed five geographically structured subpopulations. We hypothesized that marine ingressions and glaciations, which shaped the Patagonian landscape, contributed on

population differentiation. The first large screening of fermentation performance of 60 wild *S. eubayanus* strains indicated which subpopulations would be more suitable for beer production.

Introduction:

The discovery of the missing close relative of Lager-brewing yeasts, which are used for 94% of beer production worldwide, solved a mystery that had puzzled scientists and brewers for decades (Martini & Kurtzman, 1985; Kodama et al., 2005; Rainieri et al., 2006; Dunn & Sherlock, 2008; Nakao et al., 2009; Nguyen et al., 2011). Genome sequencing showed that a cryotolerant, fermentative yeast species, found in Andean Patagonia, had a DNA sequence identity of 99.56% with the previously "unknown" portion of the Lager yeast genome, and the new species was described as *Saccharomyces eubayanus* (Libkind et al., 2011). In this study, *S. eubayanus* was frequently found in association with *Nothofagus* spp. forests and the endemic parasitic fungi *Cyttaria* spp. This recently discovered species can ferment both glucose and maltose at low temperatures and produces flavor compounds of interest to the brewing industry (Libkind et al., 2011; Gibson et al., 2013; Hebly et al., 2015; Krogerus et al., 2015; 2016; 2017). As a result, many laboratories have created synthetic hybrids that mimic Lager yeasts to attempt to improve fermentation traits and create new beer flavors (Alexander et al., 2016; Hebly et al., 2015; Magalhães et al., 2017; Mertens et al., 2015; Krogerus et al., 2015; 2016). *S. eubayanus* has also been shown to be useful for the production of cider (Flores et al., 2017; Magalhães et al., 2017) and wine (Origone et al., 2017). So far, the only commercial product brewed with *S. eubayanus* is

H41 by Heineken®, which recently employed a Patagonian isolate to launch what they call a “Wild Lager”.

In recent years, new strains of *S. eubayanus* have been isolated from North America, Asia, and New Zealand, as well as Patagonia (Argentina) (Peris et al., 2014, 2016; Rodríguez et al., 2014; Bing et al., 2014; Gayevskiy et al., 2016), adding complexity to the phylogeography of this species. Population genomic studies have shown that two main populations with high genetic diversity exist in Patagonia (Patagonia A and Patagonia B/Holarctic, referred here as PA and PB/Hol, respectively), as well as two divergent subspecies from Asia (Bing et al., 2014; Peris et al., 2016). Within PB/Hol, there is a separate lineage that contains isolates from Tibet and North Carolina, which are the closest known wild relatives of Lager yeasts. Their DNA sequence identities compared to the *S. eubayanus* subgenomes of Lager yeasts are 99.82% and 99.72%, respectively (Bing et al., 2014; Peris et al., 2016). However, phylogenomic analyses suggest that none of the wild isolates of *S. eubayanus* are direct parents of the *S. eubayanus* donor to *S. pastorianus*; instead, different genetic loci have different ancestries (Peris et al., 2016).

To expand our knowledge of the genetic diversity and phylogeography of *S. eubayanus* in Patagonia, we extended our initial sampling in Andean Patagonia in terms of geography and tree species. A multilocus sequence typing approach was used to characterize the largest set of *S. eubayanus* isolates ever studied. We compared this population genetic data to comprehensive ecological data on the location and substrate of isolation. Finally, representative strains of different genetic populations were evaluated, for the first time, for their fermentation performance in brewer's wort.

Results and Discussion

S. eubayanus is well established in Patagonia

To expand our knowledge on the distribution of *S. eubayanus* in the Patagonian Andes, we significantly extended the study area by 4.9-fold in comparison to previous reports (Libkind et al., 2011, Rodríguez et al., 2014; Peris et al., 2014, 2016). Additionally, we studied samples collected from non-*Nothofagus* tree species, including both exotic and native trees.

Incubation at 30 ºC was applied mainly for Northern Patagonia samples (N=88), as well as a few Southern samples resulting in absence of yeast growth. Although previous studies in Patagonia (Libkind et al., 2011; Rodriguez et al., 2014) isolated a handful of *S. eubayanus* and *S. uvarum* strains at 30 ºC, the new samples did not yield any isolates. These results are consistent with the fact that *S. eubayanus* and *S. uvarum* are cryo-tolerant species that prefer cold habitats (Libkind et al., 2011). This part of the isolation protocol was discontinued, and the following discussions refer only to isolations at 10 ºC.

By employing a raffinose/ethanol selective protocol (Sampaio & Gonçalves, 2008), we found yeasts in all studied areas, ranging from 43% to 92% in Tierra del Fuego (Latitude 54) and Nahuel Huapi NP (Latitude 41), respectively. The low percentage of yeasts found in Tierra del Fuego samples could be due to the low temperatures recorded in this particular area (Mean Annual Temperature: 4.3 ºC). Rodriguez et al. (2014) obtained a yeast isolation percentage even lower than that reported here (25%), but their samples were from *Araucaria araucana*, whereas our samples were mainly from *Nothofagus*. *Saccharomyces* were present in all sampled

areas with a relative high incidence (average 54% of all samples per area), with values ranging from 38% to 75%. On average, these values are similar to those we previously reported in Patagonia (Libkind et a., 2011) using identical techniques, but they are higher than those found in oak forests in Europe (Koufopanow et al., 2006; Sampaio & Gonçalves, 2008), New Zealand (Zhang et al., 2010; Knight & Goddard, 2015), and North America (Sniegowski et al., 2002; Charron et al., 2014), all reporting values ranging from 7% to 33%. However, comparisons are difficult to perform given that, excepting the work of Sampaio & Gonçalves (2008), the methods used in the previous references for yeast isolation included incubation at higher temperatures and slightly different culture media. Non-*Saccharomyces* detected belonged mainly to the *Lachancea, Hanseniaspora*, and *Torulaspora* genera, which is similar to observed in bark samples from the Northern Hemisphere (Sampaio & Gonçalves, 2008; Charron et al., 2014; Sylvester et al., 2015). However, and unlike the previous reports, we found a recently described species, *Lachancea nothofagi* (Mestre et al, 2010), associated with *Nothofagus* spp., as well as two novel *Hanseniaspora* species that are currently being described formally.

