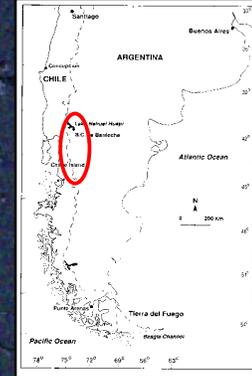


Diego Libkind

Applied Microbiology and Biotechnology Lab.
Biodiversity and Environmental Reserach Institute
(INIBIOMA), CONICET-UNComahue, Bariloche, Argentina.



?

Hi, how are you?. My name is Diego Libkind and I'm yeast scientist from Argentina. I work at the Applied Microbiology and Biotechnology Lab. in the city of Bariloche, which is located in the northwestern area of Patagonia as shown in the map at the right. I have been working on yeast biodiversity and biotechnology for more than 10 years but recently I had the luck to entered the fascinating world of Brewing science. In the lower part of the slide you can see 3 pictures, two of which you should easily recognize: a bunch of yeast cells on the top and tempting glass of Lager beer on the right. The connection between these two I believe is pretty obvious. The third picture belongs to a south hemisphere endemic fungus named «Cyttaria» (is the name of the genus). Our group has demonstrated that Cyttaria fruiting bodies (the orange gall-type structure) are a natural habitat for a wide range of yeast species. So one of my purposes today is to show you a potential connection between the Cyttaria fungi and the crispy Lager.

Bariloche, Patagonia, Argentina



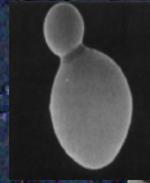
First let me show you how Bariloche looks like, so that you can imagine how boring is the place where I perform my investigations. The city is located near the Nahuel Huapi lake and the Andes mountain chain. It is one of the most important touristic cities in Argentina, and definitely from Patagonia. If you plan to visit this region of the planet sometime you must visit Bariloche, not only because of the nice sightseeing you will get but also due to our great beers. We have about 12 microbreweries in a city that has 100.000 thousand inhabitants. So probably one of the cities with highest density of breweries in Argentina. Only 150 km apart from Bariloche you can also find the most important hop production region of Argentina. So you can see that Brewing science and beer itself is quite important from where I come from.

Structure of the talk:



> Yeasts overview

- What are they?. How many?.
- Where do they live?
- Role in nature?
- What are they used for?



> Discovery of *S. eubayanus*

- Taxonomy changes
- Origin of Lager yeasts
- Insights on brewing yeasts domestication
- Impact in brewing?

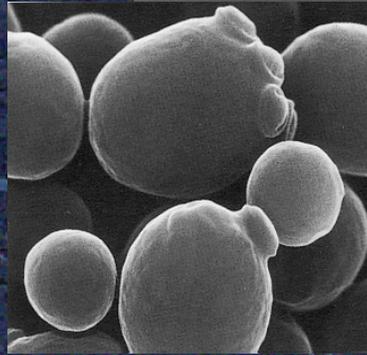
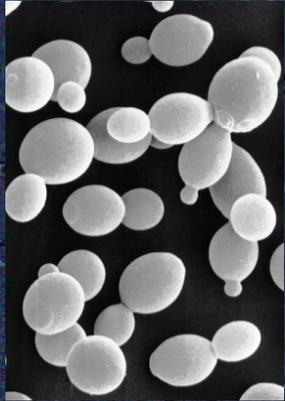
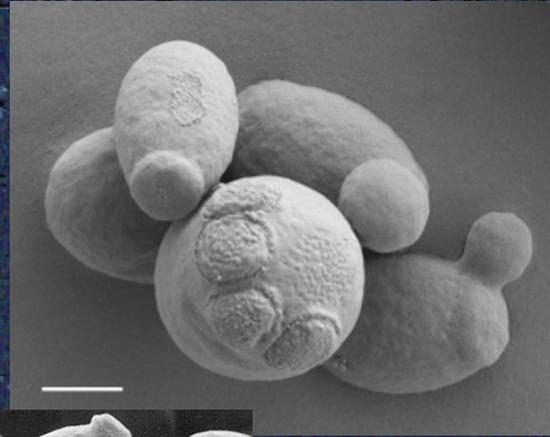


