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Pichia kudriavzevii as a representative yeast of North Patagonian winemaking terroir

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ABSTRACT

Terroir concept includes specific soil, topography, climate, landscape characteristics and biodiversity features. In reference to the last aspect, recent studies investigating the microbial biogeography (lately called 'microbial terroir') have revealed that different wine-growing regions maintain different microbial communities. The aim of the present work was to identify potential autochthonous fermentative yeasts isolated from native plants in North Patagonia, *Schinus johnstonii*, *Ephedra ochreata* and *Lycium chilense*, that could be associated to the specific vitivinicultural terroir of this region. Different *Pichia kudriavzevii* isolates were recovered from these plants and physiologically and genetically compared to regional wine isolates and foreign reference strains of the same species. All isolates were subjected to molecular characterization including mtDNA-RFLP, RAPD-PCR and sequence analysis. Both wine and native *P. kudriavzevii* isolates from Patagonia showed similar features, different from those showed by foreign strains, suggesting that this species could be part of a specific regional terroir from North Patagonia.

1. Introduction

Wine flavor is a consequence, to a considerable extent, of the vitivinicultural terroir. According to the OIV (International Organization of Vine and Wine, Resolution OIV/VITI 333/2010, 2010), 'vitivinicultural *terroir*' refers to an area in which collective knowledge of the interactions between the identifiable physical and biological environment and applied vitivinicultural practices develops, providing distinctive characteristics for the products originating from this area. *Terroir* concept includes specific soil, topography, climate, landscape characteristics and biodiversity features. In reference to the latter aspect, recent studies investigating the microbial biogeography (lately called 'microbial terroir') have revealed that different wine-growing regions maintain different microbial communities (Bokulich et al., 2014, Gilbert et al., 2014). Since 1866, when Louis Pasteur first elucidated the bioconversion of grape juice into wine, this complex biochemical process and the role of the yeast therein has been studied extensively. The yeasts, especially those belonging to *Saccharomyces* genus, are not only responsible for the bioconversion of grape sugars into alcohol and CO_2 but have also an equally important role in the formation of hundreds of secondary metabolites of importance to wine (Lambrechts and Pretorius, 2000). Nonetheless, several studies have pointed out that some non-*Saccharomyces* strains present additionally novel and interesting oenological properties that can positively influence the overall character of the wine. In fact, researchers and winemakers have nowadays recognized the importance of non-*Saccharomyces* yeasts in winemaking (Fleet, 2008; Ciani et al., 2010; Jolly et al., 2014). In this context, the continuous search for leading in the extremely competitive wine market led many research groups to explore the indigenous or autochthonous microbiota of each geographical region that could be used in starter cultures to obtain typical and distinctive wines.

The term 'autochthonous' yeast is a controversial concept among researchers; it is objectively assumed that an autochthonous yeast is that which should originate in a site and persist in it for a certain period of time (Vigentini et al., 2015). Nevertheless, this definition poses to the question if wine yeasts found in a particular wine region are in fact autochthonous (the so called native yeasts). A recent genetic study carried out with *Saccharomyces cerevisiae* strains obtained from North Patagonian wines spontaneously fermented, revealed very similar genetic features between Patagonian regional isolates and a set of European wine strains, casting doubt on the 'autochthonous' nature of our North Patagonian wine *S. cerevisiae* individuals (Peris et al., 2012). For this reason, we decided to expand sampling to recover indigenous yeasts from berries of endemic plants formerly used to manufacture fermented beverages: *Schinus johnstonii*, *Ephedra ochreata*, and *Lycium chilense* (commonly known as Molle, Solupe and Yaoyín, respectively) (Ladio, 2002; Rapoport et al., 2003).

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The aim of the present work was to identify potential autochthonous fermentative yeasts isolated from berries of the three native edible plants in North Patagonia that could be associated to the specific vitivinicultural terroir of this region. In this framework, the physiological and genetics features of *Pichia kudriavzevii* isolates recovered from these plants were compared with the same features from regional wine isolates and foreign strains of the same species. Both wine and native *P. kudriavzevii* isolates from Patagonia showed similar features, different from those showed by foreign strains, suggesting that this species could be part of a specific regional terroir from North Patagonia.

2. Materials and methods

2.1. Yeast strains

Ten *P. kudriavzevii* native strains previously isolated in our laboratory from different spontaneous wine fermentations (del Mónaco et al., 2014 and unpublished data) and ten *P. kudriavzevii* strains belonging to the ARS Culture Collection (NRRL) from different origins were analyzed. All strains are listed in Table 1.

2.2. Yeast isolation

Yeasts were obtained from fruits of three native edible plants from the North Patagonian region (latitude: 38°56′09.06″ S longitude: 68°03′36.87″ O altitude: 338 m, Neuquén Province, Argentina): *S. johnstonii*, *E. ochreata* and *L. chilense* commonly called as Molle, Solupe and Yaoyín, respectively. Sixty samples consisting in wild fruits from three different autochthonous plants from North Patagonia, Argentina (20 samples of ten *S. johnstonii* berries each, 20 samples of five *E. ochreata* berries each, and 20 samples of 15 *L. chilense* berries each) were used as source for yeast isolation. Number of berries in each sample was related to fruit size. Fruits were collected aseptically and introduced into sterile tubes containing 10 mL of a selective enrichment medium to isolate fermentative yeasts, consisting of yeast nitrogen base (YNB; Difco) supplemented with 1% *w*/*v* rafinose, 8% *v*/v ethanol and100 mg/mL chloramphenicol. This medium was proposed to be used for *Saccharomyces* isolation, due to its high ethanol concentration (Sampaio and Gonçalves, 2008). For each fruit, a sample was incubated at 30 °C for 7 days and the other at 10 °C for 30 days without shaking.

Table 1
P. kudriavzevii strains used in this study.

