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Resumo

As leveduras do género *Saccharomyces* têm sido consideradas objectos de estudo interessantes em biologia evolutiva e genética populacional dada a sua distribuição ubíqua, relevância como organismos modelo, disponibilidade de informação genómica para todas as espécies e importância nas indústrias fermentativas (fermentação da cerveja e do vinho, panificação, entre outras similares). No entanto, apesar da sua relevância como organismos modelo em inúmeras áreas da Biologia, muitas questões relacionadas com a sua ecologia e história evolutiva permanecem ainda sem resposta.

Tal como *S. cerevisiae*, frequentemente usada na fermentação do mosto de vinho e do pão, *S. uvarum* é uma levedura importante na fermentação da cidra e de alguns tipos de vinho produzidos a baixas temperaturas. Apesar da sua ligação a processos fermentativos conduzidos pelo Homem, esta levedura é frequentemente encontrada na natureza nos mais diversos substratos como cascas de árvores, cogumelos, solo e até insectos. Estudos realizados em *S. cerevisiae* indicam que a divergência genética nesta espécie está ligada à especialização ecológica. Para além disso, foram identificados eventos de domesticação ligados ao vinho e ao saké, sendo que as estirpes associadas a estas fermentações apresentam menor diversidade genética que as selvagens, para além de que derivam filogeneticamente destas. Por outro lado, a espécie filogeneticamente mais próxima de *S. cerevisiae*, *S. paradoxus*, é apenas isolada de ambientes naturais apresentando um padrão filogeográfico acentuado sendo reconhecidas várias populações geográficas.

Tendo em conta que os padrões de diversidade genética de *S. uvarum* são ainda desconhecidos, neste trabalho foram estudadas 50 estirpes provenientes de substratos e regiões muito diversificadas com o objectivo de detectar indícios de domesticação e de esclarecer os aspectos que afectam a estrutura populacional nesta espécie.

Foi realizada uma análise filogenética baseada na sequenciação parcial de três genes nucleares tendo sido identificados três grupos filogenéticos principais. Dois dos grupos corresponderam a duas regiões específicas: Australásia (*clade C*) e Patagónia (*clade B*). Todos os restantes isolados, incluindo alguns isolados da Patagónia, constituíram um grupo filogenético distinto (*clade A*) e bem separado do grupo da Australásia. A distribuição das estirpes da Patagónia ao longo de dois dos ramos filogenéticos é espelho da grande diversidade genética encontrada neste local. Quanto ao grupo das estirpes da Australásia, é caracterizado por uma elevada divergência genética relativamente aos restantes grupos. Esta divergência encontra correspondência a nível fenotípico e traduz-se também num isolamento reprodutor parcial relativamente aos indivíduos dos outros grupos filogenéticos e em evidências de diferenciação populacional. O mesmo não se verifica com as estirpes da Patagónia (*clade B*) que apesar da sua aparente divergência na análise filogenética,

apresentam características fenotípicas semelhantes às de estirpes representativas do *clade A*, inexistência de evidência de diferenciação populacional e ausência de isolamento reprodutor.

No que diz respeito ao estudo da domesticação, não foi possível encontrar o padrão observado em *S. cerevisiae* visto que quer estirpes isoladas de ambientes naturais quer estirpes provenientes de cidra ou vinho se agrupam no mesmo ramo filogenético. Além disso, a diversidade genética das estirpes domesticadas não é inferior à das estirpes selvagens. Deste modo os resultados sugerem que o processo de domesticação que ocorreu em *S. cerevisiae* não se verifica em *S. uvarum*.

É ainda de salientar que todas as estirpes provenientes do Hemisfério Sul foram isoladas ou de *Nothofagus* ou *Cyttaria,* sendo este último um fungo ascomiceta que é parasita obrigatório das árvores do género *Nothofagus*. Todas as espécies que constituem estes dois géneros povoam apenas alguns locais do Hemisfério Sul sendo que a actual distribuição está associada à separação do mega-continente *Gondwana*.

Para além dos genes nucleares, dois genes mitocondriais foram também sequenciados para um conjunto de estirpes representativo dos grupos filogenéticos obtidos com as sequências nucleares. Nesta análise, a população da Australásia surgiu como a mais divergente relativamente a um grupo constituído pelas restantes populações de *S. uvarum* e *S. bayanus* (a espécie filogeneticamente mais próxima de *S. uvarum*). Devido à incongruência verificada entre as filogenias de genes nucleares e mitocondriais, e no sentido de evidenciar possíveis fenómenos de recombinação, foi realizada uma análise baseada em *phylogenetic networks*. Com esta análise foi possível evidenciar eventos de trocas génicas entre as populações da Patagónia e do Hemisfério Norte, corroborando assim a ligação entre as populações destes dois locais. Esta aparente ligação levou a questionar se o grupo da Patagónia consiste numa população diferente do grupo do Hemisfério Norte, tendo-se efectuado uma análise para detecção de estrutura populacional no programa *Structure*. Nesta análise apenas se evidenciaram duas populações: uma correspondente às estirpes da Australásia e outra reunindo as restantes estirpes. Quando o programa foi forçado a considerar um número superior de populações a única partição encontrada consistiu na adição de mais uma população, tendo as estirpes da Patagónia sido distribuídas por dois *clusters* genéticos, sendo que um destes *clusters* é marcadamente dominante no Hemisfério Norte. Estes resultados parecem sugerir a colonização do Hemisfério Norte a partir da América do Sul.

Devido aos indícios sugeridos por estes resultados, foi realizada uma análise mais detalhada a nível populacional. Recorreu-se ao uso de três regiões microsatélite que, apresentando uma evolução mais rápida, podem ter relevância para estudos populacionais. Considerando a variação do número de repetições nas regiões microsatélite, confirmou-se a

inexistência de estrutura populacional entre a Patagónia e o Hemisfério Norte, enquanto a diferenciação da população da Australásia se manteve. Observou-se ainda a existência de substituições nucleotídicas pontuais nas estirpes da Australásia e Patagónia, relativamente às restantes, sendo que nas primeiras estas parecem estar fixas na população, algo que pode sugerir a ancestralidade da mesma. Para além disso, tanto nas estirpes da Australásia como nas da Patagónia foram encontrados alelos privados com elevada frequência na população. Esta elevada frequência aliada a uma elevada diversidade genética no caso da Patagónia, sugere uma existência antiga, tendo decorrido gerações suficientes para se propagarem alelos únicos em grande parte da população. Outro dado interessante é a existência de alelos privados característicos do Hemisfério Sul (Australásia e Patagónia). Esta associação entre estes dois locais é corroborada pelos dados de diferenciação populacional, que não sugerem uma separação tão clara destes dois grupos relativamente à clara separação encontrada entre a Australásia e o Hemisfério Norte.

Considerando os resultados obtidos no decurso deste estudo, a hipótese de trabalho para estudos futuros é a de que *S. uvarum* colonizou o Hemisfério Norte a partir de uma população original residente a Sul, provavelmente na América do Sul. Por outro lado, a ligação entre a Australásia e a Patagónia e a associação ao sistema *Nothofagus* – *Cyttaria* remete para os processos de deriva continental associada à separação do *Gondwana*. Trabalhos futuros, suportados em maior densidade de informação genética e em amostragens populacionais mais completas, serão necessários para completar o estudo da história evolutiva desta levedura, iniciado com este trabalho.

Palavras chave: *Saccharomyces uvarum*, cidra, filogeografia, genética populacional, evolução

Abstract

Yeasts belonging to the genus *Saccharomyces* are interesting model organisms for evolutionary and population genetics studies because of their ubiquitous distribution, relation to human activities and availability of complete genome sequence information for all species. Unlike S*. cerevisiae* that is closely associated to winemaking and *S. paradoxus* that is only isolated from the wild, *S. uvarum* is a cryotolerant yeast that is widely distributed in nature but can also be isolated from cider and certain wines whose fermentation is carried out at low temperatures. While in *S. cerevisiae* genetic divergence is associated with ecological variants, with two identified domestication events (wine and sake), in *S. paradoxus* geography, not ecology, drives genetic divergence. Since these aspects have not yet been studied in *S. uvarum*, a phylogeographic study of 50 strains isolated in Eurasia (both from the wild and man-driven fermentations), North and South America and Australasia was performed. Based on phylogenetic analysis of three nuclear genes, three main clades were found, two of them represented by isolates from two well-delimited regions (Patagonia, clade B and Australasia, clade C), and a third (clade A) including strains from the Northern Hemisphere and some strains from Patagonia. However, the recognition of a Patagonian population distinct from the population of the North Hemisphere was not supported in subsequent analyses where no phenotypic discontinuities were detected, no reproductive barriers were found and evidence for population differentiation through both nuclear genes and microsatellite markers was weak. Furthermore, domestication in *S. uvarum* could not be detected as, among the isolates from the Northern Hemisphere, putative domesticates could not be distinguished from wild strains. Moreover, the Australasian population showed genetic and phenotypic divergence from the remaining populations and mating studies showed partial reproductive isolation. Evidence for ancestry of the Australasian and Patagonian populations was suggested due to the presence of private alleles with high frequencies in the population. Additionally, some alleles were found to be shared by the Patagonian and Australasian populations leading to the hypothesis of a past connection between them. The ancestry signs found for the Patagonian population combined with the presence of Patagonian genotypes in the Northern Hemisphere and the absence of population structure between these populations suggest a possible migration of strains from Patagonia to North America and Eurasia. Nevertheless, this is an exploratory study that requires further analyses concerning more complete sampling and molecular data.

Keywords: *Saccharomyces uvarum*, cider yeast, phylogeography, population genetics, evolution

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Abbreviations

AMOVA	Analysis of molecular variance
bp	Base pairs
F_{ST}	Fixation index (measure of population differentiation)
HWE	Hardy-Weinberg equilibrium
k	Populations
mtDNA	Mitochondrial DNA
nuDNA	Nuclear DNA
OD_{xnm}	Optical density at x wavelength (nm)
RFLP	Restriction fragment length polymorphism
SDS	Sodium dodecyl sulfate
SNP	Single nucleotide polymorphism
TE	Tris-EDTA
YM	Yeast extract-Malt extract medium
YMA	Yeast extract-Malt extract agar medium
YNB	Yeast Nitrogen Base medium
YPD	Yeast Extract Peptone Dextrose medium
w/v	Weight per volume
ΔK	Ln (k) variation

1. Introduction

One of the most important challenges of evolutionary biology is to understand the evolutionary processes of living beings in the wild, and which factors (e.g. geography, ecology) are interfering in these processes. The study of the evolutionary history of plants and animals, although complex, is easier than the evolutionary study of microbes. That is so not only because of their macroscopic size that allows a more detailed analysis of the phenotypic diversity between individuals, but mainly because of the availability of fossils that allow the tracking of their evolutionary history since the Proterozoic Era. Microbes, like yeasts, have no fossil record, making it difficult to follow their evolutionary course. Fortunately, recent methodological and molecular advances have provided several tools to obtain and process molecular data, making it possible to test hypothesis about their evolution.