Ok, then. The presentation will be somehow structured as follows. First, a fast overview of what yeast, but from probably a different perspective to what you are used to. We will briefly discuss their diversity, habitats, role in nature and finally how humans use them. Second, I will introduce you to the details of the recent discovery of the new species *Saccharomyces eubayanus*, and its implications in brewing yeast taxonomy. Also, I will like to introduce you to the concept of microbe domestication and to the new hypothesis of the origin of the unique Lager yeasts. Finally, I will share with you some unpublished data on the diversity of *S. eubayanus*, in fact you are the first to which I will be discussing these new results. I hope you enjoyed.

What are Yeasts?



- = «foam», «To rise»
- = Fermentative ascomycetous?
- = *Saccharomyces*
- Fungi that asexually reproduce by budding or fission, which results in growth that is comprised mainly of single cells.

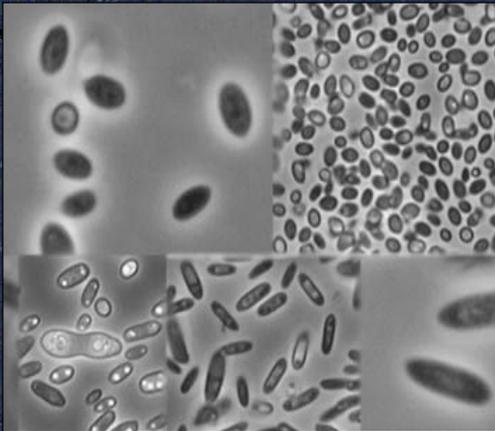


As you may already know the English word yeast and its equivalents in many other languages are based on words meaning “foam” and “to rise”, which are a direct reference to the fermentation processes that produce beer and bread. For this reason, yeasts are often thought of as fermentative ascomycetous fungi similar to *Saccharomyces cerevisiae*. In fact, it is not uncommon in some areas of molecular biology to treat the words “yeast” and “*Saccharomyces*” as synonyms. The discovery that some yeast taxa are basidiomycetes has broadened our perception of the nature of yeasts. As a consequence, we have come to view yeasts as fungi that asexually reproduce by budding or fission, which results in growth that is comprised mainly of single cells.

Yeast: Diversity



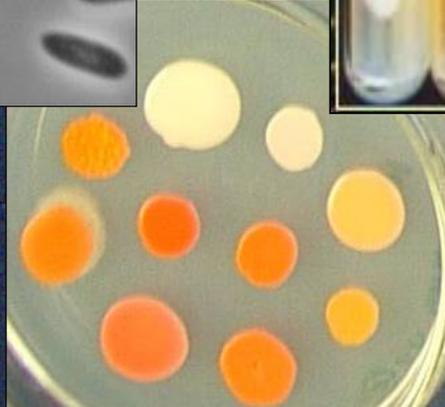
> More than 1500 yeast species are known (2011 data)



Diverse cell sizes and shapes can be found



Highly diverse in colors and colony morphology



Today, more than 1500 yeast species are known which are distributed in a wide range of taxonomic groups. In other words, yeast represents a very heterogeneous group of fungi. As can be seen from the pictures, yeasts are highly variable in cell shape, size and colony colors. Pink to orange pigmentation in the colonies are the result of the accumulation of carotenoids inside the cells (like B-carotene).

Yeasts: Ecology



> Habitat and role in nature?