Our Mini/Microsatellite PCR-fingerprinting method (MSP-PCR; Libkind et al., 2007b) allowed a rapid species assignment of a total of 206 isolates: 79 strains to *S. uvarum* and 124 to *S. eubayanus* (Fig. S1 and Libkind et al., 2011). However, it did not reveal considerable intraspecific differentiation within *S. eubayanus*, so additional molecular markers were selected for population genetic studies. *S. uvarum* and *S. eubayanus* were the only two *Saccharomyces* species found in the present study, which is in accordance with their previously reported prevalence and sympatry in Patagonia

due to ecological conditions (Libkind et al., 2011; Rodríguez et al., 2014). The low temperatures in Patagonia favor the growth of yeasts specially adapted to these climatic conditions, explaining why mesophyllic *Saccharomyces* spp. are scarce in Patagonia. The almost complete occupancy of the *Nothofagus* niche by cryotolerant species contrasts with most of other studies where *Saccharomyces* sympatric species tend to have different growth temperature preferences (Sampaio & Gonçalves, 2008; Naumov et al., 2013). As already reported (Libkind et al., 2011), the detection of a pair of cryotolerant species in Patagonia and the almost absence of thermotolerant species is unusual. Further studies focused on cold environments are required in order to determine if this is a unique characteristic of the Patagonian forest.

In general, there was no difference between Argentinean and Chilean samples in terms of the overall percentage of *Saccharomyces* (~ 54%; Table 1), but the presence of *S. eubayanus*, in Chilean samples (16%) was significantly lower than in Argentinean ones (over 33%) (Welch Two Sample t-test; p-value = 0.0007) The prevalence of *S. uvarum* over *S. eubayanus* in Chilean samples is likely because samples collected from this area were mostly associated with *N. dombeyi* trees, a host where *S. uvarum* is known to be more prevalent (Libkind et al., 2011). We previously reported that the relative proportion of *S. uvarum* and *S. eubayanus* varied markedly and depended on the tree species sampled by studying only three *Nothofagus* species (Libkind et al., 2011). These results were interpreted to suggest some degree of niche-partitioning and to explain partially the coexistence of these two sister species in Patagonia. Here by expanding our study to six *Nothofagus* trees and three other tree species, we confirmed that the deciduous trees *N. antarctica* and *N. pumilio* showed the highest rate of

isolation for *S. eubayanus* (60% and 38%, respectively; Table S1). Surprisingly, only 7% of the samples were positive for *S. eubayanus* for the other *Nothofagus* species (*N. betuloides*, *N. dombeyi*, *N. glauca*, and *N. obliqua*). For the first time, we also studied bark samples of exotic *Quercus* spp. in Patagonia, and we found higher rates of total *Saccharomyces,* as well as *S. eubayanus* isolates (76% and 71% respectively; Table S1), than in any other region of the world (Sampaio & Gonçalves, 2008; Zhang et al., 2010; Dashko et al., 2016).

(Table 1)

Among substrates studied, bark and soil samples yielded yeasts most often (> 70%), while leaves and *Cyttaria* spp. yielded yeasts least often (~ 50%) (Table S1). A small percentage of *Saccharomyces* (32%) were isolated from leaves, mainly from *N. pumilio* where 93% of isolates were *S. eubayanus*, further highlighting its preference for this tree species in comparison to *S. uvarum* (p-value = 0.0004). The rest of the sampled substrates showed no preference for which yeast species were isolated, including *Cyttaria* spp. (p-value = 0.4695), bark (p-value = 0.8469), and soil (p-value = 0.2447). We note that previous studies in the region, not based on selective enrichment methods such as that used here, were able to isolate *S. eubayanus* from water sources (Brandao et al., 2011), soil near *N. pumilio* and *N. antarctica* (Mestre et al., 2014), and in the phylloplane of *N. pumilio* leaves (Muñoz et al., 2013).

Based on our results and disregarding the isolation techniques used in each case, the Patagonian Andes region in Argentina seems to have a very high abundance of *S. eubayanus*. Specifically, our isolation success rate is between 20 to 200 times higher than other regions (Table 2) although slight differences in isolation protocols

might have affected this rate. In China, the isolation success rate of *S. eubayanus* was 2% of total samples (Bing et al., 2014); in New Zealand, it was 0.2% (Gayevskiy et al., 2016); while, in the USA and Canada, the percentage was about 0.6% (Peris et al., 2014; 2016; Sylvester et al., 2015). Thus, the Patagonian Andes is an interesting reservoir, ideal for studying the population genetics of *S. eubayanus*.

(Table 2)

Nuclear and mitochondrial markers support isolation by distance

The genetic structure of *S. eubayanus* in Patagonia was studied by sequencing highly variable genetic markers. Initially, *COX2* was used to infer mitochondrial inheritance, while the nuclear gene *DCR1* was selected because it had been proven useful in differentiating the two *S. eubayanus* populations (PA and PB/Hol) (Peris et al., 2014), the third marker, the highly variable intergenic region between *FAR8* and *RSF1* (hereafter *intFR*) (Bing et al., 2014), was also selected to examine subpopulation relationships more precisely.