Yeasts, especially those that belong to the genus *Saccharomyces*, are interesting organisms for evolutionary studies. First, they are widely distributed around the globe making it possible to perform population studies to understand their dynamics and, possibly, infer their evolution through time. Secondly, it is important to note their close relation with human activities like winemaking, cider fermentation, baking and brewing, among other human related activities. This genus, formerly known as the *Saccharomyces* sensu stricto complex, currently includes eight species (Naumov et al., 2000b; Kurtzman and Robnett, 2003) (**Fig. 1**): *S. cerevisiae,* the first yeast to be described (Rainieri et al., 2003) and mainly isolated from fermentative processes (Mortimer, 2000); *S. paradoxus,* the closest species to *S. cerevisiae* but exclusively associated with natural habitats (Johnson et al., 2004); *S. cariocanus* (Naumov et al., 2000b)*,* isolated in Brazil and closely related with *S. paradoxus*;; *S. mikatae* isolated in Japan (Naumov et al., 2000b); *S. kudriavzevii*, found in decayed leaves and soils in Japan and in oaks in Portugal (Sampaio and Gonçalves, 2008); *S. arboricolus*, recently described and isolated from oak trees in China (Wang and Bai, 2008); *S. bayanus,* associated with fermentations carried out at low temperatures; *S. pastorianus*, a natural hybrid between *S. cerevisiae* and *S. bayanus* associated with anthropic environments due to its ability to ferment at low temperatures (Libkind et al., 2011) and *S. uvarum*, the closest relative of *S. bayanus,* which is not only isolated from low-temperature fermentations of cider, wine and other beverages (Naumov et al., 2001) but also frequently isolated from the wild (Sampaio and Gonçalves, 2008).

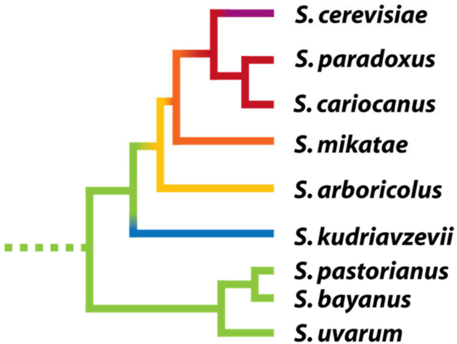


Fig. 1 – Phylogeny of the species of the genus *Saccharomyces*. The color gradient represents the growth temperature preferences of species: warm tones code for higher temperature preferences and cool tones code for lower temperature preferences (Image kindly provided by Marco Coelho).

Taking into account that species differentiation in this genus has always been difficult (Rainieri et al., 2003), the nomenclature of *S. bayanus* and *S. uvarum* has been confusing and controversial for decades and *S. uvarum* is not considered as a distinct species for many biologists. Because of the high DNA-DNA reassociation values between the type strains of these two species (Rosini et al., 1982) and the similarity of assimilation profiles of carbon sources, *S. uvarum* was initially considered a synonym of *S. bayanus* (Naumov et al., 1996), and later a variety (Naumov, 2000a). Because of the absence of a clear resolution of these taxonomic issues, the first genome to be sequenced from this complex (strain CBS 7001) was designated *S. bayanus* or *S. bayanus* var. *uvarum* instead of *S. uvarum* (Cliften et al., 2006). Libkind et al. 2011 clarified this issue by genome sequencing and mating of strains of *S. bayanus* and *S. uvarum* occurring in nature in Patagonia. The measurement of the degree of fertility is possible because of the life cycle of *Saccharomyces* spp. (**Fig. 2**), where there is no pre-zygotic barrier; therefore, all the species can mate with each other (Greig, 2009). The reproductive isolation is post-zygotic which means that F1 hybrids divide normally by asexual budding and form spores by sexual meiosis but the level of viability of these spores decreases when inter species mating occurs (Liti et al., 2006).

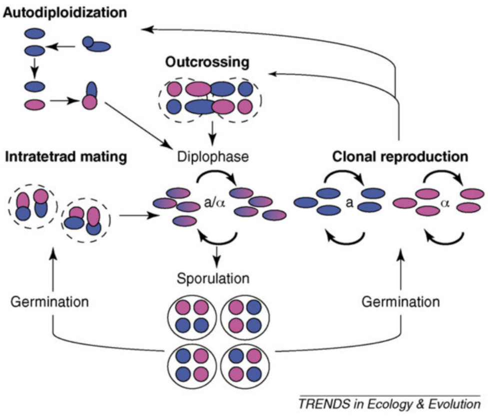


Fig. 2 - *Saccharomyces* spp. life cycle. Reproduction is defined by mating type genes (*MAT*). The presence of a *MAT**a*** allele at this locus gives a clone of mating-type **a**, and a *MAT**α*** allele gives a clone of mating type **α**. Only opposite mating types can mate (Replanski et al., 2008).

Additionally to low spore viability of hybrids (7% of viable spores), Libkind et al. (2011) found ~7% genome-wide sequence divergence between Patagonian *S. bayanus* and *S. uvarum* strains, and observed niche differentiation in the wild. Other molecular studies by Rainieri et al. (1999) and Pulvirenti et al. (2000), for instance, pointed to *S. uvarum* being a homogeneous taxonomic group, distinct from *S. bayanus*. These studies led to proposals for the reinstatement of *S. uvarum* as a separate species (Pulvirenti et al., 2000; Nguyen and Gaillardin, 2005). *S. uvarum*, along with *S. bayanus* and *S. pastorianus*, is classified as a cryotolerant yeast because it grows at temperatures lower than those preferred by *S. cerevisiae* (**Fig. 1**), being therefore adapted to low-temperature fermentations during winemaking (Naumov, 2000a) and cider production (Naumov et al., 2001). While non-cryotolerant strains are not effective in cider, lager beer and white wine fermentation, cryotolerant strains like those of *S. uvarum* are specialized in these processes. Furthermore, *S. uvarum* can also contribute to alcoholic fermentation in mixed populations with *S. cerevisiae* (Masneuf-Pomarède, 2010). In fact, it was even the predominant species, in spontaneous fermentations of must obtained from grapes cultivated in vineyards in the North of France (Massoutier et al., 1998; Naumov et al., 2001; Demuyter et al., 2004). *S. uvarum* was also isolated from natural fermentations of botrytized grape must (Naumov et al., 2000b, 2001; Sipiczki et al., 2001; Antunovics et al., 2005) and identified in natural fermentations of Recioto and Amarone wines (Tosi et al., 2009). In natural habitats, this yeast has been sporadically isolated from insects (*Mesophylax adopersus* and *Drosophila* spp.), tree fluxes of *Ulmus* and *Carpinus*, from *Nothofagus* bark, fruiting bodies of *Cyttaria*, and from mushrooms (Naumov 2003, Naumov 2006). Expeditions to Patagonia and New Zealand in 2006 and 2009 respectively (Sampaio, J.P. and Libkind, D., unpublished), allowed the collection of several strains of *S. uvarum* from *Nothofagus* trees and from fruiting bodies of *Cyttaria,* an ascomycete fungus that is an obligate parasite of *Nothofagus*. When mature, these fruiting bodies are rich in sugars, being a *Saccharomyces* habitat (Libkind et al., 2007). They are exclusively associated with *Nothofagus* spp. and therefore occur solely in South America (Argentina and Chile) and Australasia (Australia and New Zealand) (Libkind et al., 2007; Peterson et al., 2010).

Since *S. cerevisiae* is associated with winemaking and other human activities, and its closest relative *S. paradoxus* is only found in natural habitats, some authors hypothesized about the possibility of *S. cerevisiae* being a domesticated species. Domestication is therefore an important aspect to take into account in evolutionary studies of *Saccharomyces* because it can shape the evolutionary course of a population and have a strong impact in population dynamics (Johnson et al., 2004).
The selection and propagation of plants more suited to the human needs is done since the pre-Neolithic in Syria where there is early evidence for conscious cultivation and trait

selection (Hillman et al., 2001). Wheat, for instance, was one of the first crops to be domesticated more than 10,000 years ago. As a consequence, the traits that are known nowadays in domesticates are markedly different from those present in wild populations (Charmet, 2011). Given the well-established knowledge of plant and animal domestication, one can wonder if microbes were also unwittingly domesticated.

Based on the analysis of DNA extracted from ancient wine containers and molecular analysis of pottery jars found in China, it was found that yeasts have been associated with fermented beverages since 7000 BC (Mortimer, 2000). The approach for the identification of domestication processes in microorganisms is based on molecular analyses and is also based on the assumption that domesticates have a lower level of diversity when compared to wild stocks, which represent the ancestral lineages (Fay and Benavides, 2005). Molecular data such as microsatellite polymorphisms and gene sequencing indicated that *S. cerevisiae* comprises two types of populations: domesticated (associated with human environments) and non-domesticated (strains isolated from the wild) (Fay and Benavides, 2005; Legras et al., 2007). Furthermore, it was found that non-domesticated populations group according to their geographic origin. This geographic association had been already observed for *S. paradoxus* by Kuehne et al. (2007). In that study, 62 strains from several locations within two continental regions (eastern North America and eastern Europe/western Asia) were studied using nine unlinked nuclear *loci* that were sequenced. The authors were able to obtain two well-defined clades, corresponding to two different populations: North American and Eurasian. More recently, Liti et al. (2009) reinforced this geographical connection by whole genome sequencing. Two more populations were introduced, dividing therefore *S. paradoxus* in four populations: European, Far Eastern, American and Hawaiian. These studies demonstrated that geography plays a major role in *S. paradoxus* diversity and counteracted the idea that microbes, including eukaryotes, do not have biogeographies because continuous large-scale dispersal sustains an uniform and global distribution (Finlay, 2002).

Taking into account the apparent habitat and substrate preference similarities between *S. uvarum*, *S. cerevisiae* and *S. paradoxus*, and because much less is known about *S. uvarum*, the present work aims to clarify the evolutionary history and the diversity of this yeast. Therefore, a phylogeographic study was carried out in order to understand how spatial and temporal dimensions interfered with the evolution of *S. uvarum*. Phylogeography is a study that bridges many disciplines, incorporating different kinds of information from phenotypes, population ecology and molecular and evolutionary genomics to geography, climatology and ecological factors. This integration can provide valuable information on how geographical, geologic and environmental factors are interfering with species ecology, therefore shaping its evolution (Knowles, 2009). To achieve this, nuclear and mitochondrial sequence data from 50 strains of *S. uvarum* (isolated from different regions and from both wild and fermentative environments, **Table S1** in Appendix I) were used in order to infer genetic diversity and possible phylogenetic relationships. Additionally, in order to investigate if genetic diversity is related with reproductive isolation, mating tests between and within the different phylogenetic groups were performed. Furthermore, in order to clarify the partition of populations, three microsatellite *loci* were also studied.

2. Material and Methods

2.1 Yeast strains

The strains used in this study and their origins are listed in Table S1. All yeasts were grown on YPM solid medium (0.3% w/v yeast extract, 0.3% w/v malt extract, 0.3% w/v bactopeptone, 1% w/v glucose and 1.5% w/v agar) for DNA isolation, carbon source assimilation and maximum growth temperature assays.

2.2 Phenotypic assays

2.2.1 Utilization of different carbon sources

Strains were grown in 5 mL of YNB liquid medium supplemented with 1% w/v of the carbon source to be tested (glucose, fructose, melibiose, maltose and mannitol). Tubes were incubated on a shaker (150 rpm) at 25ºC for several days. Growth was evaluated in terms of turbidity.

2.2.2 Maximum growth temperature

Strains were inoculated in 5 mL of YM liquid medium (0.3% w/v yeast extract, 0.3% w/v malt extract, 0.3% w/v bactopeptone, 1% w/v glucose) and incubated at several temperatures (28ºC-36ºC) for seven days. Growth was evaluated in terms of turbidity.