Strain	Isolation source	Geographic origin
Patagonian wine		
NPCC 1146	Wine	Argentina
NPCC 1265	Wine	Argentina
NPCC 1266	Wine	Argentina
NPCC 1267	Wine	Argentina
NPCC 1391	Wine	Argentina
NPCC 1392	Wine	Argentina
NPCC 1423	Wine	Argentina
NPCC 1424	Wine	Argentina
NPCC 1425	Wine	Argentina
NPCC 1426	Wine	Argentina
Reference		
NRRL Y-5396 (T)	Fruit, berries	Russia
NRRL Y-7179	Culture, sputum	Sri Lanka
NRRL Y-7724	Unknown	Finland
NRRL Y-420	Heart blood	unknown
NRRL Y-134	Sake	Japan
NRRL YB-236	Red raspberries	L. Wickerham, NRRL, USA
NRRL YB-238	Red raspberries	L. Wickerham, NRRL, USA
NRRL Y-7550	Beverages, tea, beer	Netherlands
NRRL Y-870	Champagne	Wallerstein Labs, USA
NRRL Y-27,196	Wine	Chile

NPCC, North Patagonian Culture Collection, Neuquén, Argentina; NRRL (ARS Culture Collection) Agricultural Research Service, USA.

Aliquots (100 μL) of each suspension were plated onto GPY (*w*/*v*: 2% glucose, 0.5% yeast extract, 0.5% peptone, 2% agar) supplemented with chloramphenicol 100 mg/mL and incubated at the same temperature until colonies developed. A representative number of yeast colonies was selected according to their frequencies and morphology, and microorganisms were preserved at −20 °C in glycerol solution (20% *v*/v).

2.3. Yeast identification

Yeasts were identified by restriction analysis (PCR-RFLP) of the region encompassing the ITS1, 5.8SrRNA and ITS2 (5.8S-ITS region) as described in Lopes *et al.* (2010). PCR-RFLP patterns obtained for each isolate were compared with those of reference strains available in the www.yeast-id.com database. Yeast identifications were confirmed by sequencing both the 5.8S-ITS region and the D1/D2 domain of the 26S rRNA gene (White et al., 1990; Kurtzman and Robnett, 2003). The sequences were edited and assembled using MEGA6™ software (Tamura et al., 2013) and then subjected to a GenBank BLASTN.

2.4. Molecular characterization of *P. kudriavzevii* strains

All *P. kudriavzevii* isolates obtained from the North Patagonian region (named in this work as natural isolates) as well as ten native wine strains isolated from different spontaneously fermented wines in regional cellars (wine isolates) and ten reference strains from different origins were subsequently subjected to molecular characterization including mtDNA-RFLP, RAPD-PCR and sequence analysis. The intraspecific characterization was performed using three experimental approaches described below.

2.4.1. Mitochondrial DNA restriction analysis (mtDNA-RFLP)

Fifteen microliters of the total DNA obtained as described by Querol et al. (1992) were digested with the endonucleases *Hinf*I (Roche Molecular Biochemicals, Germany) and *Dde*I (Promega Corp., USA) according to supplier instructions. Restriction fragments were separated on 1% *w*/*v* agarose gels in 1 × TAE buffer, gels were stained with a GelRed solution (Biotium, USA) and revealed under UV light. A 50-bp DNA ladder marker (Fermentas, Lituania) served as size standard.

2.4.2. Randomly amplified polymorphic DNA (RAPD) PCR analysis

The oligonucleotides OPA 9, OPA 12, OPA 14 and OPA 15 were used for RAPD-PCR analysis (Table 2). The PCR reaction mixture (20 μL final volume) contained: 3 μL DNA sample (150 ng), 1 × *Taq* polymerase buffer, 3.5 mM Cl_2Mg, 0.1 mM dNTP mix, 2.5 μM primer, 0.5 U Taq DNA polymerase (Promega, USA). Amplifications were carried out in a Progene Thermocycler (Techne, Cambridge, UK) as follows: initial denaturing at 94 °C for 5 min, 45 PCR cycles (denaturing at 94 °C for 1 min, annealing at 36 °C – for OPA 9 – or 32 °C – for OPA 12, 14 and 15 – for 1 min and extension at 72 °C for 2 min) and a final extension at 72 °C for 10 min. Amplified products were separated by electrophoresis in 2% *w*/*v* agarose gels in TAE buffer, stained with a GelRed solution and photographed under UV light. A 50-bp DNA ladder marker (Fermentas, Lituania) served as size standard. Amplified bands were scored as present (1) or absent (0) for each primer-strain combination. Data matrixes obtained were used for calculation of band profiles based on the Jaccard coefficient. Dendrograms were generated using an unweighted pair group with arithmetic average (UPGMA) clustering algorithm. Statistical analysis was performed using NTSYS 2.2 software (Exeter Software, NY, USA) (Rohlf, 2002) and Free Tree software for bootstrapping estimation (Hampl et al., 2001).

2.4.3. Sequencing and phylogenetic analysis of NMT1 and ADE2 nuclear genes

The nuclear genes *NMT1* (*N*-Myristoyltransferase, 738 bp) and *ADE2* (phosphoribosylamino-imidazole carboxylase, 600 bp) were amplified using the primers showed in Table 2 and PCR conditions described by

Table 2
Primer sequences used in this study.

Primer	Sequence (5′–3′)	Target region	
ITS 1	TCCGTAGGTGAACCTGCGG	ITS1–5.8S-ITS2 rDNA	White et al. (1990)
ITS 4	TCCTCCGCTTATTGATATGC	ITS1–5.8S-ITS2 rDNA	White et al. (1990)
NL-1	GCATATCAATAAGCGGAGGAAAAG	D1/D2-26S rDNA	Kurtzman and Robnett (2003)
NL-4	GGTCCGTGTTTCAAGACGG	D1/D2-26S rDNA	Kurtzman and Robnett (2003)
OPA 9	GGGTAACGCC	Genomic DNA	Sangorrín et al. (2013)
OPA 12	TCGGCGATAG	Genomic DNA	Sangorrín et al. (2013)
OPA 14	TCTGTGCTGG	Genomic DNA	Sangorrín et al., 2013
OPA 15	TTCCGAACCC	Genomic DNA	Sangorrín et al. (2013)
ADE2 fw	GTCACTTCTCAGTTTGAAGC	Genomic DNA	Jacobsen et al. (2007)
ADE2 rev	ACACCATCTAAAGTAGAGCC	Genomic DNA	Jacobsen et al. (2007)
NMT1 fw	CTGATGAAGAAATCACCG	Genomic DNA	Jacobsen et al. (2007)
NMT1 rev	GCTTGATATCATCTTTGTCC	Genomic DNA	Jacobsen et al. (2007)

Jacobsen et al. (2007). PCR products were purified using the AccuPrep PCR purification kit (Bioneer, Inc., USA) and sequences obtained using Macrogen Inc. Sequencing Service (Seoul, Korea). For phylogenetic analysis, each set of homologous sequences was aligned with the MEGA6™ software following CLUSTAL-W algorithm. Trees were obtained under the Neighbor-Joining method, according to the number of differences model. Tree reliability was assessed using nonparametric bootstrap resampling of 1000 replicates. Sequences from *C. krusei* (anamorph) homozygote alleles *NMT1* and *ADE2* were added to phylogenetic analysis for comparison purposes.