A phylogenetic network was reconstructed employing *COX2* data, and 20 haplotypes (198 strains) were found (Figure 1A). This representation allowed us to better visualize reticulate evolution events. Unlike previous reports where two populations were detected using several nuclear genes and whole genome sequences (Peris et al, 2014; 2016), *COX2* analysis failed to reveal this structure on its own. Indeed, the mitochondrial *COX2* region had low genetic diversity compared to the nuclear regions, both in terms of the number of segregating sites and the nucleotide diversity (S_{COX2} = 16, π_{COX2} = 0.005; S_{DCR1} = 70, π_{DCR1} = 0.013; S_{intFR} = 31, π_{intFR} = 0.013; Table S2). However, the phylogenetic network showed that a group of mainly

Southern isolates clustered together and were genetically divergent (Figure 1A), suggesting isolation by distance, which was confirmed by the correlation between geographic and genetic distance ($r = 0.5$; $p = 0.0005$). One possible explanation for this observation may arise from a previous study of the genetic structure (also assessed using a mitochondrial marker) of the main Patagonian tree species with which *S. eubayanus* is associated, *N. pumilio* (Premoli et al., 2010). The authors argued that the eastern region of Tierra del Fuego (located along the Atlantic coast) might have acted as a refugium for *N. pumilio* during the last glaciation in Patagonia. As previously mentioned, we propose that *S. eubayanus* may have tracked its host within this refugium.

Moreover, seven *S. eubayanus* isolates with *S. uvarum* introgression in *COX2* (Figure 1A) were found in Southern Patagonia (Lake La Plata & Glaciares NP sampling sites), which add to a similar case found in the strain CRUB 1975 (yHCT105, from Puyehue) previously reported by Peris et al. (2014). Recombination between *Saccharomyces* species is common in the mitochondrial genome in both natural and industrial environments (Peris et al., 2017a, b). We investigated the available *COX2* sequences from *S. uvarum* strains to identify the source of the introgressed region but were unsuccessful because neither *S. uvarum* population varied in that specific mtDNA region (Figure S2). So far, this specific introgression (found in 7 isolates from only 3 different sampling sites) is the only evidence of reticulation between the two species in Patagonia. A similar, but not identical, introgression in this gene was found in *S. eubayanus* strains from Tibet (CDFM21L.1), from North Carolina (yHRVM108), and Lager-brewing yeasts (Peris et al., 2016, Figure S2). Future population genomic

232 analysis will shed light on the genome-wide prevalence of recombination between the
233 species. However, based on our present and previous results (Almeida et al., 2014;
234 Peris et al., 2014, 2016), recombination between *S. uvarum* and *S. eubayanus* in the
235 wild seems to be quite low, indicating that reproductive barriers limit gene flow between
236 the two species. These observations are consistent with previous data showing partial
237 genetic isolation through intrinsic postzygotic barriers and ecological prezygotic isolation
238 through tree species preference (Libkind et al., 2011; this study).

239 (Figure 1)

240 The second marker, the nuclear *Dicer* (*DCR1*) gene, partitioned the strains into
241 three clades: one containing the population PB/Hol (PB) and two others belonging to PA
242 (PA-1 and PA-2) (Figure S3, A), which is in agreement with MLST and genome
243 sequence data from a small set of strains (Peris et al., 2014; 2016). From a total of 208
244 isolates analyzed, only 29% belong to PA (23 haplotypes; 65 isolates), and these were
245 located exclusively in Northern Patagonia. The larger and more genetically variable
246 PB/Hol (42 haplotypes; 143 isolates) was distributed along the entire sampling area
247 (Figure 1B). PA was divided into two subpopulations: PA-1 was geographically
248 restricted to Nahuel Huapi ($p\text{-value} < 2.12 \times 10^{-9}$), while PA-2 was found between
249 Caviahue and Latitude 43 South. Unlike the other groups, PA-2 does not contain the
250 typical premature stop codon in *DCR1*, suggesting it could be functional in these
251 isolates (Figure S3, B). All isolates from Chile belonged to PB/Hol and formed a single
252 clade (Hap 38, 41, 42, and 43), except for isolates from Ñielol Hill (Hap 39). Lager
253 strains also clustered inside PB/Hol, forming a separate clade (Hap 44, 45, and 46).

The *intFR* nuclear marker was also able to distinguish PA and PB/Hol, and it allowed us to detect internal subgrouping through network analysis (Figure 2A). A total of 202 strains were sequenced and included 24 haplotypes. As with *DCR1*, PA was divided into PA-1 (two haplotypes), which was found near Bariloche City, while subpopulation PA-2 was found in Northern Patagonia (six haplotypes). PA-1 was recovered in association with only two tree species, the native *N. antarctica* and the exotic *Q. robur*, whereas the PA-2 group was found associated with five tree species, though mainly with *A. araucana* (73%) (Rodríguez et al., 2014). Surprisingly, only one isolate from *N. pumilio* belonged to PA, indicating a strong association between PB/Hol isolates and this tree species (p-value<1.02×10^{-7}). PB/Hol harbored considerable genetic diversity and a novel subgroup that could represent a third subpopulation (PB-3). We also recapitulated the two subgroups detected previously using a subset of the isolates (Peris et al., 2016). Specifically, five different haplotypes were distinguished in PB-1 (66 isolates), three haplotypes in PB-2 (28 isolates), and the new subpopulation PB-3 (37 isolates) had 4 haplotypes (Figure 2A; Table S1). PB-1 was the largest and had the broadest distribution throughout Andean Patagonia, while PB-2 and PB-3 were exclusively associated with the Northern (p-value<1.77×10^{-7}) and Southern (p-value<1.07×10^{-5}) Patagonia, respectively (Figure 2, B). For *intFR*, we also used sequences from Bing et al. (2014) and confirmed that Lager strains are phylogenetically closer to the Tibet strains than any of the Patagonian lineages at this locus. There were two strains, B27-1 and CR10-11, isolated from Southern Patagonia that could not be assigned to any of the subgroups of PB. The analysis of *intFR* region showed a clear structure at the subpopulation level, as indicated by low haplotype diversity and

nucleotide diversity. The genetic diversity found was higher than the genome-wide data for each population (π_A = 0.00721±0.00048 vs $\pi_{genomeA}$ = 0.00444±0.00070; π_B = 0.00622±0.00033 vs $\pi_{genomeB}$ = 0.00312±0.00030, Table S2; Peris et al., 2016), so we conclude that *intFR* performs well as an initial marker to identify new *S. eubayanus* isolates and sort them into candidate subpopulations.