Acid Environs (pH=1,5)



ICE



Oligotrophic waters



All natural habitats harbour yeasts, but... not everything is everywhere



Saccharomyces mycoses in immunocompromised patients



Mainly decomposers of organic matter, ... some can be opportunistic Pathogens

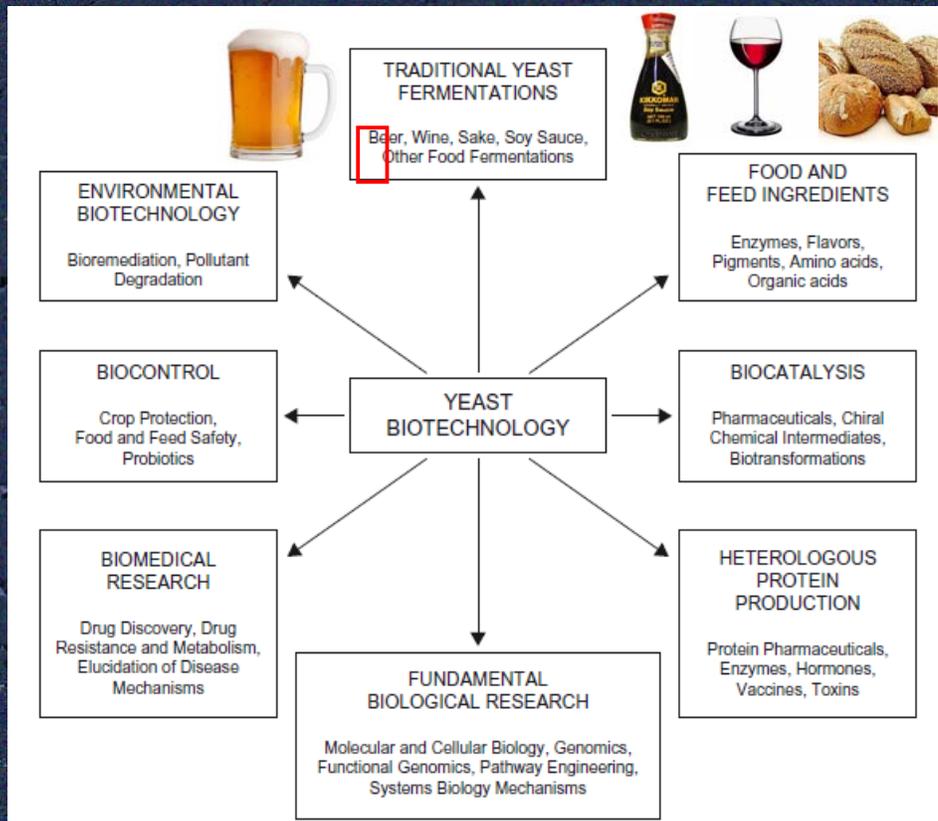
In natural environments, yeasts occur together in communities and are widely distributed throughout all biomes of the world. They have been found in the upper levels of the atmosphere (above the clouds in the stratosphere), in the deepest parts of the oceans, in ancient glacial ice, and are abundant throughout the phyllosphere. I have found them in large quantities and diversity in extreme environments of Patagonia like acidic waters of volcanic origin (pH~1,5), glacial ice, highly UV exposed tree leaves and ultra-oligotrophic waters (which is almost distilled water due to the lack of nutrients and salts).

Yeasts participate in a number of complex processes in ecosystems. Free-living yeasts are primarily decomposers and are among the earlier colonizers of nutrient rich substrates, where they are followed by a succession of organisms that degrade dead organic matter. However, yeasts are not just decomposers but can assume a diversity of forms and functions in the natural world. Along with their role in the transformation of nutrients, they can engage in intimate relationships with other organisms as mutualists, competitors, parasites, or opportunistic pathogens.

Even though *S. cerevisiae* is commonly perceived as a harmless microorganism, given its more than 1000-year association with food and beverages production, numerous reports of human infections caused by this yeast exist in the medical literature. However, these typically occur in patients with other underlying illnesses that have their immunological system compromised or suppressed. It should be noted also, that among brewing yeasts only ALE yeast might be considered potential opportunistic pathogens due to their ability to grow at 37°C (human body temp). Lagers have no record of pathogenicity so far, and probably will remain so due to their lower growth temperatures.