2.5. Enzyme screening methods

Qualitative assays of enzymatic activity detection were performed on solid media using young yeast cultures (24–48 h cultures in GPY).

2.5.1. Protease activity

Skim milk agar and BSA agar plates containing basic medium agar (*w*/*v*: 0.67% YNB, 0.2% glucose and 2% agar) were supplemented with 1% *w*/*v* skim milk powder and 1% *w*/*v* bovine serum albumin respectively, inoculated with fresh yeast cultures and incubated at 26 °C for 3 days. The appearance of clear (skim milk agar) or white (BSA agar) halos around the yeast streak was indicative of proteolytic activity (González et al., 2004).

2.5.2. Pectinase activity

Pectinase activity was evaluated using basic medium supplemented with 1% *w*/*v* citric pectin (pH 4.5). Plates were incubated at 26 °C for 5 days. Enzyme activity was evidenced by the formation of a clear zone around the colonies against a purple-brown background on pectin plate after Lugol's solution addition (Fernandes-Salomão et al., 1996).

2.5.3. Xylanase activity

This evaluation was performed using plates containing basic medium agar with 1% *w*/*v* xylane (pH 5). Yeast cultures were streaked on medium surface and plates were incubated at 26 °C for 5 days. Xylanase activity was detected as a clear zone around the streak in the opaque medium.

2.5.4. Glycosidase activities

Tubes (1.5 mL) containing 500 μL of basic medium agar added with 1 mM *p*-nitrophenyl-β-D-glucoside (pNPG), *p*-nitrophenyl-β-D-xyloside (pNPX) and *p*-nitrophenyl-α-L-rhamnoside (pNPR) were used to evaluate β-glucosidase (βGl), β-xylosidase (βXy) and α-rhamnosidase (αRh) activities, respectively (Rodríguez et al., 2004). Cultures were incubated at 26 °C for 3 and 7 days to βGl/βXy and αRh, respectively. Yeast strains showing enzymatic activities developed a yellow coloration in the culture medium after addition of 250 mM Na_2CO_3 buffer (100 μL). Agar esculin medium (*w*/*v*: 0.67% YNB, 0.5% esculin,0.1% glucose, 0.02% ferric ammonium citrate 2% agar; pH 5) was also tested to confirm βGl activity. In this case, strains showing βGl activity produced a dark brown halo around the colony (Hernández et al., 2003).

2.6. Antagonistic activity

Antagonistic activity was evaluated using an agar-plate assay (Sangorrín et al., 2002). Sensitivity of the *P. kudriavzevii* isolates to the antagonic activity of a set of four reference killer strains (*S. cerevisiae* YAT 679, K1 type; *S. cerevisiae* NCYC 738, K2 type; *Wickerhamomyces anomalus* NCYC 434, K5 type; *Williopsis saturnus* var. *mrakii* NCYC 500, K9 type) as well as five regional wine strains showing antagonistic capacity (*W. anomalus* NPCC 1027, *Torulaspora delbrueckii* NPCC 1032, *T. delbrueckii* NPCC 1033, *Metschnikowia pulcherrima* NPCC 1144 and *Pichia kluyveri* NPCC 1148). Antagonistic capacity of *P. kudriavzevii* isolates was evaluated against the collection killer sensitive strains *S. cerevisiae* P351 (PROIMI yeast collection) and *Candida glabrata* NCYC 388.

2.7. Principal coordinates analysis

Molecular (mtDNA-RFLP and RAPD-PCR patterns) and physiological (killer biotypes) qualitative variables were used to perform a principal coordinates analysis (PCoA) as described by Lopes et al. (2006). For each isolate a matrix value of 1 or 0 was assigned denoting the presence or absence of a band (molecular profile), a hydrolytic activity or a sensitivity to a particular killer strain. The Simple Matching coefficient (SM) (Sokal and Michener, 1958) was used to compute the similarity between pairs. Relationships among the 36 isolates were investigated by principal coordinates analysis (PCoA) and depicted in a 2D scatter plot using the NTSYS software 2.2 (Exeter Software, NY, USA) (Rohlf, 2002).

Table 3
Number of samples showing yeast growth at 10 °C and 30 °C and detected yeast species.

Substrate	Samples with yeasts (%)[a]		Yeast species[b]	
	10 °C	30 °C	10 °C	30 °C
Schinus johnstonii	–	10 (1)	–	*P. kudriavzevii* (3)
Ephedra ochreata	–	70 (7)	–	*P. kudriavzevii* (11)
Lycium chilense	20 (2)	20 (2)	*P. kudriavzevii* (2) *S. saitoi* (1)	*P. kudriavzevii* (6)

[a] Of a total of 10 samples analyzed at 10 °C and 30 °C of each substrate (60 samples in total).
[b] Number of isolates recovered are indicated between parenthesis.

3. Results

3.1. Yeast isolation and identification

In order to investigate potential yeasts that could be part of the North Patagonian winemaking terroir, an experimental methodology developed for fermentative yeast isolation was employed (Sampaio and Gonçalves, 2008). Yeast development was observed in 33.3% and 6.6% of the selective media containing fruit samples after incubation at 30 °C and 10 °C, respectively (Table 3). Only for the case of *L. chilense* fruits, yeast could be recovered from the two temperatures tested. Colonies developed in GPY agar plates showed similar morphology; however, 3, 11 and 9 isolates were recovered from *S. johnstonii*, *E. ochreata* and *L. chilense*, respectively (Table 3). All isolates were identified using 5.8S-ITS PCR-RFLP and confirmed by sequencing both 5.8S-ITS and the D1/D2 domains of the 26S rRNA gene as belonging to *P. kudriavzevii* species, except for one isolate from *L. chilense* fruits incubated at 10 °C which was identified as *Saturnispora saitoi* (Table 3). All *P. kudriavzevii* amplicons showed 100% nucleotide similarity among them and with those available in Genebank database for strain *Issatchenkia orientalis* (*P. kudriavzevii*) NRRL Y-5396^T (for D1/D2 domains of the 26S rRNA gene) and ATCC 24210^T (for 5.8S-ITS). Similarly, the amplicons obtained for the only isolate of *S. saitoi* showed 100% nucleotide similarity with those available for the type strain *S. saitoi* NRRL Y-6671.