2.2.3 Specific growth rates

Strains were previously inoculated in YNB liquid medium with 2% w/v of glucose (pre-inoculum). When the pre-inoculum reached OD_{640nm} =2-2.5 (middle of the exponential growth phase) it was transferred to a 500 mL Erlenmeyer flask with 100 mL of YNB liquid medium supplemented with 2% w/v of carbon source (glucose or melibiose). These flasks were incubated at three different temperatures (10ºC, 18ºC and 25ºC) and turbidy (OD_{640nm}) was measured at 2-6 hours intervals.

2.3 Mating tests

Strains were plated on sporulation medium (0.1% w/v glucose, 0.25% w/v yeast extract, 1% w/v potassium acetate, 1.5% w/v agar) and incubated at 25ºC for 3 -5 days in order to induce sporulation. Tetrads were recovered and incubated for 15 minutes in the presence of lyticase (0.4 mg/mL) in order to weaken the cell wall. Crosses were performed on YPD medium (1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose, 2% w/v agar). Tetrads were dissected and individual spores were crossed with a micromanipulator Zeiss-Axio Scope.A1. Observations with a microscope were performed after 4-6h in order to detect formation of zygotes. In order to measure the F1 fertility, spores from 50-60 tetrads (200-240 spores) were placed individually in YPD plates with the aid of a micromanipulator and the plates were incubated for three days at 25ºC. The viable spores were those that formed a visible colony. Hybrids were confirmed either by sequencing or RFLPs.

2.4 DNA sequencing

2.4.1 DNA extraction

Yeast cells were grown in YMA medium (YM with 1.5% w/v agar) for two days at 25ºC. About 2 g of cells were harvested and washed with sterile distilled water and placed in a microtube with microspheres overnight at -20 ºC. The resulting pellet was resuspended in 500 µl of lysis buffer (10 mM Tris-base, 1 mM EDTA, 100 mM NaCl, 2% w/v Triton X-100 and 1% w/v SDS), 250 µL of chloroform and 250 µL of phenol on TE buffer. This solution was vortexed for 20 min and centrifuged for 20 min at 16000 g. The aqueous portion was transferred to a new tube, a new phenol-chloroform extraction was performed and the DNA was precipitated with 1 mL of 100% ethanol. The solution was incubated at -20ºC for 30 min, centrifuged for 5 min at 16000 g and the resulting pellet was resuspended in 50 µL of TE buffer with RNAse.

2.4.2 PCR amplification and product purification

A total of six unlinked genes were amplified. Four nuclear genes: *EST2* (encodes for a reverse transcriptase subunit of the telomerase holoenzyme), *MET2* (encodes for a L-homoserine-O-acetyltransferase, catalyzes the conversion of homoserine to O-acetyl homoserine), *HIS3* (encodes for a imidazoleglycerol-phosphate dehydratase that catalyzes the sixth step in histidine biosynthesis), *FSY1* (encodes for a specific fructose symporter*)* and two mitochondrial genes*: SSU1* (encodes for the small subunit ribosomal of RNA) *and COXII* (encodes for the cytochrome c oxidase II). A volume of 2 µl of DNA was added to 48 µl PCR reaction mix containing 0.2 mM dNTP, 1X Taq Buffer with 2 mM $MgCl_2$, 0.2 µM of each *primer* (sequences on **Table S2**) and 1U of *DreamTaq*. Reactions were run on a *Biometra TGradient* thermocycler programmed as follows: an initial denaturation step at 95ºC for 5 min, followed by 40 cycles of denaturation at 95ºC for 1 min, annealing at 49ºC (*HIS3* and *EST2*) or 52ºC (*FSY1* and *SSU1*) or 53ºC (*COXII*) or 57ºC (*MET2*) for 1 min and polymerization at 72ºC for 1 min. The polymerization was completed by an additional 7 min of incubation at 72ºC. PCR products were purified using the *Ilustra GFX* purification kit, following the manufacturer's instructions.

2.5 DNA sequence analyses

2.5.1 Phylognetic trees

The six genes (*COXII*, *EST2*, *HIS3*, *FSY1*, *MET2* and *SSU1*) were sequenced by STABVIDA (http://www.stabvida.com/frontpage/eng/index.php).

Sequence comparisons and alignments were obtained using *BioEdit* v. 7.053 (http://www.mbio.ncsu.edu/bioedit/bioedit.html). When necessary, manual corrections were made. Phylogenetic trees were obtained using *MEGA* 5 (http://www.megasoftware.net/).

2.5.2 Phylogenetic networks

Split Networks were constructed using the algorithm Neighbour-Net available on *SplitsTree* v4 (Huson and Bryant, 2006).

2.5.3 Molecular diversity and DNA divergence between populations

Molecular diversities between strains were calculated in *DnaSP* v.5.10.01 (Librado and Rozas, 2009) using the Jukes and Cantor correction (Jukes and Cantor, 1969).

DNA divergence between populations was also calculated in *DnaSP* v.5.10.01 and was estimated taking into account the average number of nucleotide substitutions per site between populations (Dxy) using Nei's equation (Nei, 1987).

2.5.4 Population structure

Inference of population structure was made using the program *Structure* 2.1, assuming the admixture model, k=6 subpopulations and correlated allele frequencies with 1000000-iteration burn in, and 500000 iterations of sampling. Additionally, 15 interactions per run were performed. This software can be applied to most of the commonly-used genetic markers, including microsatellites, RFLPs and SNPs in order to investigate population structure (Pritchard et al., 2000). A model in which there are k populations (where k may be unknown) is assumed, each of which is characterized by a set of allele frequencies at each locus. It is assumed that within populations, *loci* are at Hardy-Weinberg Equilibrium, and linkage equilibrium and no particular mutation process is assumed. In this study, an admixture model was assumed which means that each individual could draw some fraction of its genome from each of the k populations, meaning that they may have mixed ancestry.

Distruct 1.1 software (Rosenberg, 2004) was used to graphically display the results produced by the genetic clustering obtained with *Structure* 2.1.

2.6 Microsatellite analyses

2.6.1 Microsatellite amplification and typing

The six primers for amplification of the three *loci* containing microsatellites were designed by Masneuf-Pomarède et al. (2005). Microsatellites are short DNA sequence stretches in which a motif of one to six bases is tandemly repeated. These sequences can differ in repeat number among individuals of the same species, and based in some studies that have been carried out in humans, *Drosophila* (Schlotterer and Harr, 2000) and mice (Rubinsztein et al., 1995; Makova et al., 2000) it seems that its mutation rates can range from 10^{-6} to 10^{-2} per generation being therefore significantly higher than base substitution rates which can range from 10^{-9} to 10^{-6} (Moryiama and Gojobori, 1992; Strachan and Read, 1999; Xue et al., 2009). Their hypervariability and ubiquitous occurrence made microsatellites one of the most popular choices for population genetic studies (Manel et al., 2003) since they have the potential to answer ecological and evolutionary questions (Selkoe and Toonen, 2006).

Amplification conditions were also adapted from Masneuf-Pomarède et al. (2005). Allelic diversity of the microsatellite loci was first observed in 3% agarose gel and molecular typing was performed by gel analysis on *Gelcompare* software. In order to confirm the number of repeats, 20% of the representatives of each group obtained were sequenced. In *locus* where low definition was obtained in the agarose gel, all the samples were sequenced.

2.6.2 Statistical Analysis

2.5.2.1 F-statistics and population distinctiveness

FSTAT (v. 2.9.3.2; Goudet, 2002) was used to examine Hardy-Weinberg Equilibrium and to calculate private alleles. The Hardy–Weinberg principle states that both allele and genotype frequencies in a population remain constant—that is, they are in equilibrium—from generation to generation unless specific disturbing influences are introduced. The testing for this parameter is commonly used as a quality control filter in for markers with experimentally determined genotypes.

2.5.2.2 Population differentiation

Exact tests for population differentiation, molecular diversities, analysis of molecular variance (AMOVA; 10 000 permutations) and pairwise F_{ST}s were performed using *Arlequin* 3.5.1.2 (Excoffier et al., 1992; Laval, 2005). AMOVA is a statistical model for studying molecular variation within a species and for estimating population differentiation directly from molecular data and testing hypotheses about such differentiation (Excoffier et al., 1992). A variety of molecular data such as direct sequence data may be analyzed using this method (Excoffier et al., 1992). The basic principle assumed is an extension of Wright's (1951) F-statistics to include multiple hierarchical levels of population structure (Weir and Cockerham, 1973) and to account for evolutionary distances (usually sequence divergence) among alleles (Excoffier et al., 1992). This analysis is widely used for quantifying the contribution of various levels of population structure to patterns of genetic variation. The AMOVA implementation in *Arlequin* uses permutation tests to evaluate the null hypotheses of no population structure within groups and between groups. If the genetic variance is higher within groups than between the same groups, one can say that the groups are not isolated from each other, being therefore a continuous identity and not two distinct populations. On the other hand, if the variance among groups is higher than within the groups, probably these groups are well isolated and therefore two populations can be recognized.

The exact test for population differentiation takes into account genotype or haplotype frequencies and is based in Markov chains (10.000 steps during the burnin and 100.000 steps after the burnin). The molecular diversity indices are calculated based on number of different alleles.

One of the most used tools to access to population differentiation is the F_{ST} (fixation index) parameter defined by Wright 1969 (Balloux and Lugon-Moulin, 2002). This parameter represents a measure of the diversity of randomly chosen alleles within the same sub-population relative to that found in the entire population. It is often expressed as the proportion of genetic diversity due to allele frequency differences among populations. This parameter varies from 0 to 1 where 0 indicates absence of differentiation and 1 indicates complete differentiation (Raymond and Rousset, 1995).

3 Results and Discussion

3.1 Phylogenetic analyses

The phylogenetic analysis (**Fig. 3**) of the partial sequences of three nuclear genes (*FSY1*, *HIS3* and *MET2*) provided information about strain relationship within *S. uvarum*. Three clades were formed, two of them corresponding to two specific geographic locations: Patagonia (clade B) and Australasia (Tasmania and New Zealand, clade C). The remaining strains grouped in a clade that included all the strains from the Northern Hemisphere and also some strains isolated in Patagonia (clade A). It is important to note the lower divergence between clade B (Patagonian group) and clade A (supported by a lower bootstrap value: 57%) when compared with the divergence found between clade A and clade C (Australasian group), which was supported by a high bootstrap value (99%). Still regarding the Australasian strains, it is possible to observe some genetic diversity between them (eight variable sites, four of which are parsimony informative). Although this diversity is much lower than the diversity found in the remaining populations (see **Table 1**) it is possible to differentiate the strains from Tasmania that group together in a separate branch (dark blue dots on phylogenetic tree in **Fig. 3**) from the strains collected in New Zealand (light blue dots). The Patagonian population presents the highest level of diversity (32 variable sites, 23 of which are parsimony informative) being found both on clade A and B presenting almost two times more nucleotide diversity (0.0041) than all the Northern Hemisphere strains (21 variable sites, 10 of which are parsimony informative) which present a nucleotide diversity of 0.0023 (**Table 1**). Therefore, two types of genotypic background were identified in Patagonia: one is unique to this region (clade B) and the other which is also found in the Northern Hemisphere (clade A).