Ironically, despite the huge importance of *S. cerevisiae* and related species, relatively little is known about its origins and natural lifestyle.

Yeasts: What are they used for?



Yeasts are currently applied in a wide range of biotechnological disciplines as shown in this slide: besides traditional yeast fermentations, yeast are employed to obtain food and feed ingredients, and used as biocatalysts for the production of for example pharmaceuticals molecules. Yeasts are also used for the production of heterologous proteins such as enzymes, hormones, vaccines, etc. In other words, genes from other organisms are inserted into yeast and these are used as bio factories. They are also used for fundamental research in multiple biological disciplines. Also applied for Biomedical research, Bio control strategies and environmental biotechnology.

Note that brewing is just one of many applications that yeasts have (as a group, not meaning *Saccharomyces* species).

Yeasts: What are they used for?



TABLE 3.1 Representative Yeast Genera and Species Associated with Fermented Foods and Feeds

Food	Yeast Species Involved	Food	Yeast Species Involved
Beers and ales	<i>Saccharomyces cerevisiae</i> <i>Saccharomyces bayanus</i> <i>Saccharomyces pastorianus</i> (= <i>S. carlsbergensis</i>) <i>Saccharomyces bayanus</i> var. <i>uvarum</i>	ment olives and cucumbers	<i>Candida valida</i> <i>Saccharomyces bayanus</i> <i>Yarrowia lipolytica</i> <i>Debaryomyces</i> spp. <i>Candida</i> spp. <i>Kluyveromyces marxianus</i> <i>Lachancea cidi</i> <i>Pichia</i> spp. <i>Rhodotorula</i> spp. <i>Saccharomyces</i> spp. <i>Candida</i> spp.
Breads and bakery products	<i>Saccharomyces cerevisiae</i> <i>Torulopsis delbrueckii</i> <i>Candida krusei</i> (sourdough) <i>Candida milleri</i> (sourdough) <i>Kazachstania exiguua</i> (sourdough) <i>Zygosaccharomyces rouxii</i>	Kenkey (African fermented maize)	<i>Debaryomyces</i> spp. <i>Kluyveromyces</i> spp. <i>Saccharomyces</i> spp. <i>Candida</i> spp. <i>Trichosporon</i> spp.
Cachaça	<i>Saccharomyces cerevisiae</i> <i>Schizosaccharomyces pombe</i>	Kimchi	<i>Debaryomyces</i> spp. <i>Trichosporon</i> spp. <i>Candida</i> spp. <i>Cryptococcus</i> spp. <i>Debaryomyces</i> spp. <i>Saccharomyces</i> spp. <i>Kluyveromyces</i> spp. <i>Pichia</i> spp. <i>Rhodotorula</i> spp. <i>Saccharomyces</i> spp.
Cheeses	<i>Debaryomyces hansenii</i> <i>Candida</i> spp. <i>Kluyveromyces marxianus</i> <i>Kluyveromyces lactis</i> <i>Yarrowia lipolytica</i> <i>Geotrichum candidum</i> Other <i>Geotrichum</i> spp. <i>Rhodotorula</i> spp. <i>Trichosporon</i> spp. <i>Saccharomyces cerevisiae</i>	Lao Chao	<i>Kluyveromyces lactis</i> <i>Kluyveromyces marxianus</i> <i>Candida kefir</i> <i>Candida famata</i> <i>Candida krusei</i> <i>Debaryomyces hansenii</i> <i>Geotrichum candidum</i> <i>Yarrowia lipolytica</i> <i>Saccharomyces cerevisiae</i> <i>Saccharomyces cerevisiae</i> <i>Saccharomyces bayanus</i>
Other dairy products (e.g., kefir, yoghurt, fermented milk)	<i>Kluyveromyces lactis</i> <i>Kluyveromyces marxianus</i> <i>Candida kefir</i> <i>Candida famata</i> <i>Candida krusei</i> <i>Debaryomyces hansenii</i> <i>Geotrichum candidum</i> <i>Yarrowia lipolytica</i> <i>Saccharomyces cerevisiae</i> <i>Saccharomyces cerevisiae</i> <i>Saccharomyces bayanus</i>	Soy paste (Chiang Miso)	<i>Zygosaccharomyces malanga</i> <i>Zygosaccharomyces</i> spp. <i>Candida</i> spp. <i>Zygosaccharomyces rouxii</i> <i>Candida famata</i> <i>Candida etchellsii</i> <i>Candida versatilis</i> <i>Debaryomyces</i> spp. Other <i>Candida</i> spp.
Ciders	<i>Saccharomyces cerevisiae</i> <i>Saccharomyces bayanus</i>	Soy sauce (Jiang yu; Shoyu)	<i>Zygosaccharomyces kombuchaensis</i> <i>Candida</i> spp. <i>Saccharomyces cerevisiae</i> <i>Saccharomyces bayanus</i> <i>Saccharomyces bayanus</i> var. <i>uvarum</i> <i>Saccharomyces kudriavzevii</i> "Saccharomyces sake" Other <i>Saccharomyces</i> spp. <i>Hanseniaspora uvarum</i> <i>Kloeckera apiculata</i> <i>Candida</i> spp. <i>Cryptococcus</i> spp. <i>Pichia</i> spp. <i>Saccharomyces</i> spp. <i>Trichosporon</i> spp.
Cocoa	<i>Hanseniaspora uvarum</i> <i>Kloeckera apiculata</i> <i>Kluyveromyces marxianus</i> <i>Pichia fermentans</i> <i>Kluyveromyces marxianus</i> <i>Saccharomyces bayanus</i> <i>Schizosaccharomyces</i> spp. <i>Candida boidinii</i>	Tea fungus	"Saccharomyces boulardii" <i>Saccharomyces cerevisiae</i> <i>Kluyveromyces marxianus</i> <i>Candida</i> spp.
Coffee	<i>Debaryomyces hansenii</i> <i>Candida</i> spp. <i>Cryptococcus</i> spp. <i>Rhodotorula</i> spp. <i>Saccharomyces cerevisiae</i> <i>Yarrowia lipolytica</i>	Wines	
Fermented meats and sausages	<i>Kluyveromyces</i> spp. <i>Candida parapsilosis</i> <i>Candida krusei</i>	Silage	
Fermented milks		Probiotics	