(Figure 2)

Geography and ecology might explain population differentiation

To investigate potential factors explaining the population structure found in Patagonia for *S. eubayanus*, we incorporated both ecological and spatial variables into our database and analyses. Given the clear population differentiation around Latitude 43 (Figure 1B), this area was of special interest. Near that latitude, similar population distributions have been previously observed in the closest relative of *S. eubayanus*, *S. uvarum* (Almeida et al., 2014), as well as for plants, such as *Podocarpus nubigena* (Quiroga & Premoli, 2010), and multiple species of the genus *Nothofagus* (Mathiasen & Premoli, 2010), which are the main trees with which both *S. uvarum* and *S. eubayanus* are associated in Patagonia. The allopatric evolution and peculiar phylogeography of *Nothofagus* trees in this region was postulated to be the result of a combination of geographical barriers (at Latitude 43) caused by extensive marine inflows from the Pacific Ocean during the Miocene (Mathiasen & Premoli, 2010; Premoli et al., 2012) and multiple refugia generated by recent glaciations (Mathiasen & Premoli, 2010, Premoli et al., 2010; Premoli et al., 2012). Given the sympatric nature of both psycrophilic yeast species in association to Patagonian *Nothofagus* and the similar phylogeographic patterns shown by all of them, a yeast-tree co-evolutionary hypothesis

300 can be speculated where the same geological events shaped their population structure.
301 Extensive genomic and biogeographical studies are necessary to test this hypothesis.
302 To be noted, the PB/Hol successfully established itself along the Andean
303 Patagonia and across several continents (Bing et al., 2014; Gayevskiy et al., 2016;
304 Peris et al., 2014, 2016), but PA was restricted to North Patagonia. Since *S. eubayanus*
305 is of considerable technological importance for the brewing industry (Libkind et al.,
306 2011; Gibson et al., 2013; Hebly et al., 2015; Krogerus et al., 2015, 2016, 2017), we
307 performed a first fermentation performance assessment of a large set of wild strains.
308
309 **Fermentation performance of different *S. eubayanus* lineages**
310 The fermentation performance of *S. eubayanus* in wort has not yet been fully
311 studied; all previous studies have examined a single isolate, the type strain (Gibson et
312 al., 2013; Hebly et al., 2015; Krogerus et al., 2015, 2016, 2017). That same strain has
313 also been used to make several interspecific hybrids with *S. cerevisiae* (Alexander et
314 al., 2016; Hebly et al., 2015; Magalhães et al., 2017; Mertens et al., 2015; Krogerus et
315 al., 2015, 2016). To summarize these studies, the type strain showed faster growth than
316 most Lager yeasts in laboratory media containing glucose or maltose as a carbon
317 source at 10°C (Gibson et al., 2013), while at 20°C, it was still competitive (Walther et
318 al., 2014). The type strain displayed reduced growth at temperatures of 25°C or higher
319 (Walther et al., 2014; Mertens et al., 2015). Attenuation values (ca. 65%) in all the
320 experiments were low due to the inability to ferment one of the main sugars of beer
321 wort, maltotriose (Gibson et al., 2013; Krogerus et al., 2015, 2016; Mertens et al., 2015).

322 Alcohol values ranged between 5.0% and 5.7 % (Gibson et al., 2013; Krogerus et al.,
323 2016), and flocculation was generally low (Krogerus et al., 2015, 2016).

324 Here 60 isolates were selected based on genetic (*DCR1* haplotypes) and
325 geographic data and tested in micro-fermentations of a malt extract medium (Table S1).
326 The average attenuation for the species was 52% (compared with 85% for a typical
327 Frohberg Lager yeast) with a range from 36% to 70% (Figure 3A). Our results with the
328 type strain confirmed the results of previous studies (65% attenuation, Gibson et al.,
329 2013; Krogerus et al., 2016), and this value was among the highest found for the
330 species. In terms of populations, the highest average attenuation was observed for
331 PB/Hol, 53.8±7.5%, while the average for PA was significantly lower: 49.5±5.3% (p-
332 value<10^{-2}). This variable was correlated with total CO_2 production (Figure 3E). No
333 detectable differences were found in attenuation among subpopulations (Figures 3B; F).
334 The differences in attenuation and CO_2 production between PA and PB/Hol could be the
335 result of differential maltose fermentation capabilities, but further studies are needed to
336 test this hypothesis. The low attenuation observed in general for the species has been
337 proposed to be the result of the inability of *S. eubayanus* to ferment maltotriose (Gibson
338 et al., 2013; Hebly et al., 2015; Magalhães et al., 2016). Gibson et al. (2013) measured
339 the uptake of radiolabelled maltose (99%) and maltotriose (nd) for the *S. eubayanus*
340 type strain and found it to be similar to Lager yeasts of the Saaz group. Similar results
341 were obtained by other authors (Hebly et al., 2015; Magalhães et al., 2016). In Lager
342 yeasts (and some Ale strains), maltotriose assimilation occurs due to the presence of
343 genes encoding permeases, including *MTT1* and *AGT1,* which were thought to come
344 from the *S. eubayanus* parental strain (Cousseau et al., 2013). However, none of the

345 available *S. eubayanus* genomes contain *MTT1*, although deposited reads contain
346 sequences closely related to *AGT1* (Baker et al., 2015; Magalhães et al., 2016; Peris et
347 al., 2016). In any case, none of the tested strains, including those in the present work,
348 ferment maltotriose.