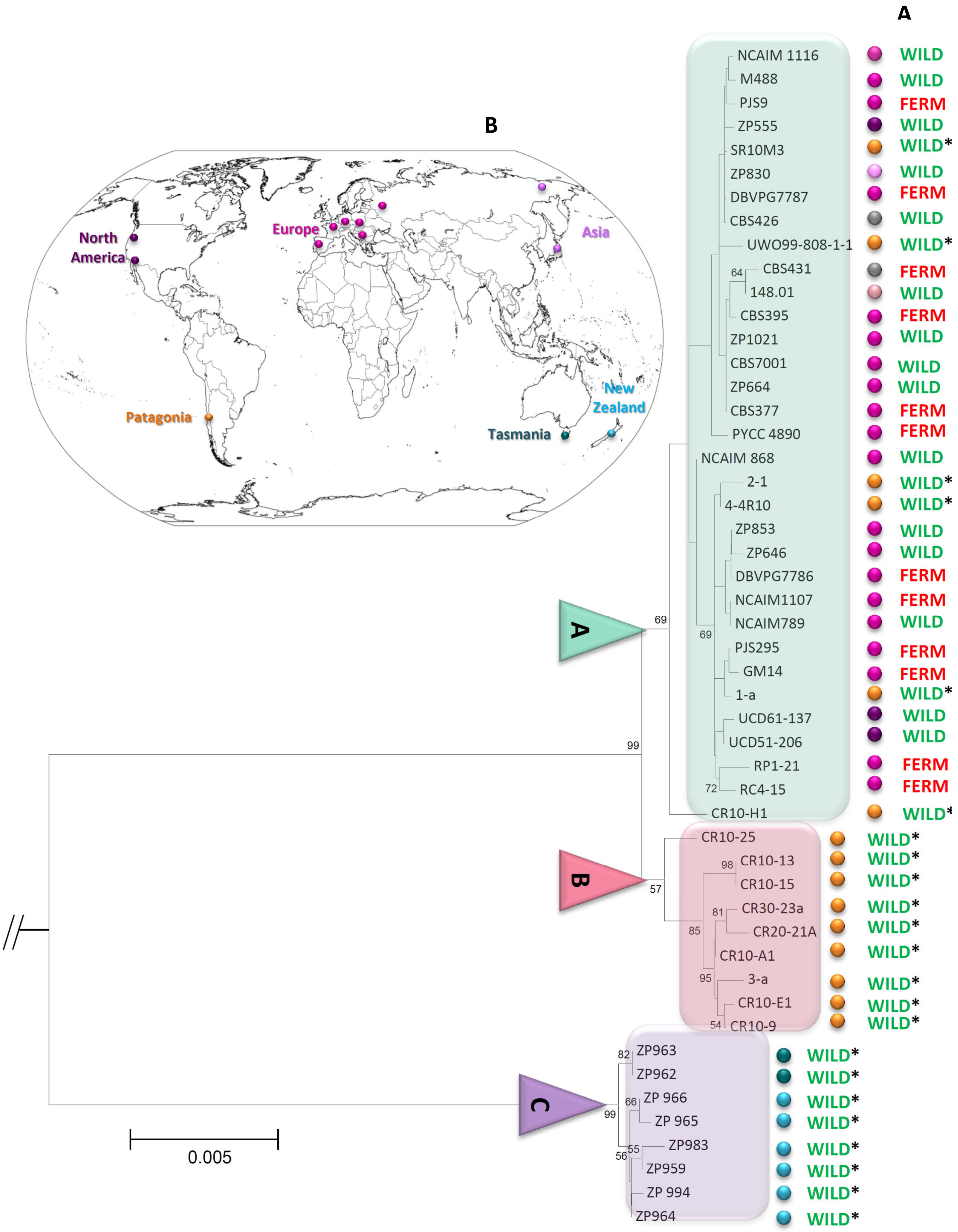

A
NCAIM 1116 WILD
M488 WILD
PJS9 FERM
ZP555 WILD
SR10M3 WILD*
ZP830 WILD
DBVPG7787 FERM
CBS426 WILD
UWO99-808-1-1 WILD*
64
CBS431 FERM
148.01 WILD
CBS395 FERM
ZP1021 WILD
CBS7001 WILD
ZP664 WILD
CBS377 FERM
PYCC 4890 FERM
NCAIM 868 WILD
2-1 WILD*
4-4R10 WILD*
ZP853 WILD
ZP646 WILD
DBVPG7786 FERM
NCAIM1107 FERM
NCAIM789 WILD
69
PJS295 FERM
GM14 FERM
1-a WILD*
UCD61-137 WILD
UCD51-206 WILD
RP1-21 FERM
72
RC4-15 FERM
CR10-H1 WILD*
69
A
99
CR10-25 WILD*
98
CR10-13 WILD*
CR10-15 WILD*
81
CR30-23a WILD*
CR20-21A WILD*
85
CR10-A1 WILD*
95
3-a WILD*
CR10-E1 WILD*
54
CR10-9 WILD*
B
57
82
ZP963 WILD*
ZP962 WILD*
66
ZP 966 WILD*
ZP 965 WILD*
99
C
55
ZP983 WILD*
56
ZP959 WILD*
ZP 994 WILD*
ZP964 WILD*
0.005
B
North America
Europe
Asia
Patagonia
New Zealand
Tasmania

Fig.3 – A) Concatenated Neighbor-Joining phylogeny of the three nuclear genes (*FSY1*, *MET2*, *HIS3)*. The bootstrap consensus tree inferred from 1000 replicates (only bootstrap values above 50% are displayed). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. All ambiguous positions were removed for each sequence pair. There were a total of 2465 positions in the final dataset, 128 of which are parsimony-informative. Major clades are depicted in different colors and marked with a triangle. Dots indicate the geographical origin of the isolates (grey dots correspond to unknown geographical location). The type of isolation substrate is described as WILD or FERM, depending on whether the strain was isolated from nature or fermentative processes, respectively. The WILD strains marked with * were isolated from the *Nothofagus /Cyttaria* system. B) World map indicating the major sampling areas. Geographical regions are marked with dots with different colors.

All the strains from the Southern Hemisphere were isolated from *Nothofagus* trees (either bark or soil next to the tree) or from *Cyttaria* but no species-specific association was found. Although the wild strains isolated in the Northern Hemisphere were isolated from more diverse substrates it is not yet known if *S. uvarum* is strictly associated with the *Nothofagus* – *Cyttaria* system in the Southern Hemisphere.

To investigate the possible domestication of *S. uvarum*, several strains isolated from fermentation processes were included in the study. Thus, concerning the substrate of isolation it is possible to divide the strains in two major groups: WILD (strains isolated from nature) and FERM (strains isolated from fermentation processes as winemaking, cider making and other related fermentations). In order to avoid biased results, only the wild strains from Europe (EUR: WILD, **Table 1**) were compared with those obtained from man-driven fermentations (EUR: FERM, **Table 1**) since all the fermentation-related strains were isolated in Europe (see **Fig. 3** or **Table S1** in Appendix I).

Table 1- Molecular diversity for each population represented as nucleotide diversity of nuclear genes (Jukes Cantor algorithm). Populations were geographically delimited (AUST: Australasia; PAT- Patagonia and NH- strains from Northern Hemisphere) or separated by the substrate of isolation (EUR:WILD-European strains isolated from nature; EUR:FERM-European strains isolated from fermentations).

Populations	Jukes Cantor Nucleotide Diversity (Nuclear genes)
AUST	0.0012
PAT	0.0041
EUR:WILD	0.0017
EUR:FERM	0.0028
NH	0.0023

Taking previous studies into consideration, domesticated strains are expected to have low genetic diversity and to phylogenetically derive from “wild” stocks (Fay and Benavides, 2005). In the phylogenetic tree of **Fig. 3** there is no clear relationship between the strains isolated from fermentations (FERM) as they are distributed along clade A. Furthermore, it is not possible to infer any ancestry of the European WILD group relatively to the FERM group. Moreover, the genetic diversity of FERM strains is higher than that of WILD strains (**Table 1**), which suggests that domesticated strains are absent in *S. uvarum*. It is important to note that information has to be accurate in order that the results of such a study are meaningful. For example, wild strains isolated from fermentations as contaminants will falsify the results. Although these preliminary results require confirmation, they suggest some similarities with what is known for *S. paradoxus* where geography, instead of ecology, plays the main role in driving genetic diversity.

Additionally to nuclear genes, two unlinked mitochondrial genes were sequenced (*COXII* and *SSU1*) for 27 strains representative of the clades obtained with nuclear genes. Concerning the mitochondria, it is known that mtDNA might not have the same evolutionary history as its nuclear counterpart due to its peculiar inheritance pattern. It is also known that in *Saccharomyces* yeasts, when mating occurs the resulting hybrid inherits both parental sets of chromosomes but mtDNA from only one parent (Marioni et al., 1999). Therefore, because of the different evolution rates and evolutionary histories between the two types of DNA, an independent phylogenetic analysis was performed (**Fig. 4**). The nuclear and mitochondrial phylogenetic trees were not totally congruent. The Australasian population seems to be more related to *S. bayanus* than to the remaining populations of *S. uvarum*. Furthermore, strains from the Northern Hemisphere and Patagonia are now grouped into one clade.

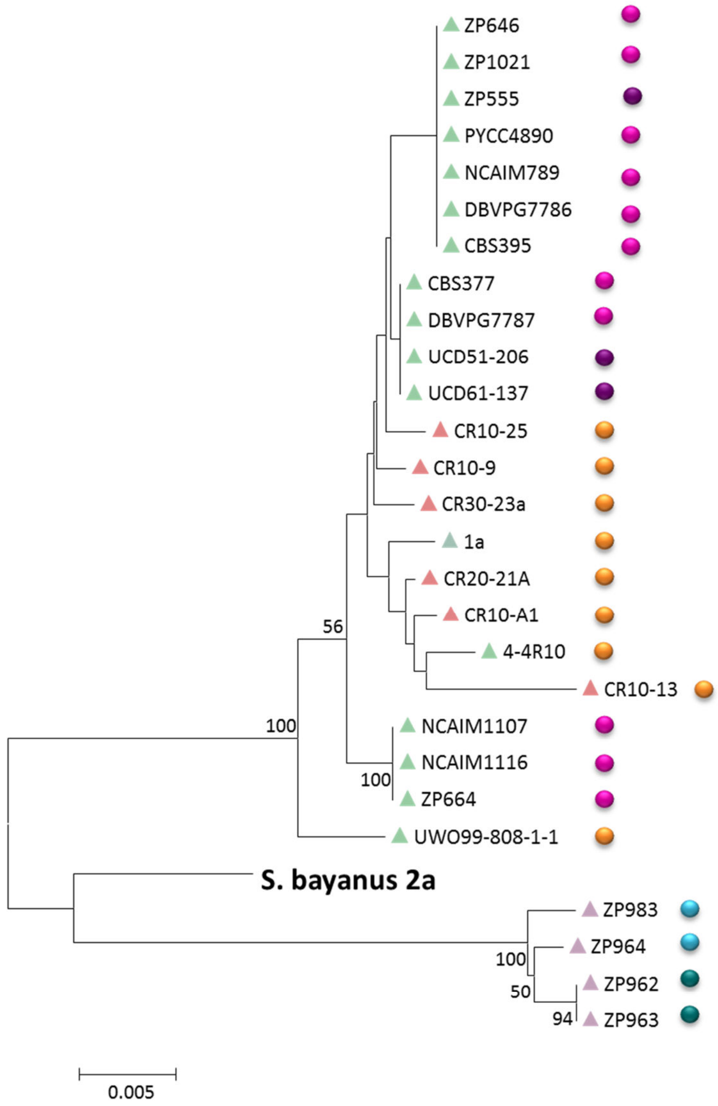
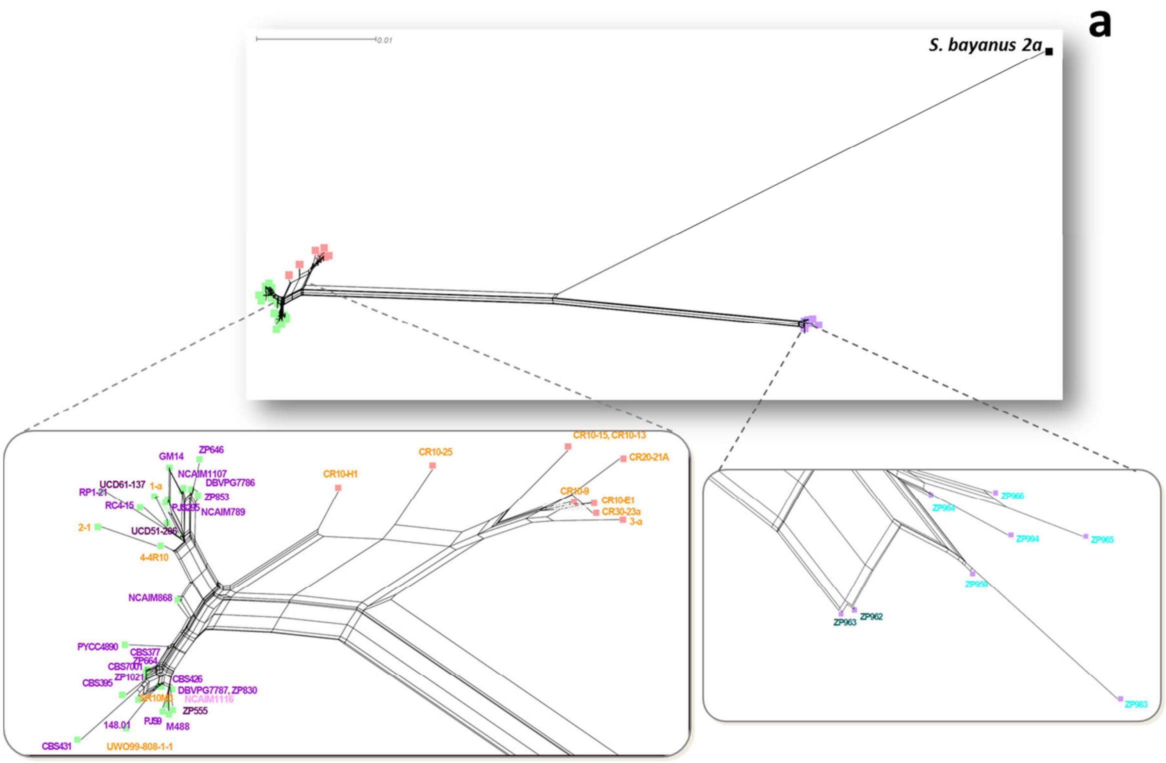