Saccharomyces = brewing yeasts

Why?



If we only focus on the yeast species used in fermented foods and feeds you can see the large number of different yeast species involved. However, when focusing on beer *Saccharomyces* leads the scene. Considering that more than 300 species correspond to fermentative species, do any one know or guess why *Saccharomyces cerevisiae* and related species are the ones chosen for brewing. And please do not answer because it comes in a fancy package as a very clever argentinean homebrewer did.

Brewing yeasts: why Saccharomyces?



> More than 300 fermenting known yeast species

TABLE 61.3 Fermentation Power (w/v) of Some Yeast Species¹

<2%	2–5%	5–8%	>8%
<i>Candida ernobii</i>	<i>Hanseniaspora uvarum</i>	<i>Candida stellata</i>	<i>Saccharomyces bayanus/</i>
<i>C. guilliermondii</i>	<i>Pichia fermentans,</i>	<i>Lachancea thermotolerans</i>	<i>uvarum S. cerevisiae</i>
<i>C. melinii</i>	<i>Lachancea kluveri</i>	<i>Saccharomyces kudriavzevii</i>	<i>S. pastorianus</i>
<i>C. parapsilosis</i>	<i>Schwannomyces</i>	<i>S. mikatae</i>	<i>S. paradoxus</i>
<i>C. sake</i>	<i>occidentalis</i>	<i>Saccharomyces ludwigii</i>	
<i>C. tropicalis</i>	<i>Torulaspora pretoriensis</i>	<i>Schizosaccharomyces pombe</i>	
<i>C. valida</i>	<i>Zygorulaspota mrakii</i>	<i>Torulaspora delbrueckii</i>	
<i>Debaryomyces castellii</i>		<i>Zygosaccharomyces bailii</i>	
<i>Lindnera satumus</i>		<i>Z. rouxii</i>	
<i>Metschnikowia pulcherrima</i>		<i>Zygorulaspota florentinus</i>	
<i>M. reukafii</i>			
<i>Pichia membranifaciens</i>			
<i>Schwanniomyces polymorphus</i>			
+>300 ca. fermenting species, including about 50 that exhibit slow or retarded fermentation			