3.2. Molecular characterization

All *P. kudriavzevii* isolates from different origins were subjected to molecular characterization including mtDNA-RFLP, RAPD-PCR and sequence analysis. These three different molecular typing methods allowed us to evaluate the intraspecific genetic variability as well as to assess the possible relationship within Patagonian native (natural and wine isolates) and foreign reference strains. Eight different mtDNA-RFLP patterns were detected among the total number of isolates with each particular endonucleases (either *Hinf*I or *Dde*I). However, the analysis of mtDNA-RFLP combining both enzymes allowed us to identify ten combined patterns (column "Co" in Table 4), nine of them associated with the reference strains and only one common to all (native and wine) regional isolates (Table 4, Fig. 1).

RAPD-PCR patterns were obtained using four different primers (OPA 9, OPA12, OPA14 and OPA15). The number of bands and the polymorphisms observed were different for each primer. The oligonucleotides OPA 12 and OPA 15 produced a scarce band number as well as a very low degree of reproducibility (data not showed). In contrast, OPA 9 and OPA 14 yielded a higher number of bands (18 and 12 respectively) and the greatest level of discrimination evidencing 11 and 12 haplotypes, respectively (Table 4). Independently from the analyzed primer, the smallest genetic variability was observed among the native isolates (coincidentally four haplotypes were detected with OPA9 and four with OPA14). The combined

Table 4
Molecular and physiological typing of native and reference strains of *P. kudriavzevii*.

Isolates[a]		Patterns															
		mtDNA-RFLP			RAPD-PCR			Antagonistic activity[b]									
		Hinf I	*Dde* I	Co	OPA 9	OPA 14	Co	K_1	K_2	K_5	K_9	Wa	Td_1	Td_2	Mp	Pk	Co
Native	NPCC 1368^{Lc}	A	1	I	1	a	I	−	+	+	+	−	−	−	+	−	A
	NPCC 1369^{Lc}	A	1	I	2	a	II	−	+	+	+	+	+	−	+	−	B
	NPCC 1370^{Lc}	A	1	I	2	a	I	−	−	−	+	−	+	−	+	−	C
	NPCC 1371–73^{Lc}	A	1	I	2	a	I	+	+	+	+	+	+	−	+	−	D
	NPCC 1372^{Lc}	A	1	I	2	b	III	−	−	+	+	+	+	−	+	−	E
	NPCC 1374–75^{Lc}, 1379^{Eo}	A	1	I	2	a	I	−	−	+	+	+	+	−	+	−	E
	NPCC 1376^{Lc}	A	1	I	2	a	I	−	−	−	+	+	+	−	+	+	F
	NPCC 1377^{Eo}	A	1	I	2	a	I	−	−	+	+	−	+	−	+	−	G
	NPCC 1378^{Eo}	A	1	I	2	a	I	−	−	+	+	+		−	+	−	H
	NPCC 1380^{Eo}	A	1	I	2	a	I	−	−	−	+	−	−	−	+	−	I
	NPCC 1381^{Eo}	A	1	I	2	b	III	−	−	−	+	+	+	−	+	−	J
	NPCC 1382^{Eo}	A	1	I	2	a	I	−	−	−	+	−	+	−	+	−	C
	NPCC 1383^{Eo}	A	1	I	2	a	I	−	−	−	+	−	−	−	+	−	I
	NPCC 1384^{Eo}	A	1	I	2	a	I	−	+	+	+	+	+	−	+	−	B
	NPCC 1385^{Eo}	A	1	I	2	a	I	−	−	+	+	+	+	+	+	−	K
	NPCC 1386^{Eo}	A	1	I	3	c	IV	+	+	+	+	+	+	−	+	−	D
	NPCC 1387^{Eo}	A	1	I	3	a	V	+	−	+	+	+	+	−	+	−	L
	NPCC 1388^{Sj}	A	1	I	2	a	I	−	−	+	+	+	+	−	+	−	E
	NPCC 1389^{Sj}	A	1	I	2	a	I	−	+	+	+		+	−	+	+	M
	NPCC 1390^{Sj}	A	1	I	2	a	I		+	+	+	+	+		+		B
	NPCC 1391, 1423–25^{W}	A	1	I	2	d	VI	−	−	+	+	+	−	−	+	−	H
	NPCC 1392, 1426^{W}	A	1	I	4	a	VII	+	−	+	+	+	+	+	+	−	N
	NPCC 1146, 1265–67^{W}	A	1	I	2	a	II	−	+	+	+	+	+	−	+	+	Ñ
Reference	NRRL Y-5396 (T)	B	2	II	2	e	VIII	+	−	+	+	+	+	+	+	+	O
	NRRL Y-134	C	3	III	5	f	IX	−	−	+	+	+	−	−	+	−	H
	NRRL YB-236	C	4	IV	6	g	X	−	−	+	+	+	−	−	−	−	Q
	NRRL YB-238	D	5	V	7	h	XI	−	−	+	+	+	−	−	+	−	H
	NRRL Y-420	E	6	VI	6	i	XII	+	−	+	+	+	−	−	−	−	P
	NRRL Y-870	F	6	VII	8	j	XIII	−	−	+	−	+	−	−	+	−	S
	NRRL Y-7179	G	7	VIII	9	i	XIV	−	−	+	+	+	−	−	+	−	H
	NRRL Y-7550	D	8	IX	10	i	XV	−	+	+	+	+		−	+	−	R
	NRRL Y-7724	H	8	X	11	k	XVI	−	−	+	+	+	−	−	+	−	H
	NRRL Y-27196	D	8	IX	10	l	XVII	−	+	+	+	+	+	+	+	−	T
Total patterns		8	8	10	11	12	17										21

c-Co: combined pattern.