Fig.4 - Neighbor-Joining phylogeny of *SSU1* and *COXII* genes for 27 representative strains of *S. uvarum*. The bootstrap consensus tree was inferred from 1000 replicates (only bootstrap values above 50% are displayed) taking into account the specific evolutionary history of the data (yeast mitochondria). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. All ambiguous positions were removed for each sequence pair. There were a total of 1094 positions in the final dataset, 60 of which were parsimony-informative. Triangles represent the phylogenetic clades according to Fig. 3. Circles indicate the geographical origin of the isolates according to the code color on Fig. 3.

To overcome the phylogenetic incongruences and the low bootstrap values of some branches in both phylogenetic trees (**Fig. 3** and **Fig. 4**), a Neighbor-Net Network was constructed (**Fig. 5**). Phylogenetic networks are useful to detect more complex evolutionary histories, including reticulate events, such as hybridization, recombination and horizontal gene transfer. The Neighbor-Net is a distance based method for constructing split phylogenetic networks that is based on the Neighbor-Joining algorithm (Bryant and Moulton, 2003). The resulting networks of both mtDNA and nuDNA are displayed on **Fig. 5,** where clearer phylogenetic relationships are shown. The interpretation of these results requires a clarification of the assumptions behind the design of a split network. The parallel edges are used to represent the splits computed from the data and each split is a divider of the taxa into two subsets, being therefore a bipartition of the taxon set (which could be compared with the partition obtained when a branch is removed from a phylogenetic tree). Its length is proportional to the weight of the associated split, being analogous to the length of a branch in a phylogenetic tree. It is often necessary that these networks contain nodes (that not always represents ancestral individuals) so that the incompatible splits may be accommodated. Therefore it is possible to find a number of parallel edges associated with each split (Huson and Bryant, 2006). Split networks make an explicit representation of evolutionary history, as the internal ‘‘nodes’’ represent ancestral species (but not all the nodes), nodes with a single parent correspond to absence of reticulated events (represented by parallel lines in the network) and nodes with more than two parents (represented by perpendicular lines in the network) correspond to reticulate events such as recombination, hybridization or lateral gene transfer (Huson and Bryant, 2006). Taking this into account, in the expanded images of **Fig. 5** multiple reticulate events involving strains from Patagonia and Northern Hemisphere can be observed. Furthermore, reticulate events between clade C (Australasian strains) and the remaining strains were few which reinforces the evidence for strong isolation of the Australasian population.



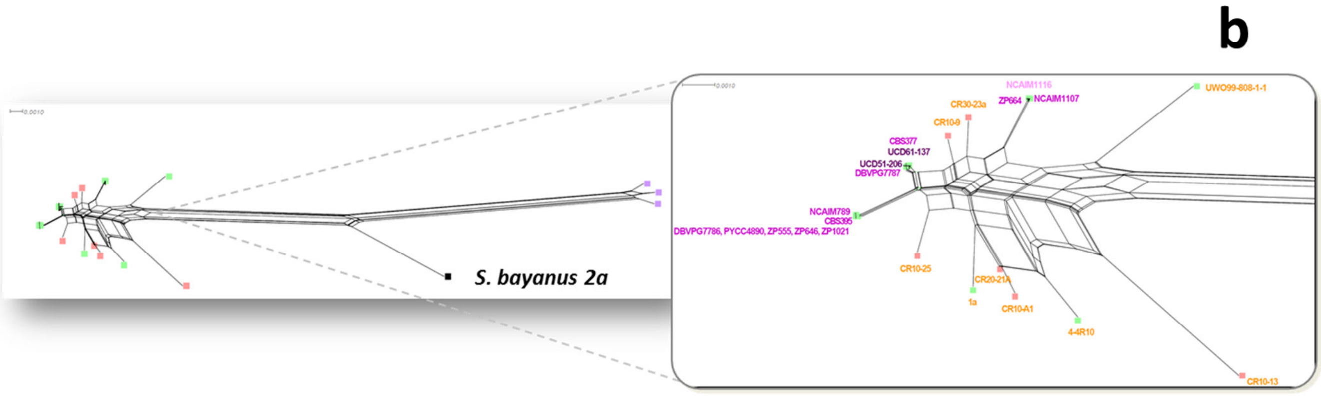


Fig.5 – Neighbor-Net networks of nuDNA and mtDNA. a) nuclear DNA network constructed with *FSY1*, *HIS3* and *MET2* sequences data; b) mtDNA network constructed with *SSU* and *COXII* sequence data. The circles in the map represent the major clades according to Fig 3. Network details are shown in lined rectangles (expanded images). The color codes for strain names on the expanded images refer to the geographical origin according to Fig. 3.

The divergence of the Australasian population is in agreement with the results of the phenotypic tests (**Table S4** and **S5** in Appendix I). Specifically, there are no differences in carbon source assimilation and growth temperatures between Patagonian and Northern Hemisphere strains but the isolates from Australasia showed a lower maximum growth temperature and a general inefficiency of growth in the majority of the carbon sources (**Table S4**). Furthermore, the Australasian isolates displayed an expanded period of latency when grown in melibiose or did not grow at all. As the fermentation of melibiose is one of the distinctive features of *S. uvarum* (Naumov, 2000a), these results raised some doubts about the identity of this population. To address this topic, mating tests involving Australasian strains and strains from other geographical locations were performed in order to determine the existence of post-zygotic isolation.

3.2 Reproductive isolation

The process of speciation occurs by two general mechanisms and it is central to biological sciences in general and evolutionary studies, in particular. The first mechanism is ecological speciation and it is defined as the evolution of reproductive isolation between populations, or subdivisions of a single population, as a result of ecologically-based divergent natural selection. Under this process, natural selection acts differently between environments, driving the fixation of different alleles; each one advantageous in its own environment (Schluter and Conte, 2009). The other mechanism is called mutation-order speciation, where the populations diverge as they accumulate different sets of mutations under similar selective pressures. Natural selection drives alleles to fixation in both speciation mechanisms, but selection favors divergence only under ecological speciation (Schluter and Conte, 2009).

Therefore, the level of reproductive isolation can be used as a measure of biological distance between populations and in the case of *Saccharomyces* yeasts it can even be represented as a percentage of viable F1 hybrid spores, since there is no premating reproductive isolation. What compromises the success of hybridization is the postmating reproductive isolation which can cause hybrid sterility, causing a certain quantity of the gametes produced by F1 progeny to be unviable (Greig, 2009). There were two major questions that were addressed through this analysis. First, the degree of reproductive isolation of the Australasian population was investigated and secondly reproductive isolation between the Patagonian and the Northern Hemisphere populations was analyzed.

Table 2- Crosses and percentage of spore viability of F1 hybrids. Colored cells represent the phylogenetic group to which each strain of *S. uvarum* belongs according to Fig. 3. Specific geographical location is displayed on the cells (A- Australasia, P-Patagonia, E-Europe; F- Far East; N- North America). Sequence divergence of *EST2* gene between the individuals was also calculated. The black cells represent the *outgroup* species (*S. bayanus* 2a). In order to make a comparison, results from previous works were also included (Sp- *S. paradoxus; Sc- S. cerevisiae*).

Crosses			Viable Spores (%)	No. of spores analyzed	Polymorphic Sites	Sequence divergence[a]
Su ZP964 x Su 4-4R10	A	P	35.8	400	70	0.06926
Su ZP1021 x Su 4-4R10	E	P	96.8	218	0	0.00000
Su ZP1021 x Su CR10-15	E	P	96.7	212	15	0.01431
Su CR10-15 x Su 4-4R10	P	P	97.2	204	15	0.01431
Su 4-4R10 x Sb 2a	P	B	7.3[b]	362[b]	99	0.09995
Su CR10-15 x Sb 2a	P	B	10.3	212	100	0.10103
Su ZP964 x Sb 2a	A	B	18.8	204	80	0.07970
Su ZP1021 x Sb 2a	E	B	15.6	218	99	0.09995
Sp N44 x Sp YPS125	F	N	36.3[c]	424[c]	36	0.03503
Sp NBRC1804 X Sp YPS125	F	N	32.1[c]	352[c]	*	*
Sp N44 X Sp NBRC 1804	F	F	86.8[c]	280[c]	*	*
Sp CBS432 X Sp YPS125	E	N	40.4[c]	416[c]	*	*
Sc S288c X Sp CBS432	N	E	0.67[c]	892[c]	*	*

[a] Nucleotide diversity of *EST2* gene using the Jukes and Cantor correction.

[b] Values taken from Libkind et al., 2011.

[c] Values taken from Liti et al., 2006.