Saccharomyces show the highest fermentation power and alcohol tolerance

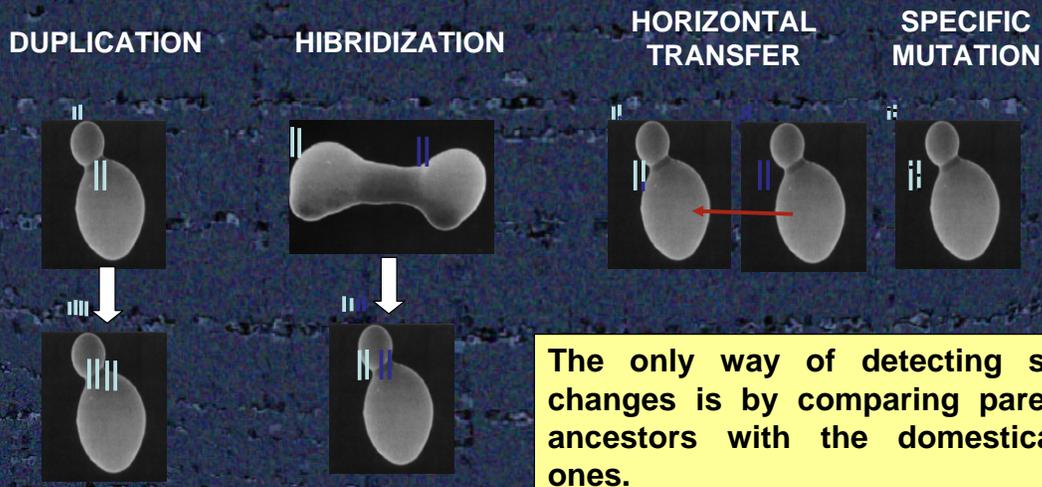
The factor that unites all species of *Saccharomyces* is the ability to produce significant amounts of alcohol by fermenting simple sugars. However, there are many other species that can ferment simple sugars to ethyl alcohol, but the quantitative expression of this ability is extremely variable. It becomes clear from the present table that only certain species of *Saccharomyces* are able to produce (and thus also tolerate) above 8% alcohol, and this answers our open question.

Yeasts: wild vs domesticated



> Domestication process:

The accumulation of genetic changes which result in improved properties of a biological organisms due to the selective pressure imposed by humans.



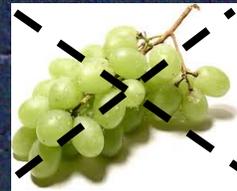
As you already know, modern brewing strains are not exactly equal to those found in nature. Though they derive from once wild yeasts, they have suffered many genetic changes becoming at the end a refined fermentative machine, in other words “wort fermentation specialists”. This is the result of a process called domestication. For example, domestication of crops and livestock selects for desirable characteristics through directional breeding so that domesticated lineages become genetically distinct from their wild ancestors in ways that make them more useful to Humans. Microbial domestication was different since only in the late nineteenth century yeasts were considered as responsible for the fermentation process, Even yeast were not know as important in brewing, re-utilization from batch to batch and also selection of the best batches by brewmasters definitely contributed to the shaping of the genetics and consequently to the brewing characteristics of the strains used today to produce Ales and Lagers.