349 (Figure 3)

350 The fermentation rate was observed to be quite similar between PA and PB/Hol
351 (Figure 3C), although within PA, PA-1 was lower than PA-2 (not significantly different)
352 (Figure 3D). The latter subpopulation showed a higher average fermentation rate than
353 the other subpopulation, which is a relevant industrial trait, but almost all the isolates of
354 this group showed low attenuation. The strains with the highest fermentation rates were
355 CRUB 1946 and CRUB 2032, which belonged to PB-1 and PA-2 respectively. From the
356 first fermentation screening of a broad panel of natural isolates of *S. eubayanus* from
357 Andean Patagonia, we conclude that PB/Hol generally has the most potencial features
358 for brewing purposes.

359 **Conclusions**

360 This work represents the most comprehensive study of *Saccharomyces* spp. in
361 Patagonia, with 400 samples processed to isolate strains and 563 strains analyzed. We
362 obtained an average isolation rate of 31%, which is the highest isolation success rate
363 for *S. eubayanus* described in Patagonia (Libkind et al., 2011). We obtained more *S.*
364 *eubayanus* isolates than all isolates ever recorded worldwide (Peris et al., 2014, 2016;
365 Rodríguez et al., 2014; Bing et al., 2014; Gayevskiy et al., 2016). The use of *intFR* as
366 genetic marker led us to sort the isolates of *S. eubayanus* into five Patagonian

subpopulations, including one not previously detected. These subpopulations are geographically structured and supported by ecological data. PA was located in the Northern Patagonia, with PA-1 restricted to the Nahuel Huapi NP and PA-2 distributed from Latitude 43 to 37. Population PB/Hol was widely distributed along Andean Patagonia and was divided in three subpopulations; PB-1 was found from 54º to 37º and is the closest relative to the Holartic lineage that includes domesticated hybrid strains, while PB-2 and PB-3 were mainly located in Northern and Southern Patagonia, respectively. The patterns of diversification and differentiation between *S. eubayanus* populations are remarkably reminiscent of those described recently for its sister species, *S. uvarum* (Almeida et al., 2014), which strongly suggests that shared changes in Patagonian landscape, such as those produced by glaciations and marine introgressions from the Pacific Ocean, drive the observed population structures and biogeographies of these species. The abundance and diversity found in Patagonia, coupled with the recent evidence of a population expansion in China (Peris et al., 2016), support the hypothesis of a dispersion from South America to the Northern Hemisphere, although further investigation is needed. Recent phylogenomic studies using > 1000 *S. cerevisiae* isolates, suggest and Asian origin for the whole *Saccharomyces* species complex (Peter et al., 2018).

The first screen of native *S. eubayanus* fermentation potential in wort suggested that some subpopulations would be more suitable for beer production. These results are opening the door to future research on the use of *S. eubayanus* and its application in the brewing industry by creating new lager hybrids or by directly employing wild or improved *S. eubayanus* strains.

Experimental Procedures

Sampling areas

Sampling areas were located in the Andes region of Patagonia, specifically along the Andes Mountains in Argentina and in Araucanía (IX Region) in Chile. From 2010 to 2013, a total of 400 new samples were collected from different sources (bark, *Cyttaria* spp., leaves, and soil) associated with diverse tree species, mainly of the endemic genus *Nothofagus*. *Cyttaria* spp. stromata were harvested during the Southern Hemisphere summer between November and February. For bark, leaves, and soil, at least four independent samples were obtained from each sampling site. All samples were collected in sterile plastic bags and stored at 4ºC until processing following previously reported procedures (Libkind et al., 2007a; Sampaio & Gonçalves, 2008).

S. eubayanus isolation and identification

Saccharomyces isolations were performed using ethanol 8% v/v and raffinose (10 g/L) as the sole carbon sources, as previously described (Sampaio & Gonçalves, 2008; Libkind et al., 2011). Samples were mostly incubated at 10ºC; in some cases, incubation temperatures of 20ºC, 25ºC, and 30ºC, were also applied. Tubes showing turbidity, suggesting microbial growth, were processed. Purified isolates showing typical *Saccharomyces*-like ascospores were characterized by the Mini/Microsatellite PCR-fingerprinting method (MSP-PCR) using the $(GAC)_5$ primer (Libkind et al., 2007b). The sequence of the ITS region (Schoch et al., 2012) was obtained from strains that had atypical or ambiguous MSP-PCR results. The occurrence of yeasts in all samples was calculated (as percentages), as well as for the specific occurrence of *Saccharomyces*

yeasts and *S. eubayanus*. All yeast isolates were preserved at -80ºC in a glycerol solution (20% v/v).

Phylogenetic analyses

In addition to the newly isolated strains (N=144), *S. eubayanus* yeast strains isolated (N=75) in previous studies from different locations in Andean Patagonia were included in this paper (Libkind et al., 2011; Rodríguez et al., 2014). *S. eubayanus* strains isolated (N=21) from other parts of the world were included in the DNA sequence analyses (Bing et al., 2014; Gayevskiy et al., 2015; Peris et al., 2014, 2016).