*assay not performed in this study

Concerning the cross between strains from New Zealand (ZP 964, clade C) and Patagonia (4-4R10, clade A), a total of two F1 hybrids were obtained and analyzed in terms of spore viability. It was found that only 36% (**Table 2**) of the spores were viable, which means that there is, indeed, partial reproductive isolation between strains from clade C (Australasia) and strains from clade A. Liti et al. (2006) searched for a correlation between reproductive isolation and sequence divergence in some *Saccharomyces* species, including *S. paradoxus* and *S. cerevisiae*. In that study, inter and intra-species crosses were performed and some correlation between reproductive isolation and sequences divergence was found, as the highest spore viabilities were found when strains with less genetic divergence were crossed (**Table 2**). In the case of *S. paradoxus* this is directly correlated with geographical distance, therefore strains that from geographically closer populations originate F1 hybrids with higher spore viabilities. For instance, values of 32.1-36.3 % of spore viability of F1 hybrids were found for *S. paradoxus* populations from different continents. Furthermore, concerning for inter-species crosses much lower values (7% *S. uvarum* x *S. bayanus* and 0.67% *S. cerevisiae* x *S. paradoxus*) are typically obtained. Therefore, this type of reproductive isolation found for the Australasian strain is not enough to consider this group as a different species.

Additionally, phylogenetic distinct Patagonian strains were also crossed (**Table 2**), giving rise to a highly viable progeny (97.2% of viable spores). Taking these results into consideration, although the phylogenic analyses (**Fig. 3**) showed some differentiation of the Patagonian group (clade B) and some sequence divergence was also found (**Table 2**), this differentiation does not correlate to reproductive isolation. These results are consistent with those obtained previously with the network analysis (**Fig. 5**) where evidence for genetic contact between these two clades was found.

Another interesting observation is that there are different degrees of reproductive isolation between *S. uvarum* and *S. bayanus*. When Patagonian strains of *S. uvarum* were crossed with Patagonian strains of *S. bayanus* the percentage of spore viability of F1 hybrids was lower (7% and 10%, **Table 2**) than that of crosses involving strains of *S. uvarum* from other regions (19% for the Australasian strain and 16% for the cross involving European strain). This might be related with reinforcement, a process by which natural selection increases reproductive isolation between sympatric populations (Nosil et al., 2002; Servedio and Noor, 2003).

3.3 Population Differentiation

A population can be considered as a group of individuals that is well separated from other groups of individuals of the same species. Separation between populations may be geographical or genetic (Xu, 2006). Through phylogenetic analyses it was found that the Patagonian strains have two types of genotypic background and were grouped in A and B clades (**Fig. 3**). However, network analysis did not support this separation and a connection between clade A and clade B was suggested. Moreover, mating tests revealed no reproductive isolation between strains from these two groups.

To investigate in more detail the relationship between the South American strains and those from the Northern Hemisphere, sequence data from the three nuclear genes was analyzed in *Structure*, a software that allows the inference of distinct populations and assign each individual to the populations that were formed (Pritchard et al., 2000). Therefore, this analysis could be useful to understand the structure of the populations of *S. uvarum* as well as to identify migrant or admixed individuals. The most probable number of populations (k) is determined based in Ln (k) variation where the k that presents the higher value of Δk is the one that most likely represents the number of existing populations (**Fig. 6a**). The *Structure* analysis found evidence for two genetic clusters, one corresponding to the Eurasian, North American and Patagonian isolates and the other corresponding to the Australasian isolates (**Fig. 6b**). Therefore, this analysis rejected the hypothesis of a genetic separation between Patagonia and the Northern Hemisphere. Since the identification of migrants or mosaic individuals could be useful, a higher number of genetic clusters was subsequently explored. When the program was forced to consider a higher number of clusters ($3\leq k \leq 6$), only three clusters were identified (**Fig. 6c**).

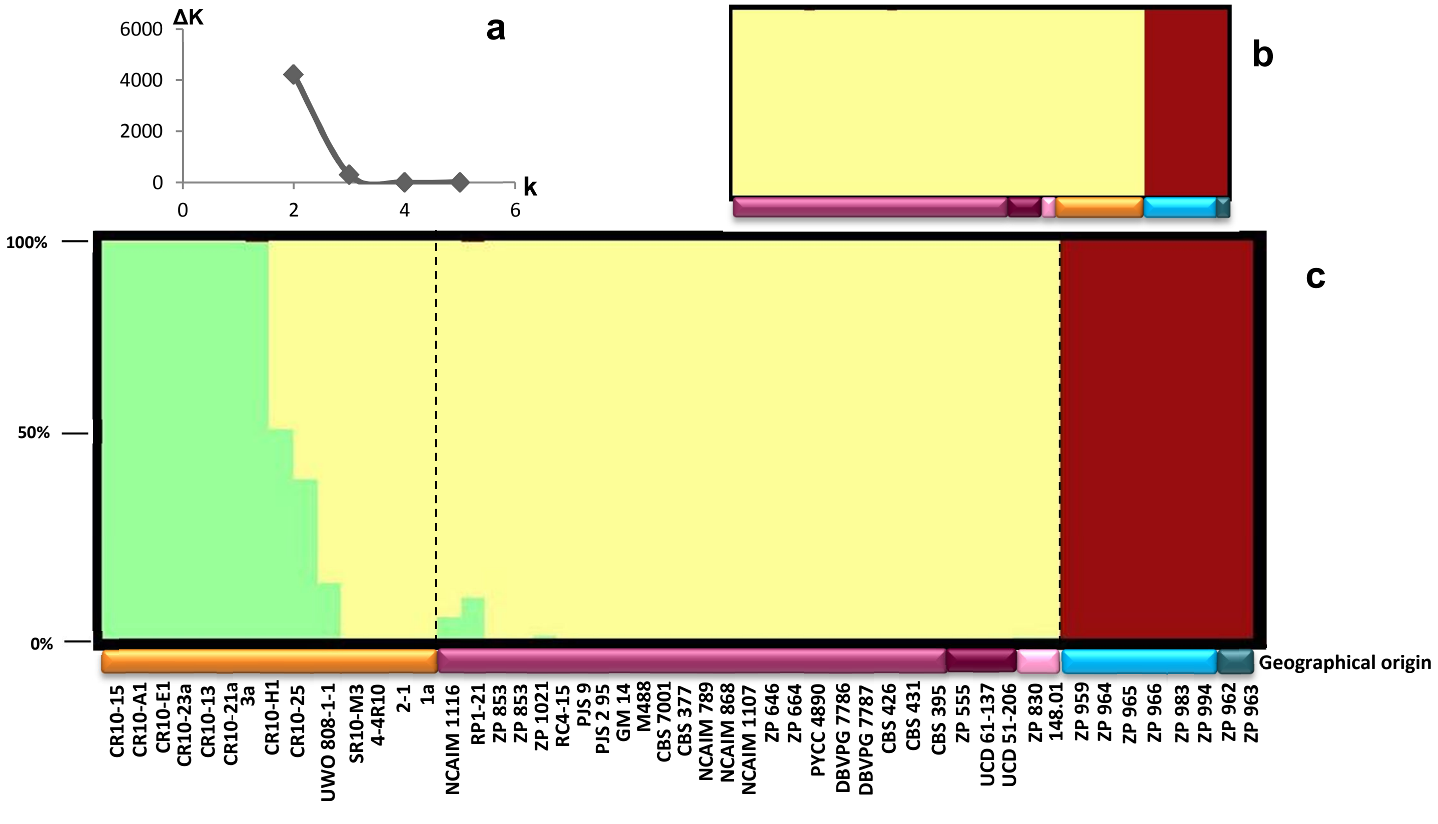


Fig. 6- Inference of population structure of *S. uvarum* using DNA sequences of the three nuclear genes (*FSY1*, *HIS3* and *MET2*)**.** a) *Structure* software ΔK plot representing the variation of Ln (k) in each k test. The higher ΔK value corresponds to the most probable number of populations (k); b) Plot displaying the two populations found by *Structure* software. Each color in the plots is associated with the different clusters formed; c) Resulting plot for k≥ 3. Each color in the plots is associated with the different clusters formed. Names of the strains and their geographical origin (color-coded according to the map on Fig.3) are displayed.

In this case, the Patagonian population was found to contain two clusters (represented by green and yellow colors in **Fig. 6c**), with some strains having admixture genotypes. This pattern naturally contributed to the high diversity of Patagonian strains. Strain CR10-H1 presents an apparent equal mixture of the two clusters. Two more Patagonian strains present complex genotypes with different degrees of admixture (CR10-25 and UWO-808-1-1). In the Northern Hemisphere, the yellow genotype is dominant. However two European strains (NCAIM 1116 and RP1-21) present a minor contribution of the green cluster. This indicates that these individuals might have a mixed ancestry, having inherited fractions of their genome from Patagonian migrants.

The next step of the study involved microsatellite genotyping. Three microsatellite *loci* were amplified, typed and sequenced (for approximately 70% of the strains). These sequences can differ in repeat number among individuals of the same species and it seems that mutation rates of microsatellites are significantly higher than substitution rates of coding sequences (Masneuf-Pomarède et al., 2007). Therefore, their hypervariability and ubiquitous occurrence made microsatellites one of the most popular choices for population genetic studies (Manel et al., 2003). Several tests were performed taking into account the groups previously defined (see **Table 1**). First, an AMOVA (Analysis of MOlecular VAriance) was performed for each pair of groups using the software *Arlequin*. In **Table 3** can be observed that when Northern Hemisphere and Patagonian populations are considered, the variance is higher within groups (68.87%) than among groups (31.13%) which points to an absence of population structure between Patagonian (PAT) and Northern Hemisphere (NH) strains, as already suggested. On the other hand, it can be seen that the Australasian (AUST) population is isolated from the Northern Hemisphere (NH) group as 65% of the molecular variance is explained by differences between groups and only 35% is explained by differences within groups. Interestingly, the variance within the Australasian and Patagonian populations was similar to the variance among these populations, which suggests that genetic divergence was lower than that found between Australasian population and the population of the Northern Hemisphere.

Table 3- Analysis of molecular variance for pairs of populations. Percentage of molecular variance explained by differences among and within populations.

Pairs of Populations	Variance Among Populations	Variance Within Populations
NH-PAT	31.13 %	68.87 %
NH-AUST	64.80 %	35.20 %
AUST-PAT	46.85 %	53.15 %

To consolidate population differentiation analysis, exact tests for population differentiation were performed (**Table 4**). This analysis suggested that the Patagonian, Australasian and Northern Hemisphere populations are differentiated.

Table 4 - P-values for population differentiation. Evidences for differentiation were found when p<0.005. Values in bold are those that are more relevant for the study.

	AUST	PAT	WILD	FERM	NH
AUST		**Differentiation**	Differentiation	Differentiation	**Differentiation**
PAT	**0.0002±0.0001**		Differentiation	Differentiation	**Differentiation**
WILD	0.0008±0.0003	0.0004±0.0001		No differentiation	No differentiation
FERM	0.0028±0.0009	0.0030±0.0007	0.1482±0.0057		No differentiation
NH	**0.0001±0.0001**	**0.0002±0.0001**	0.9919±0.0012	0.9972±0.0002	

Since differentiation between the Northern Hemisphere and Patagonian populations had not been detected before, this particular aspect was analyzed with more detail. Therefore, pairwise F_{ST}s were calculated (**Table 5**). This analysis compares the diversity of alleles in the subpopulation relatively to the entire population. The value of 0.495 obtained for the comparison of Australasian and Patagonian populations suggests little differentiation since a value of 0 means no differentiation and 1 means total differentiation. It can, therefore be anticipated that these two groups are not well separated from each other. In addition, the lack of differentiation between Patagonia and the Northern Hemisphere was confirmed (F_{ST}=0.3301). Furthermore, a value of ~0.6 was obtained between NH and AUST, indicating some population differentiation, which is congruent with all the previous results. Concerning the comparison between wild strains (WILD) and fermentation-related strains (FERM), a value of 0.1025 was obtained, confirming the lack of differentiation between the two groups.