[a] Superscripts Lc, Eo, Sj and W indicate the origin of the isolate (*Lycium chilense*, *Ephedra ochreata*, *Schinus johnstonii* and wine respectively). (T) Type strain.

[b] K_1: *Saccharomyces cerevisiae* YAT679; K_2: *S. cerevisiae* NCYC738; K_5: Wickerhamomyces anomalus NCYC434; K_9: Williopsis saturnus var. mrakii; Wa: W. anomalus NPCC1027; Td_1: Torulaspora delbrueckii NPCC1032; Td_2: T. delbrueckii NPCC1033; Mp: Metschnicowia pulcherrima NPCC1144 and Pk: Pichia kluyveri NPCC388.

analysis of the patterns obtained with the two primers allowed us to detect only seven different haplotypes within the 26 Patagonian isolates (Table 4). Contrarily, all the reference strains showed different genetic polymorphisms (10 distinct haplotypes) including the two strains with the same mtDNA-RFLP pattern (NRRL Y-7550 and NRRL Y-27,196). All reference strains polymorphisms were different from those presented by Patagonian isolates. Using RAPD combined patterns of OPA 9 and OPA 14 a UPGMA cluster analysis of Patagonian and reference strains was performed (Fig. 2).

The third molecular analysis consisted of the sequencing of two nuclear gene fragments that are rich in single-nucleotide polymorphisms (SNPs): NMT1 (*N*-myristoyltransferase, 11 SNPs) and ADE2 (phosphoribosylamino-imidazole carboxylase, 9 SNPs), with the main purpose of carrying out a phylogenetic study of all the strains analyzed. The selected genes were previously described in a population structure study of *C. krusei* (*P. kudriavzevii* anamorphic form) from different geographical sources (Jacobsen et al., 2007). Phylogenetic trees obtained from the sequence analysis of both *ADE2* and *NMT1* genes, analyzed both individually and concatenated, were found to be untrustworthy from the statistical viewpoint. The bootstrap values for the nodes in these trees were low (<50%) and did not help to robustly distinguish the internal structure of the populations of Patagonian and reference *P. kudriavzevii* strains (data not shown). Nevertheless, the detailed analysis of the *NMT1* sequence alignment, particularly on the region of the variable sites, allowed us to observe some differences. As shown Table 5, the native isolates were mainly distributed in two groups differentiated only by a nucleotide substitution at position 144 (G → A), except *P. kudriavzevii* isolates NPCC 1369, 1377 and 1384 which also evidenced other changes in the nucleotide sequence compared with the strain used as standard (NPCC 1388, isolate belonging to the largest group). Moreover, all the *P. kudriavzevii* reference strains showed nucleotide variants (alleles) not observed among the Patagonian isolates highlighting the genetic differences between the two groups.

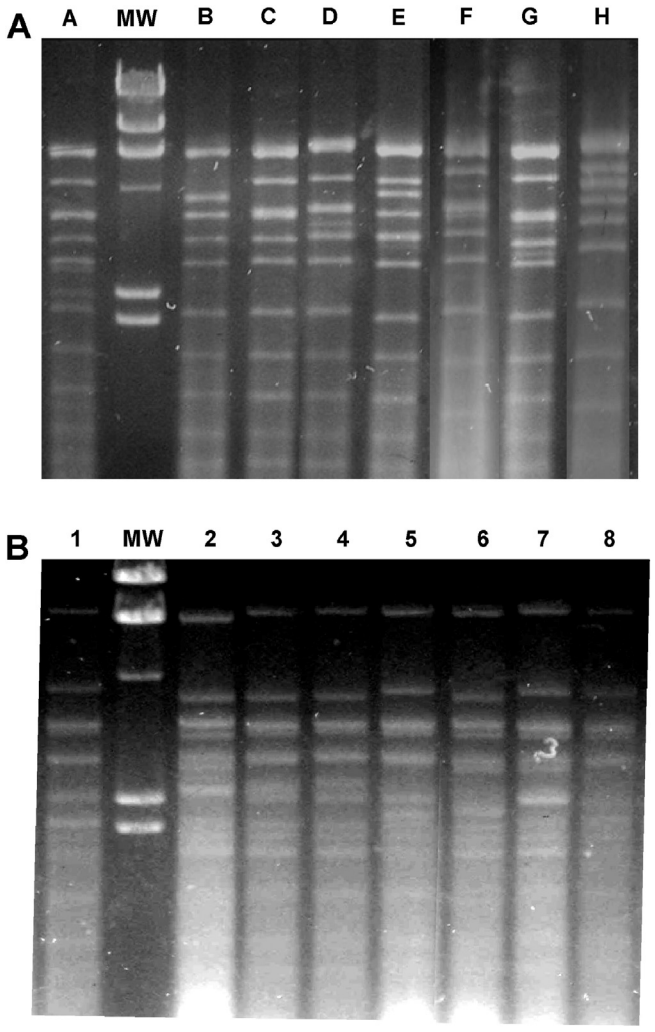

Fig. 1. Intraspecific characterization of *P. kudriavzevii* strains by RFLP-mtDNA analysis. mtDNA-RFLP patterns obtained for native and reference strains digested with *Hin*fI (A) and *Dde*I (B). MW = lambda DNA digested with *Hin*dIII molecular marker. Pattern identity is indicated in the top of each lane and corresponded to those in Table 4.

3.3. Physiological characterization

We evaluated some physiological traits relevant from the enological point of view, such as the production of diverse enzymatic activities (including protease, pectinase, xylanase, α-rhamnosidase, β-xylosidase and β-glucosidase activities) as well as the antagonistic activity. Sensitivity patterns were also used as an additional intraspecific discriminatory method. Protease activity was evaluated using two different substrates: skim milk and BSA. All the *P. kudriavzevii* isolates were able to hydrolyze both substrates after 16 h of incubation, except the reference strains NRRL Y-5396 and NRRL Y-27,196, in which no degradation was observed (Table 6). β-Glucosidase (βGl) activity was also evaluated using two different substrates: ρ-nitrophenyl-β-glucopyranoside (pNPG) and esculin. Surprisingly the results were very contrasting between assays. Almost all the Patagonian and reference isolates showed capability to hydrolyze esculin after 72 h of incubation evidencing β-glucosidase activity but none of them showed a positive signal when pNPG was used as substrate. The exceptions were isolates NPCC 1374 and NRRL YB-236 that showed no β-glucosidase activity at all on either of the substrates tested. None of the Patagonian and reference isolates showed pectinase, xylanase, β-xylosidase or α-rhamnosidase activity under the assayed conditions.