The mitochondrial gene *COX2* (502 bp) (Peris et al., 2014; 2017a), the nuclear gene *DCR1* (859 bp) (Peris et al., 2014), and an intergenic region between the nuclear genes *FAR8* and *RSF1* (*intFR*; 547 bp) (Bing et al., 2014) were amplified and sequenced for phylogenetic studies. Sequences were aligned using ClustalW in MEGA 6.0 (Tamura et al., 2013) and corrected manually. Sequences were deposited in GenBank (Accessions MF479762 - MF479947; MF459967 - MF460120; MF460121 - MF460298; and MF416138 - MF416149).

Single-gene phylogenies (*COX2*, *DCR1*, *intFR*) were reconstructed using the Maximum Likelihood (ML) method in MEGA 6.0, as well as Bayesian Inference (BI) with BEAST v1.7.5 (Drummond et al., 2007). The best-fit model of evolution for each gene fragment in the data set was determined using the Akaike Information Criterion (AIC) in MEGA 6.0 (Tamura et al., 2013). A Phylogenetic Neighbor-Net network was reconstructed from each gene alignment using Network 4.613 with the Neighbor Joining method (Bandelt et al., 1999).

In order to focus on the phylogeography of *S. eubayanus* in Patagonia, phylogenetic network analysis of *COX2* gene sequences was performed in Network 4.613 (Bandelt et al., 1999) using only strains isolated in Patagonia (198 strains). A ML phylogenetic tree was constructed using the *DCR1* gene sequences of 211 global strains of *S. eubayanus* with *S. uvarum* (CBS7001) as the outgroup; Lager-brewing yeasts (CBS1503; SafLager-023, W34/70, WLP802, Wyeast2001) and brewing contaminant hybrid strains (CBS380, CBS424, CBS425, NBRC1948) were also included in the analysis. ML phylogenetic network analysis of *intFR* gene sequences of 242 *S. eubayanus* strains from Patagonia, North America, New Zealand, Tibet, and West China (Peris et al., 2014, 2016; Rodríguez et al., 2014; Gayevskiy et al., 2016; Bing et al., 2014) was performed with *S. uvarum* (CBS7001) as the outgroup.

Genetic and ecological diversity between populations

To study mechanisms of population differentiation, we generated a database (see Table S1) of isolates with the source coordinates, altitude, type of substrate, host tree, and other informative ecological parameters, such as precipitation, radiation, and mean temperatures extracted from WORLDCLIM database (Hijmans et al., 2005) by using DIVA-GIS (Hijmans et al., 2004). Mantel's test, implemented in the *vegan* package of R (Oksanen et al., 2013), was used to detect the correlation between genetic distances (Phi_{ST}), corrected by the Kimura 2-parameter model, and the geographic distance matrix generated using Geographic Distance Matrix Generator v1.2.3 (http://biodiversityinformatics.amnh.org/open_source/gdmg/index).

In addition, we tested for a correlation between genetic distances and ecological (altitude, mean temperature, precipitation, and UV radiation) distance matrixes. In these

458 analyses, we defined 20 distinct regions from this isolation data. DnaSP v5 (Librado et
459 al., 2009) was used to calculate genetic diversity statistics for each locus, such as
460 number of Haplotypes, Haplotype diversity (Hd), nucleotide diversity (π), and the
461 number of segregating sites (S). Similarly, we performed Tajima's D (Tajima, 1989) and
462 Fu's Fs (Fu, 1997) tests to detect selection or unusual demography.

463 **Micro-fermentation experiments**

464 Micro-fermentations were conducted in malt extract wort for 60 strains chosen to
465 maximize genetic diversity. The fermentation assay was carried out at 10ºC in 10mL in
466 wort with an Original Gravity of 12 ºBrix (125.8 g/L of sugar). Fermentation was
467 monitored by weight loss until a constant weight was reached (Lopes et al., 2007). We
468 used an inoculation rate commonly used for Lager-brewing yeasts: 1.5 x 10^7 cell/mL
469 (White & Zainasheff, 2010). Immediately after fermentations ended, yeast cells were
470 removed by centrifugation, and the Final Gravity was measured. Attenuation or sugar
471 consumption was calculated as the difference between the Original Gravity and Final
472 Gravity, as described by White & Zainasheff (2010).

473 The monitored mass loss was corrected to the percent of sugar consumed, as in
474 Pérez-Través et al. (2015). Curve fitting was carried out using the reparametrized
475 Gompertz equation proposed by Zwietering et al., 1900:

$$y = D * \exp\{-\exp[((\mu max * e)/D) * (\lambda - t) + 1]\}$$

476 where y is the % of consumed sugar; D is the maximum sugar consumption value
477 reached (the asymptotic maximum, %); μ_{max} is the maximum sugar consumption rate (h^{-1};
478 here fermentation rate), and λ is the lag phase period during which sugar
479 consumption was not observed (h). Data were fitted using the nonlinear regression

480 analysis of GraphPad Prism version 6.00 (GraphPad Software, La Jolla, CA, USA)
481 minimizing the sum of squares of the difference between experimental data and the
482 fitted model. In order to compare these variables between populations we performed a
483 Wilcoxon Test, and then we corrected for multiple testing with Bonferroni to compare
484 variables between subpopulations.
485
486 **Statistical analyses**
487 The ecological and kinetic parameters were analyzed using GraphPad Prism version
488 6.00 (GraphPad Software, La Jolla, CA, USA) to conduct nonparametric Wilcoxon tests
489 to compare means. Fisher's exact tests were performed in the R statistical package (R
490 Development Core Team).
491
492 **Acknowledgments**
493 This work was accomplished with financial aid from Agencia Nacional de
494 Promoción Científica y Tecnológica (PICT2014-3677, PICT2014-2542), Universidad
495 Nacional del Comahue (Project B199), and CONICET (International Bilateral
496 Cooperation CONICET–NSF andPIP392) granted to DL, as well as grants PICT 1198,
497 PIP 555, and PIN04-A128 to CL. This material is based upon work supported by the
498 National Science Foundation under Grant No. DEB-1253634 (CTH), the Robert Draper
499 Technology Innovation Fund from the Wisconsin Alumni Research Foundation (CTH),
500 and funded in part by the DOE Great Lakes Bioenergy Research Center (DOE Office of
501 Science BER DE-FC02-07ER64494 to Timothy J. Donohue). CTH is a Pew Scholar in
502 the Biomedical Sciences, supported by the Pew Charitable Trusts. DP is a Marie