Table 5 - Population pairwise F_{ST}s. The (+) or (-) signals indicates whether the F_{ST} values are statistically significant or not. Bolded values are those that are more relevant for the study.

	AUST	PAT	WILD	FERM	NH
AUST		**0.4949**	0.6748	0.6223	**0.5950**
PAT	**+**		0.3837	0.3153	**0.3301**
WILD	+	+		0.1025	0.0178
FERM	+	+	-		-0.0004
NH	**+**	**+**	-	-	

It was not possible to infer population structure with microsatellite information in *Structure* software since the data could not be stabilized due probably to the insufficient number of *loci* studied.

3.4 Inference of ancestry

In preliminary analyses it was found that the Australasian strains presented base substitutions in microsatellite sequences that seemed to be fixed in the population (**Fig. S1**). On the other hand, base substitutions were also identified in some strains from Patagonia, although they were not found to be fixed in the population. These events were found much less frequently in the Northern Hemisphere strains. The apparent fixation of these substitutions raised the hypothesis of the ancestry of the Australasian population. And therefore, additional analyses were carried out in order to explore this possibility. The number of unique alleles in a population can be used as a measure of genetic distinctiveness (Castric and Bernatchez, 2003) and can help to infer ancestry since ancestor genotypes tend to have a higher number of private alleles with high frequency in the population, while low frequencies could be indicative of incipient differentiation. It was found that $(TA)_5$ was completely fixed in the Australasian population suggesting that this population may be ancestral and isolated. It was also found that the Patagonian population presented the highest number of private alleles with high frequencies (>30%) (**Table 6**). Most of the private alleles found in the Northern Hemisphere had low frequencies in the population (<30%) which suggests a relatively recent origin. Moreover, when the Northern Hemisphere is divided in four subpopulations (North America, Central Europe, Eastern Europe and Asia), no private alleles are found in any of these populations (data not shown).

Table 6- Private alleles found in each population. Number (N_A); probability of finding ($p(N_A)$) ; and frequency in the population (%) of private alleles.

Populations	***Locus***	**N_A**	**$p(N_A)$**	**%**
AUST	$(TA)_5$	1	0.20	100.0%
	$(ATT)_7$; $(ATT)_8$	2	0.33(3)	12.5% ; 12.5%
PAT	$(CTG)_{11}$	1	0.16(6)	30.8%
	$(TA)_6$	1	0.20	69.2%
NH	$(TA)_{11}$	2	0.33(3))	14.3%
	$(CTG)_7$; $(CTG)_8$	2	0.33(3)	4.8% ; 4.8%
	$(TA)_{13}$	1	0.16(6)	33.3%
NH + PAT	$(CTG)_9$	1	0.16(6)	35.1 %
	$(TA)_{11}$	1	0.20	22.6%
	$(ATT)_{10}$; $(ATT)_{10}$	2	0.33(3)	19.6% ; 50.2%
AUST + PAT	$(CTG)_{12}$	1	0.16(6)	61.9%
	$(ATT)_9$	1	0.16(6)	57.1%

It is important to notice that two alleles were found to be shared by the Patagonian and Australasian populations and both are present in the majority of strains of each population**.** This result, combined with the molecular variance analysis (**Table 3**) and the results for population differentiation (**Table 5**), suggests that Patagonia and Australasia have some genetic relatedness. Furthermore, several private alleles were found to be shared between the Patagonian and Northern Hemisphere populations suggesting a genetic relatedness that was already discussed.

3.5 An evolutionary hypothesis

Drawing evolutionary histories for microorganisms is complicated as inferences from the past are limited to the study of existing genomes and phenomes. In this work several approaches were used, ranging phylogeographic analyses to phenotypic characterizations and population genetic analyses. The results obtained suggest that the highest molecular diversity of *S. uvarum* is found in Patagonia. The Patagonian population seems to be related to the population of the Northern Hemisphere due to the lack of population structure between these two broad geographical locations, low level of molecular divergence, absence of reproductive isolation and because genotypes found in Patagonia are also found in North America and Eurasia. Additionally, evidence of ancestry was found in Patagonia due to the existence of two private alleles with high frequencies (**Table 6**). This allows to put forward the hypothesis of the migration from Patagonia to the Northern Hemisphere, and a bottleneck effect associated with a loss of diversity during this migration.

It was also found that the Australasian population was homogenous but divergent (**Table 1**) from to the remaining groups. Signs of ancestry were also found for this population due to the existence of a unique and fixed allele. Additionally, evidence of contact with the Patagonian population was found due to the presence of unique alleles common to this two groups (**Table 6**), and due to the weak evidence for population structure in AMOVA and F_{ST} analysis (**Table 3** and **Table 5**). Therefore it seems possible that there was ancient contact between the two populations, but that their subsequent isolation led to partial reproductive isolation. Another aspect that links these two populations is the association with the *Nothofagus*–*Cyttaria* system. These two genera are found solely in the Southern Hemisphere and their present distribution is associated with the Gondwana break up (Li and Zhou, 2007; Peterson et al., 2010). Although there is no evidence that *S. uvarum* is strictly associated with *Nothofagus* in the Southern Hemisphere, this association found for the South American and Australasian *S. uvarum* populations is noteworthy.

4 Concluding Remarks and Future Perspectives

The apparent habitat and substrate preference similarities between *S. uvarum*, *S. cerevisiae* and *S. paradoxus*, and the lack of phylogenetic and population genetic information for *S. uvarum* at a global scale, led us to set out an exploratory study in order to clarify the natural history of this yeast. Unlike what was found for *S. cerevisiae* (Fay and Benavides, 2005; Legras et al., 2007), no ecological specialization was found in *S. uvarum* and no evidence of domestication events could be obtained. Instead, it was found that genetic divergence is more associated with the geography, similarly to what was found for *S. paradoxus*. However, in the case of *S. paradoxus* the geographical specialization operates at a continental scale (Liti et al., 2009) and partial reproductive isolation was found between strains from North America and Europe (Liti et al., 2006). In the case of *S. uvarum*, the geographical influence is solely found for the Australasian strains. Moreover, the reproductive isolation found for the Australasian strain was comparable to that found for *S. paradoxus* strains from different continents (**Table 2**).

It is important to note that the results presented here have to be viewed as preliminary and exploratory because a deeper sampling is needed to cover adequately the various regions that were considered. This would allow a more robust study of the populations and gene flow between them, which is important for the understanding of the possible migrations in *S. uvarum*. Concerning the results obtained with microsatellite data it is important to refer that these analyses are highly influenced by sample size and number of alleles studied. In fact, larger populations can present higher number of alleles and higher molecular diversity (allelic richness). In the present study only three *loci* were analyzed, therefore it would be recommendable to increase the number of microsatellite *loci*. Ideally, complete genome data would of interest to conclusively overcome most of the uncertainties of the work presented here. Nevertheless, this work represents the first attempt to study *S. uvarum* at the population level at a continental scale, representing the initial step to clarify, at a global scale, the natural history of this yeast.

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Appendix I

Table S1- Strains studied in this work with respective locality and substrate information.

Species	Strain	Locality	Substrate
S. uvarum	CBS 377	Germany	Pear wine
S. uvarum	CBS 395^T	Netherlands	Juice of *Ribes nigrum*
S. uvarum	CBS 426	unknown	Honey
S. uvarum	CBS 431	Unknown	Fermenting juice of Marx pears
S. uvarum	CBS 7001	Spain	*Mesophylax adopersus*
S. uvarum	NCAIM 789	Hungary	Exudate of *Carpinus betulus*
S. uvarum	NCAIM 868	Hungary	Slimy material on a stump
S. uvarum	NCAIM 1107	Hungary	Canned sour cherry
S. uvarum	NCAIM 1116	Russia	Grape berries
S. uvarum	DBVPG 7786	Czech Republic	Wine
S. uvarum	DBVPG 7787	Slovakia	Wine
S. uvarum	PYCC 4890	Portugal	Less of wine
S. uvarum	UCD 51-206	California, Porcupine flat	*Drosophila persimiles*
S. uvarum	UCD 61-137	California, Berryessa Hills	*Drosophila pseudoobscura*
S. uvarum	ZP 555	Canada	*Quercus. garryana*
S. uvarum	ZP 646	Germany	Cider
S. uvarum	ZP 664	Germany	*Quercus robur*
S. uvarum	ZP 830	Japan	*Quercus glauca*
S. uvarum	ZP 853	Germany, Bochum	*Fagus sylvatica*
S. uvarum	ZP 1021	Portugal	Soil from *Castanea sativa*, Douro
S. uvarum	UWO 99-807-1-1	Patagonia	*Nothofagus* sp.
S. uvarum	4-4R10	Patagonia, Rucahuenu	*Cyttaria* on *Nothofagus dombeyi,* 2006
S. uvarum	2-1	Patagonia, Rucahuenu	*Cyttaria* on *Nothofagus dombeyi,* 2006
S. uvarum	1-a	Patagonia, Los Rapidos	*Cyttaria on Nothofagus dombeyi, 2006*
S. uvarum	3-a	Patagonia, Rucahuenu	*Cyttaria on Nothofagus dombeyi, 2006*
S. uvarum	SR10-M3	Patagonia, Colonia Suiza, Bariloche	*Cyttaria on Nothofagus Dombeyi, 2007*
S. uvarum	CR10-H1	Patagonia,Los rápidos	*Cyttaria on Nothofagus dombeyi, 2007*
S. uvarum	CR10-A1	Patagonia,Cerro Otto, Bariloche	*Cyttaria on Nothofagus pumillio , 2007*
S. uvarum	CR10-E1	Patagonia,Tronador road	*Cyttaria on Nothofagus antarctica, 2007*
S. uvarum	CR30-23a	Patagonia, Camino a Frias	*Nothofagus dombeyi*, 2008
S. uvarum	CR20-21a	Patagonia	*Nothofagus antarctica, 2008*
S. uvarum	CR10-25	Patagonia	*Nothofagus antarctica, 2008*
S. uvarum	CR10-15	Patagonia	*Nothofagus pumillio, 2008*
S. uvarum	CR10-13	Patagonia, Perito Moreno	*Nothofagus pumillio,* 2008
S. uvarum	CR10-9	Patagonia	*Nothofagus alpina, 2008*
S. uvarum	ZP 959	New Zealand, Lewis Pass	*Cyttaria gunni* on *Nothofagus menziesii*
S. uvarum	ZP 962	Tasmania, Mount Field National Park	*Nothofagus cunninghamii*
S. uvarum	ZP 963	Tasmania, Mount Field National Park	*Nothofagus cunninghamii*
S. uvarum	ZP 964	New Zealand, Lewis Pass	*Nothofagus solandri* var. *solandri,*
S. uvarum	ZP 965	New Zealand, Lewis Pass	*Nothofagus solandri* var. *solandri*
S. uvarum	ZP 966	New Zealand, Lewis Pass	*Nothofagus solandri* var. *solandri*
S. uvarum	ZP 983	New Zealand, Lewis Pass	*Nothofagus solandri* var. *solandri*
S. uvarum	ZP 994	New Zealand, Lewis Pass	*Nothofagus solandri* var. *solandri*
S. uvarum	148.01	Russia, Blagoveshchensk	Exudate of the elm Ulmus pumila
S. uvarum	GM 14	France, South West	Fermented Must
S. uvarum	PJS 9	France, Sancerre	Fermented Must
S. uvarum	RC4-15	France, Alsace	Fermented Must
S. uvarum	M 488	Moldova	Grapes, monosporic clone, GI Naumov
S. uvarum	RP1-21	France, Alsace	Fermented Must
S. uvarum	PJS 2-95	France , Sancerre	Fermented Must
S. bayanus	2-a	Patagonia	*Cyttaria* in *Nothofagus dombeyi*, 2006

Table S2- Sequences and annealing temperatures of the primers used in this study. For the ones which were not design in the study the reference is shown.