Regarding antagonistic activity, we evaluated both sensitivity of all *P. kudriavzevii* isolates against a panel of nine yeasts strains with demonstrated antagonistic capacity and antagonistic capacity against two reference sensitive strains. Twenty-one sensitivity patterns were detected among isolates, 15 patterns among Patagonian isolates and 7 among reference strains (Table 4). The pattern H was the only sensitivity pattern found in both reference and Patagonian group of isolates and it was the most frequent pattern among the reference strains. It is interesting to note that only 20% of the isolates were sensitive to K1 and K2 *S. cerevisiae* killer strains and almost all were sensitive to species *W. saturnus* var. *mrakii* NCYC 500 and *M. pulcherrima* NPCC 1144. On the other hand, antagonistic activity was not observed among analyzed isolates, independently from the sensitive reference strain used in the assays.

Finally, molecular and physiological qualitative data were used to perform a unified analysis in order to detect potential relationships among isolates from different origins. For that purpose, combined mtDNA-RFLP, RAPD-PCR and killer patterns were converted into binary data matrices and subjected to PCo analysis (Fig. 3). The first two coordinated, explaining 76% of total variability in data, were used to perform a 2D scatter plot. Three clusters were evident in this plot; cluster I grouped all the Patagonian (native and wine) yeasts and clusters II and III included only reference foreign strains (Fig. 3).

4. Discussion

The area of the upper valley of Negro and lower valley of Neuquén rivers, located in Northwestern Patagonia, is one of the most southern winemaking regions in the world. This region possesses ecological (soil, climate, humidity) characteristics favorable for the culture of high quality vines that are responsible for the distinctive characteristics of resulting wines. This particular *terroir*, makes winemaking one of the main economic activities in this region, and the search for new

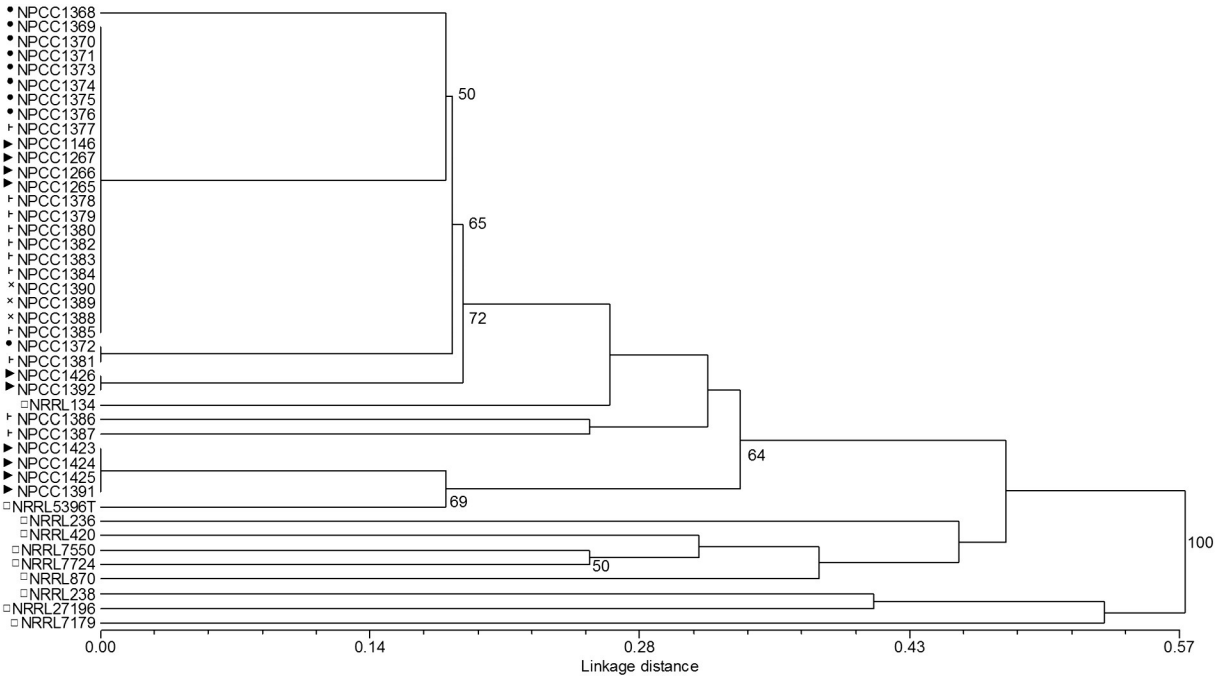

Fig. 2. UPGMA cluster analysis of Patagonian and reference *P. kudriavzevii* based on comparison of RAPD combined patterns of OPA 9 and OPA 14. *P. kudriavzevii* yeasts from (□) Reference, (►) wine, (●) *Lycium chilense*, (⊢) *Ephedra ochreata* and (×) *Schinus johnstonii* origin. Numbers at the nodes correspond to bootstrap values (percentages over 50%) based on 1000 replicates.

technologies to improve and diversify wines has become one of the main purposes of regional winemakers. Among these technologies, during the last decade the development of local yeast starter cultures, mainly based in *S. cerevisiae* strains selected among the autochthonous microbiota, became an interesting approach. This practice potentially generates wines with distinctive characteristics of the *terroir* (Lopes et al. 2002 and 2007). A more recent study about *S. cerevisiae* comparative genetics analyzed the phylogenetic relationships of 142 wine yeast strains of this species obtained from different countries, including *S. cerevisiae* isolates from North Patagonian region, demonstrated that all strains from Argentina had a close genetic proximity to wine strains from Europe (Peris et al., 2012). This genetic proximity is not surprising because vine plants, and surely its associated biota, came to the new world from Europe after colonization.