Sklodowska-Curie fellow of the European Union's Horizon 2020 research and innovation programme, grant agreement No. 747775. We thank the authorities of Argentina National Park administration for providing permission for sample collection within National Parks; Miriam Gobbi, Hernan Pastoriza, and other colleagues from MABB for helping collecting samples in Patagonia; A. B. Hulfachor for editing.

Conflicts of Interest

The authors declare that they have no conflicts of interest with the content of this article.

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513

514

Area	Code	Nº of samples	% Yeast	% *Saccharomyces*	% *S. eubayanus*
Caviahue[†]	Cav	20	20	20	20
Lircay	Chi[&]	25	88	60	4
Nahuelbuta	Chi[&]	31	87	61	26
Ñielol Hill	Chi[&]	12	67	58	17
Lanín NP	Lan	64	66	42	22
Lanín NP*[†]	Lan	60	68	52	18
Nahuel Huapi NP	NH	40	92	75	52
Nahuel Huapi NP*	NH	83	90	64	43
El Bolsón	Bol	8	63	38	38
Alarces NP	Ale	31	61	61	32
La Plata Lake	LLP	31	65	55	45
Glaciares NP	Gla	70	81	61	43
Tierra del Fuego NP	TdF	88	43	40	26
TOTAL		**563 (400)**	**70 (69)**	**54 (54)**	**31 (31)**

Table 1. Distribution of samples per site and the percentages that yielded yeasts, *Saccharomyces* spp., and *S. eubayanus*. Data presented correspond only to samples incubated at 10 ºC. Data from previous studies in Patagonia: *Libkind et al., 2011; [†]Rodríguez et al., 2014. The numbers in parentheses indicate the total samples and percentages obtained specifically in this study. Chilean sampling areas are marked as Chi[&].

Region/Area	% of positive samples out of total analyzed	Isolates	References
Patagonia	31	>200	Libkind et al., 2011 Rodríguez et al., 2014 This work
USA/Canada	<1	10	Peris et al., 2014; 2016 Sylvester et al., 2015
China	2	10	Bing et al., 2014
New Zealand	0.2	1	Gayevskiy & Goddard, 2016

Table 2. *S. eubayanus* occurrence worldwide and comparison of sampling efforts and Nº of isolates.

523

Figure 1: A) Neighbor-Net Phylogenetic Network reconstructed from *COX2* sequences. Each circle corresponds to a unique haplotype, while circle size represents the number of individuals with that haplotype. Colors represent the region where each strain was isolated. 92% of strains below the red dotted line come from south of Latitude 43. The light blue rectangle marks the isolates with *S. uvarum* introgressions. Cav: Caviahue, Chi: Chile, Lan: Lanin, NH: Nahuel Huapi, Bol: Bolson, Ale: Alerces NP, LLP: La Plata Lake, Gla: Glaciares, TdF: Tierra del Fuego. B) Georeferenced isolations are shown in the map with corresponding colors for each region. The red dotted line indicates Latitude 43. Red and blue lines on the left represent the distribution of populations A and B, respectively.

524

Figure 2. A) Neighbor-Net Phylogenetic Network reconstructed from *intFR* sequences. Each circle corresponds to a unique haplotype, while the circle size represents the number of individuals in the haplotype. Colors represent the region where each strain was isolated; white and striped colors correspond to non-Patagonian strains. Each subpopulation is highlighted and circled in black. *S. uvarum* (CBS7001) was used as outgroup. B) Relative frequency of each subpopulation in each sampling area. Colors represent the regions where the strains were isolated. C) Population genetic summary statistics as extracted from DnaSP v5.10. The number of strains are in parentheses. bp: fragment length in base pairs. S: number of segregating sites. π: nucleotide diversity.

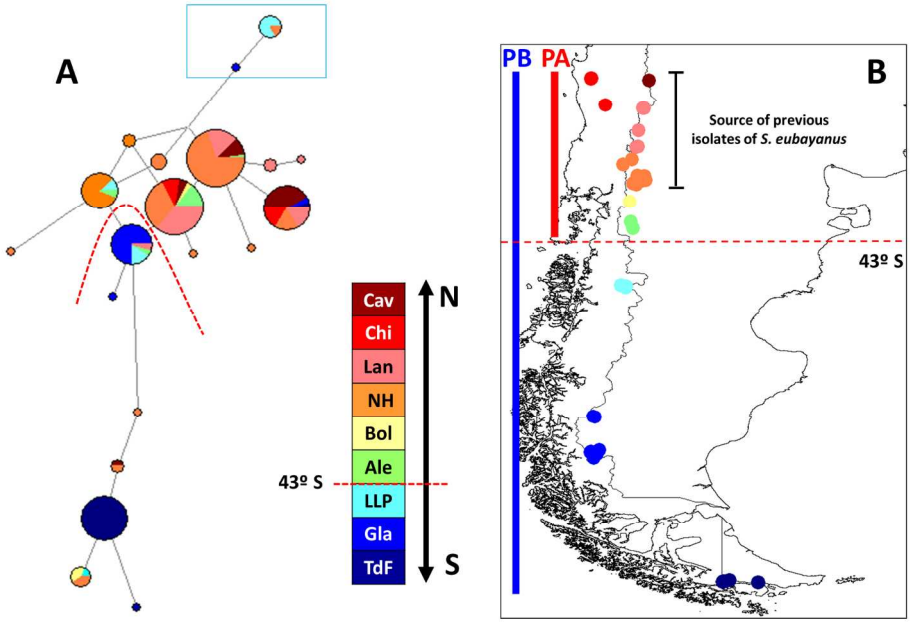
525

Figure 3. Box Plot showing fermentation parameters. Attenuation was measured refractometrically,

fermentation rate was calculated from micro-fermentations (see Methods), and the weight loss was measured periodically.