Gene	Primer	Sequence (5' to 3')	Ta	Reference
MET2	**MET2-cerF**	CGAAAACGCTCCAAGAGCTGG	**57ºC**	Sampaio and Gonçalves, AEM 2008
	MET2-cerR	GACCACGATATGCACCAGGCAG		
HIS3	**SuHIS3-F**	ATGTCAGAGCAAAAGGCCCTA	**49ºC**	Rainieri et al., AEM 2006
	SuHIS3-R	CATGAGAACACCCTTTGTGGA		
FSY1	**FSY-230F**	GGATCYTCRACAAGCGTTTCTC	**52ºC**	Designed in this work
	FSY-1650R	AAGGCAAACAYGTAAAGCAAAG		
EST2	**EST2uva-80F**	AATATGGTCAATTCAATGGC	**49ºC**	Designed in this work
	EST2uva-1265R	TAACTGTTGTGGTTTCTACA		
COXII	**COXIIuva-F**	CAGCAACACCAAATCAAGAAG	**53ºC**	Designed in this work
	COXIIuva-R	CATATTAGCATGGCCAGT		
SSU1	**SSUuva-1F**	CGAAAGATTGATCCAGTTAC	**52ºC**	Designed in this work
	SSUuva-680R	ATGCTCCACTGCTTAAGTC		
(TA)n	**(TA)n_F**	GTATGCATCACTATTTTTCG	**52ªC**	Masneuf-Pomarède et. al.,SAM 2007
	(TA)n_R	AATTTGGTAATTTGAATGTG		
(CTG)n	**(CTG)n_F**	GGACACTAGAGTTCGTCTCG	**52ªC**	Masneuf-Pomarède et. al.,SAM 2007
	(CTG)n_R	GCCACCACTATCAGTTCG		
(ATT)n	**(ATT)n_F**	TGCCCTTCTTATTCTTGT	**46ªC**	Masneuf-Pomarède et. al.,SAM 2007
	(ATT)nR	GAAAATATCAACGCATTAAA		

Table S3- Number of repeats of each microsatellite locus for all the strains used in this study.

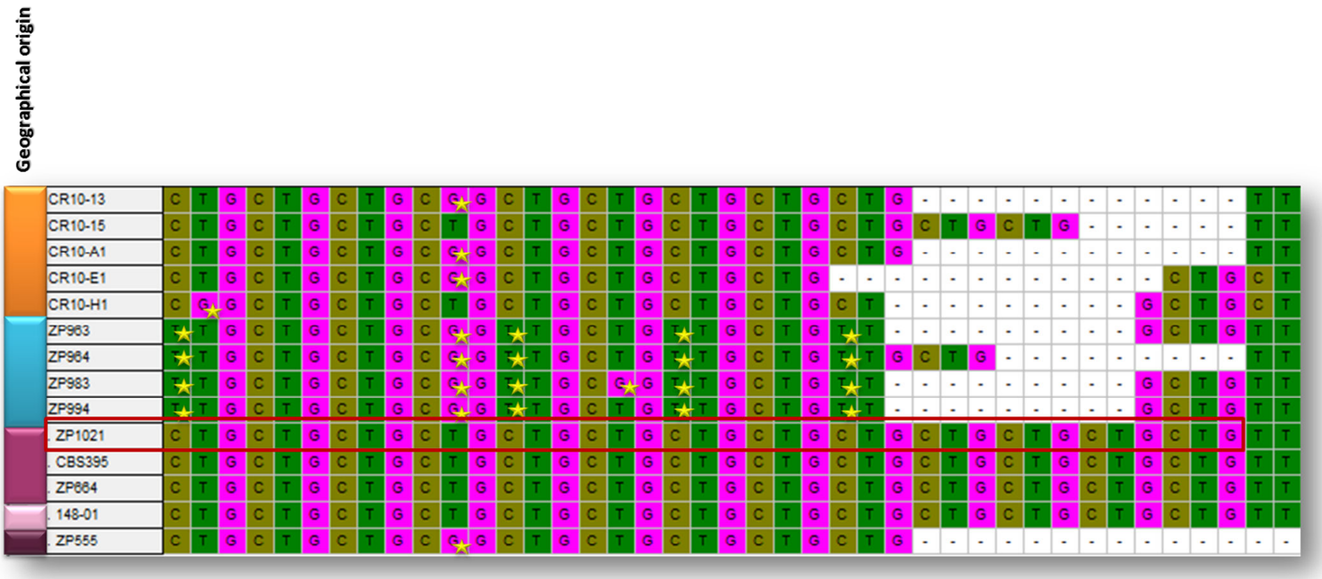
Strain	(CTG)n	(TA)n	(ATT)n
CBS 7001	13	13	11
CBS 395	13	8	11
CBS 377	13	8	10
CBS 426	8	8	11
CBS 431	13	11	11
ZP 555	9	11	10
ZP 646	9	8	11
ZP 664	13	8	10
ZP 830	13	8	11
ZP 853	13	13	10
ZP 1021	13	13	11
NCAIM 789	9	8	10
NCAIM 1107	9	8	11
NCAIM 1116	13	13	11
DBVPG 7786	13	13	11
DBVPG 7787	9	8	11
RC4-15	9	11	11
RP1-21	9	8	15
GM 14	13	8	15
M488	9	13	10
PJS 9	7	8	11
PJS 2 95	8	13	11
148.01	13	11	11
CR10-13	9	6	9
CR10-15	11	6	9
CR10-25	12	6	9
CR10-A1	9	6	9
CR10-E1	12	6	10
CR10-H1	12	6	11
CR30-23A	9	6	9
CR20-21A	12	6	9
CR10-9	12	6	9
SR10-M3	11	6	11
4-4R10	11	11	11
2-4R10	11	11	11
3a	12	6	10
1a	9	11	11
UWO 99-808-1-1	11	11	11
UCD 61-137	13	13	11
UCD 51-206	13	8	15
ZP 959	12	5	8
ZP 962	12	5	9
ZP 963	12	5	9
ZP 964	12	5	9
ZP 965	12	5	9
ZP 966	12	5	8
ZP 983	12	5	7
ZP 994	12	5	9

Table S4- Results for carbon source assimilation (Mel.- Melibiose; Gluc.- Glucose; Malt.- Maltose.; Man.- Mannitol; Fruct.- Fructose and Galact.- Galactose) and maximum grouth temperature (MGT). Geographical origins of the strains are also displayed (AUST- Australasia; EUR- Europe; A- Asia; NA- North America; PAT- Patagonia). Growth was evaluated in terms of turbidity (- no growth; + growth; D delayed growth; D* signs of growth appeared 15 days after inoculation).

	Strains	Mel.	Gluc.	Malt.	Man.	Fruct.	Galact.	MGT
AUST	ZP 959	D*	+	D*	-	+	+	32ºC
	ZP 962	D*	+	-	D	+	+	31ºC
	ZP 964	D*	+	D*	D	+	+	31ºC
	ZP 965	D*	+	D*	D	+	+	32ºC
	ZP 983	D*	+	D*	-	+	+	32ºC
EUR	ZP 1021	+	+	+	-	+	+	35ºC
	DBVPG 7786	+	+	+	-	+	+	35ºC
	ZP 853	+	+	+	-	+	+	35ºC
	ZP 646	D	+	+	D	+	+	35ºC
	NCAIM 868	D	+	+	D	+	+	35ºC
	NCAIM 1107	+	+	D	D	+	+	35ºC
A	ZP 830	+	+	+	D	+	+	35ºC
NA	UCD 51-206	+	+	D	-	+	+	35ºC
	ZP 555	+	+	+	D	+	+	35ºC
PAT	CR10-13	+	+	+	-	+	+	35ºC
	CR30-23a	+	+	+	D	+	+	35ºC
	4-4R10	+	+	+	D	+	+	35ºC

Table S5 - Results for growth rate and latency in two different carbon sources (Glucose and Melibiose). The test was performed using one strain from Patagonia (4-4R10) and one from Australasia (ZP 964).

Carbon Source	Temperature	Strains	Growth Rate (h^{-1})	Doubling Time (h)	Latency (h)
Glucose	**25ºC**	ZP 964	0.240	2.9	5
		4-4R10	0.270	2.5	5
	18ºC	ZP 964	0.150	4.6	8.5
		4-4R10	0.170	3.9	8.5
	10ºC	ZP 964	0.036	9.8	13>X>27.5
		4-4R10	0.037	9.4	13>X>27.5
Melibiose	**25ºC**	ZP 964	0.064	11.4	25
		4-4R10	0.114	6.3	5
	18ºC	ZP 964	0.048	13.7	25
		4-4R10	0.091	9.7	5
	10ºC	ZP 964	0.018	36.6	40
		4-4R10	0.044	15.2	5



CR10-13	C	T	G	C	T	G	C	T	G	C	G★	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	-	-	-	-	-	-	-	-	-	-	-	-	T	T
CR10-15	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	-	-	-	-	-	-	T	T
CR10-A1	C	T	G	C	T	G	C	T	G	C	G★	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	-	-	-	-	-	-	-	-	-	-	-	-	T	T
CR10-E1	C	T	G	C	T	G	C	T	G	C	G★	G	C	T	G	C	T	G	C	T	G	C	T	G	-	-	-	-	-	-	-	-	-	-	-	-	C	T	G	C	T
CR10-H1	C	G★	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	-	-	-	-	-	-	-	-	-	G	C	T	G	C	T
ZP963	T★	T	G	C	T	G	C	T	G	C	G★	G	T★	T	G	C	T	G	T★	T	G	C	T	G	T★	T	-	-	-	-	-	-	-	-	-	G	C	T	G	T	T
ZP964	T★	T	G	C	T	G	C	T	G	C	G★	G	T★	T	G	C	T	G	T★	T	G	C	T	G	T★	T	G	C	T	G	-	-	-	-	-	-	-	-	-	T	T
ZP983	T★	T	G	C	T	G	C	T	G	C	G★	G	T★	T	G	C	G★	G	T★	T	G	C	T	G	T★	T	-	-	-	-	-	-	-	-	-	G	C	T	G	T	T
ZP994	T★	T	G	C	T	G	C	T	G	C	G★	G	T★	T	G	C	T	G	T★	T	G	C	T	G	T★	T	-	-	-	-	-	-	-	-	-	G	C	T	G	T	T
. ZP1021	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	T	T
. CBS395	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	T	T
. ZP664	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	T	T
. 148-01	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	T	T
. ZP555	C	T	G	C	T	G	C	T	G	C	G★	G	C	T	G	C	T	G	C	T	G	C	T	G	C	T	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Fig. S1- Partial alignment of $(CTG)_n$ locus for representative strains of Australasia, Northern Hemisphere and Patagonia. Side bars represent geographical location for each strain (color-coded according to the map on Fig. 3). Sequence with no base substitutions is outlined in red. Base substitutions are marked with yellow *.