In this context, we believe that other natural environments probably constitute a source of truly autochthonous yeasts, representative of the Patagonian region. With this aim, we sampled sugar-rich fruits of three endemic plants commonly found in this region: *E. ochreata*, *S. johnstonii* y *L. chilense* (popularly known as Solupe, Molle y Yaoyín, respectively). Some ethnobotanical studies report that the fruits of these native edible plants have been used by ancient populations of the Patagonian region for the elaboration of fermented beverages (Rapoport et al., 2003; Ladio, 2002). Using the selective methods for fermentative yeast isolation proposed by Sampaio and Gonçalves (2008) and successfully used by several researchers for the recovery of *Saccharomyces* yeasts from natural substrates (Lopes et al., 2010; Libkind et al., 2011; Rodríguez et al., 2014), we could not recover any yeasts belonging to *Saccharomyces* genus. This result is an additional evidence about the fact that *Saccharomyces* genus is not cosmopolitan but rather occupy certain ecological niches either artificial and natural (Demuyter et al., 2004; Csoma et al., 2010; Lopes et al., 2010; Libkind et al., 2011; Rodríguez et al., 2014; Naumov et al., 2000, 2002, 2011, Hittinger, 2013).

To our surprise, almost 100% of the recovered yeasts in selective medium at both 10 °C to 30 °C isolation temperatures belonged to the species *P. kudriavzevii*. This species has been described in various places around the world. It has been isolated from a wide variety of substrates including soil (Limtong et al., 2009), fruits (Arias et al., 2002; Clavijo et al., 2010; Li et al., 2010; Nyanga et al., 2012) and musts (Clemente-Jimenez et al., 2004; Koh and Suh, 2009; Daniel et al., 2009; Papalexandratou and De Vuyst, 2011; Vogelmann and Hertel, 2011; Sharma et al., 2012; Bezerra-Bussoli et al., 2013; Freire et al., 2014). Recent studies carried out in our laboratory detected *P. kudriavzevii* as a frequent yeast species in several North Patagonian spontaneous wine fermentations (del Mónaco et al., 2014).

It is clear that the exclusive recovery of the *P. kudriavzevii* species from the fruits of endemic plants does not mean that this species is the only yeast species colonizing the studied surfaces, but rather, it was the result of a selective methodological approach. The wide variety of substrates from where this species has been isolated in North Patagonia (fruit surfaces exposed to climatic conditions and culture medium containing 8% *v*/v ethanol at a temperature of both10 °C and 30 °C, fermenting grape musts) is directly related to the ability of this species to withstand stress conditions. The tolerance of *P. kudriavzevii* to different stress conditions has been previously mentioned by some researchers,

Table 5
Nucleotide divergence in the variable region of the *NMT1* gene sequence between native and reference strains.

Isolates[a]		Variable positions (in vertical) 1123 684880 644061
Native (NPCC)	1388^{Sj}†	GTGCTT
	1146, 1265-67^{W}; 1370-72, 75-76^{Lc}; 1378, 80, 82-83, 85-87^{Eo}; 1389^{Sj}; 1391-92, 1423-26^{W}	.....
	1368, 1373-74^{Lc}; 1379, 1381^{Eo}; 1390^{Sj}	..A...
	1384^{Eo}	.....G
	1369^{Lc}	..A..G
	1377^{Eo}	..A.GG
Reference (NRRL)	5396T, 238	...T..
	870	..AT..
	7179, 7550, 7724, 27196	.CA...
	134, 420	ACA...
	236	AC.T..

a- Superscripts Lc, Eo, Sj and W indicate the origin of the isolate (*Lycium chilense*, *Ephedra ochreata*, *Schinus johnstonii* and wine respectively). (T) Type strain.
† Strain highlighted in grey was used as reference for comparison.

Table 6
Enzymatic characterization of native and reference *P. kudriavzevii* strains.

Isolates[a]		Enzymatic activities (substrate)[b]			
		Protease (milk)	Protease (BSA)	β-Gl (esculin)	β-Gl (pNPG)
Native (NPCC)	1146, 1265–67^{W}, 1368–73, 1375-76^{Lc}; 1377-87^{Eo}; 1388-90^{Sj}; 1265–57, 1391–92, 1423-26^{W}	+	+	+	−
	1374^{Lc}	+	+	−	−
Reference (NRRL)	5396^{T}, 27,196	−	−	+	−
	236, 238, 420, 870, 7179, 7550, 7724	+	+	−	−
	134	+	+	+	−

[a] Superscripts Lc, Eo, Sj and W indicate the origin of the isolate (*Lyciumchilense*, *Ephedra ochreata*, *Schinus johnstonii* and wine respectively). (T) Type strain.
[b] BSA: bovine serum albumin; β-Gl: beta-glucosidase. Positive enzymatic activity is indicated with +. Negative enzymatic activity is indicated with −.

that have even appointed this species as a multi-stress tolerant yeast (Kwon et al., 2011; Isono et al., 2012; Yuangsaard et al., 2013).

Additionally, some authors have also studied the possible role and use of *P. kudriavzevii* in winemaking (Kim et al., 2008; del Mónaco et al., 2014). Nowadays, the significance of non-*Saccharomyces* yeasts in wine production is well known as an integral part of wines authenticity (Jolly et al., 2014). Considering the above, we decided to deepen the study of this species in order to know if these yeasts are truly autochthonous of North Patagonian region. For this purpose, we worked in the genetic and physiological characterization of both natural (isolated in this study) and wine isolates from Patagonia as well as of foreign strains of this species available in culture collections.

Several techniques like RAPD-PCR, nuclear genes PCR-RFLP, karyotype and gene sequencing analyses have been described for intraspecific discrimination in different *non-Saccharomyces* species including *P. kudriavzevii* (Carlotti et al., 1994; Dassanayake and Samaranayake, 2000, Jacobsen et al., 2007; Martorell et al., 2006; Agnolucci et al., 2009). Nevertheless, this is the first work reporting the use of mtDNA-RFLP, RAPD-PCR with OPA primers and *MNT1* gene sequence analyses for *P. kudriavzevii* strains characterization. To our surprise, all the Patagonian isolates (native and wine isolates) of *P. kudriavzevii* showed the same unique mtDNA-RFLP pattern regardless of the endonuclease used (*Hinf* I and *Dde* I), while the RAPD analysis with two OPA primers (OPA9 and OPA14) discriminated at least seven different haplotypes, completely distinctive to those observed in reference foreign strains. The use of OPA series primers has been previously reported for the molecular typing of isolates belonging to species *Dekkera bruxellensis* and *Pichia guilliermondii* by Martorell et al. (2006) and Sangorrín et al. (2013). These authors found that the combination of patterns obtained with different primers, including OPA 9 and 14, allowed the discrimination of strains with the same mitochondrial restriction profile. These results concur with those obtained in the present study.