A, C, E) Differences between populations, Wilcoxon's Test. B, D, F) Differences between subpopulations, Wilcoxon's Test corrected by Bonferroni (p-value: *<0.05; **<10^{-2}; ***<10^{-3}; ****<10^{-4}). subP is an abbreviation for subpopulations.

526



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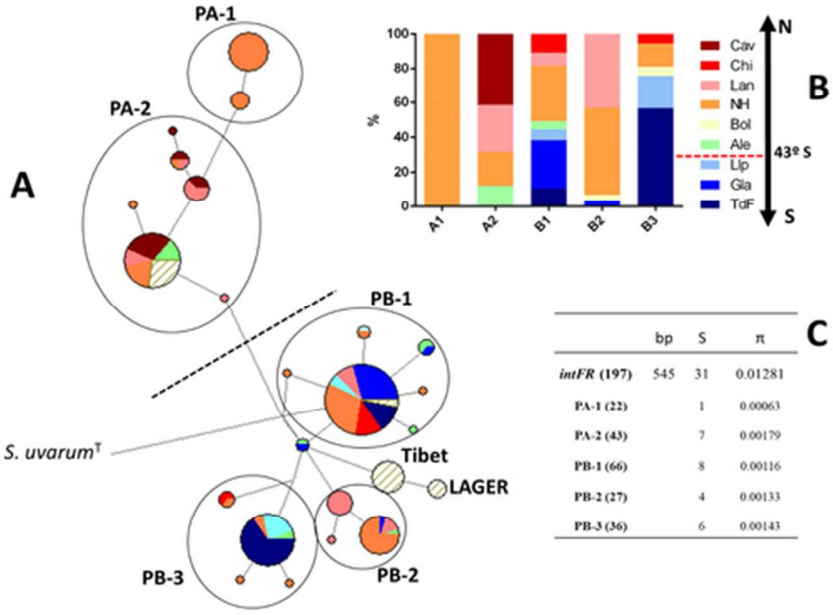
160x101mm (300 x 300 DPI)



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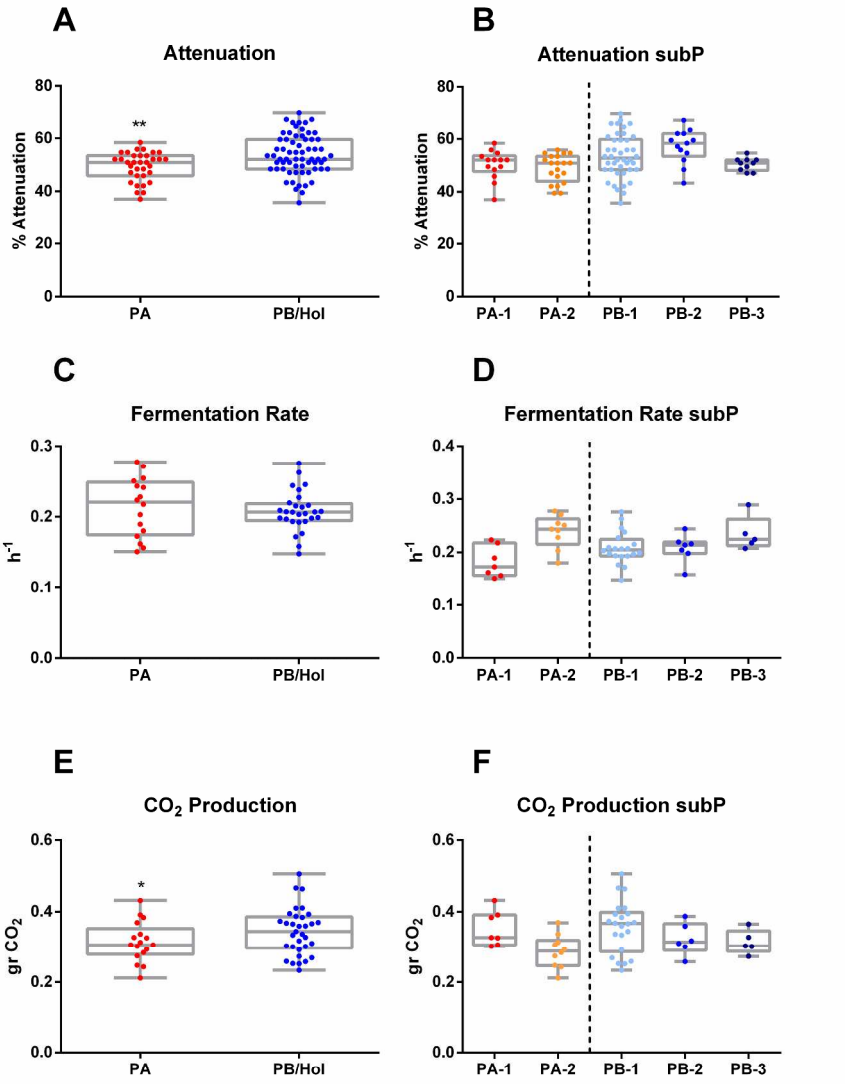
50x32mm (300 x 300 DPI)



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263x361mm (300 x 300 DPI)