Similar to that observed with RAPD-PCR assays, the sequence analysis of a *MNT1* nuclear gene region evidenced the existence of five haplotypes among Patagonian isolates and also five haplotypes (different from those present in Patagonian isolates) among reference strains. However, most Patagonian isolates (all wine isolates and 14 out of 23 isolates from fruits) showed the same allele according to the partial sequence of *MNT1* gene. All these molecular approaches evidence a high relationship between regional isolates of *P. kudriavzevii*, supporting the idea that this species is representative of a regional autochthonous biota that could be part of the North Patagonian *terroir*. This idea was also supported by the PCoA that evidenced that all Patagonian *P. kudriavzevii* isolates, from both wine and natural origin, grouped together and clearly distanced from the reference strains.

Additionally, sensitivity patterns against a panel of diverse yeasts showing antagonistic capacity, is a physiological method that has been reported to be a good diversity index for both *Saccharomyces* and no-*Saccharomyces* yeasts when used in combination with molecular markers as mtDNA-RFLP or RAPD analyses (Lopes et al., 2006, 2009, Buzzini et al., 2007). In this work, the combined use of sensitivity patterns (using a panel of nine reference and regional antagonistic strains) and RAPD polymorphisms also increased the discriminatory capacity to differentiate *P. kudriavzevii* native isolates to more than twice the one exhibited by molecular methods themselves (15 vs 7patterns respectively).

Non-Saccharomyces yeasts like *P. kudriavzevii* can be employed as mixed starter cultures in combination with an ethanol-tolerant *S. cerevisiae* strain. In that context, antagonistic behavior including killer activity is considered a relevant technological attribute within yeast selection protocols for winemaking (Rainieri and Pretorius, 2000). Different antagonistic interactions between starter cultures and wild yeast strains naturally present during wine fermentation could be established affecting the normal evolution of the process. These

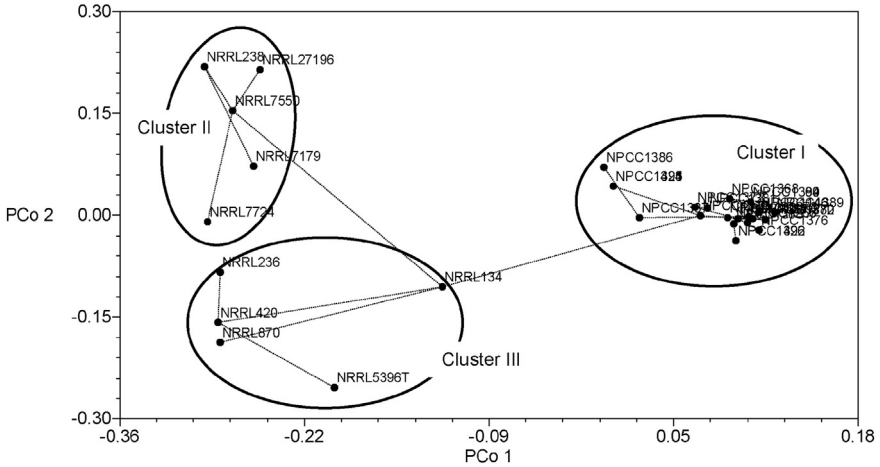

Fig. 3. Principal coordinates analysis (PCoA) of molecular (mtDNA-RFLP, RAPD-PCR) and physiological (sensitivity to antagonistic yeast strains) patterns.

interactions become relevant in areas where yeasts with antagonistic capacity, as those producing killer toxins, are widespread such as the North Patagonian region (Sangorrín et al., 2001). Interestingly, all Patagonian *P. kudriavzevii* isolates lacked of antagonistic capacity (they could not affect the growth of neither *S. cerevisiae* starter cultures nor wild yeast biota) and the great majority of them were resistant to the antagonistic activity of *S. cerevisiae* strains (K1 and K2 in this work).

Finally, we evaluated additional physiological properties typically present in non-*Saccharomyces* yeasts that could reinforce the potential use of *P. kudriavzevii* in winemaking. We evaluated the production of a set of enzymes related to the improvement of wine aroma (xylanase, α-rhamnosidase, β-xylosidase and β-glucosidase and pectinase activities) or wine stabilization (protease activity) (Günata et al., 1985, 1988; Ganga et al., 1999; Manzanares and Vallés, 2005, Ugliano, 2009). Our results showed that most regional isolates showed protease and β-glucosidase activities. Strong protease activity had been already associated with *P. kudriavzevii* (Trindade et al., 2002; Mugula et al., 2003; Ogunremi et al., 2015). However, the presence of β-glucosidase in this species seems to be uncommon (Trindade et al., 2002). Strikingly, whereas the protease activity was detected in all the strains regardless the substrate employed (BSA or skim milk), the β-glucosidase activity was detected only using esculin as substrate, but was absent with *p*-nitrophenyl-β-*D*-glucopyranoside (pNPG), even though this is routinely used for β-glucosidase screenings (Rosi et al., 1994; Gueguen et al., 1995, Manzanares et al., 1999; Rodríguez et al., 2007). Our results highlight the importance of using at least two types of substrates for enzymatic screening studies thus minimizing the risk of obtaining a false-negative result caused by the specific selectivity of the enzyme by the substrate. These results are consistent with those reported by Hernández et al. (2003) that used different substrates (arbutine, esculine and pNPG) and culture media composition for the detection of glucosidase activity.

In conclusion, we want to emphasize the importance of this study in our winegrowing region. Our results show that the North Patagonian isolates of *P. kudriavzevii* would be endemic of this region and, unlike *S. cerevisiae* local isolates that showed to be related to European strains, local *P. kudriavzevii* probably truly represent part of the North Patagonian regional *terroir*. This discovery, added to the convenient enological properties exhibited by wine *P. kudriavzevii* Patagonian strains as described in a previous study (del Mónaco et al., 2014), turns them into an invaluable potential tool for the development of regional mixed starter cultures.

Acknowledgements

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