Some of the mechanisms that generate such genetic modifications include genome duplication, which involves duplicating the complete set of genes and thus to have gene products produced twice as much. Hybridization between two closely related species, which, for certain traits result in the merge of both species characteristics in a single strain. Also incorporation of other species DNA through horizontal gene transfer and specific mutations that occur randomly in the genome. It should be noted that any of these events has a reduced probability of occurrence and even less if we consider those events that significantly change something that might affect the cell fitness or adaptation to the brewing environ. But, the stress situation imposed by the wort gravity and the high alcohol among others; and the huge number of cells produced in a single batch, help to increase the odds.

Brewing yeasts: *Saccharomyces*, Taxonomy and Ecology?



S. cerevisiae ALE



S. uvarum (*S. bayanus* var. *uvarum*)



S. bayanus (*S. bayanus* var. *bayanus*) } LAGER



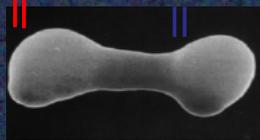
S. pastorianus (*S. carlsbergensis*)



LAGER YEAST:

(*S. pastorianus*)

Hybrid: *S. cerevisiae* + psychophilic *Saccharomyces*



S. bayanus??

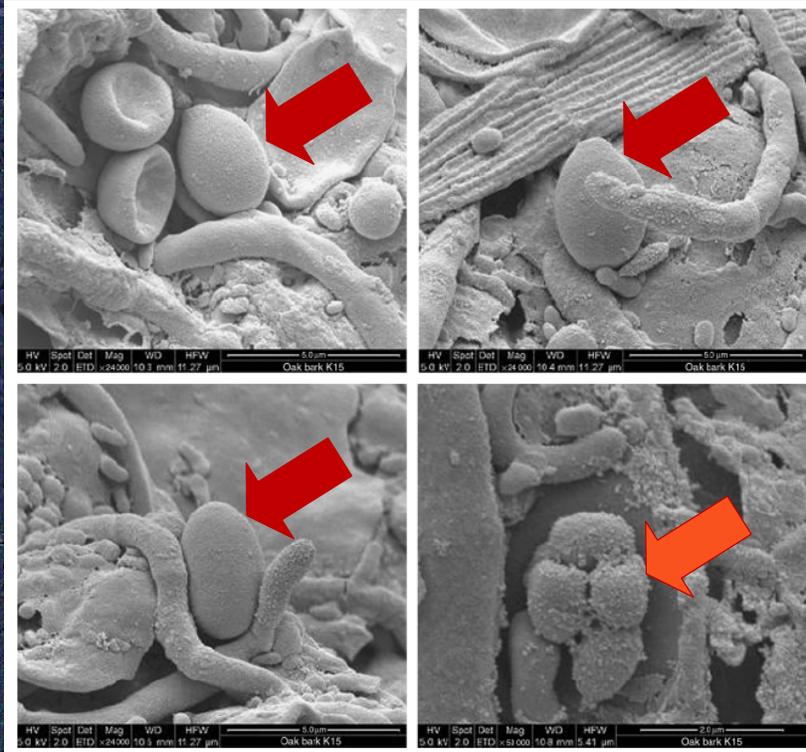
The taxonomy of brewing yeasts, especially that of Lager yeasts, is quite confusing. Even for me who I'm a member of the Systematics and Nomenclature Committee of the International Commission on Yeasts. I apologize in the name of all my colleagues that we couldn't solve this earlier. So, until recently our general view was that *S. cerevisiae* was responsible of Ale production as well as most wines. That Lager depending on the authors and year of publication could be any of these three species, *S. uvarum*, *S. bayanus* and *S. pastorianus*. Two of which it was not clear if they were different species or different varieties of a single species. Both *S. uvarum* and *S. bayanus* are frequent in white wines, cider and Champagne production but have been also related to Lager brewing.

The habitat of *S. cerevisiae* was believed to be the surface of grapes or equivalent sugary rich fruits, but it has recently become clear that the bark and soil of certain tree species is the primary habitat of this species (mainly oaks). *S. uvarum* has also been found in such environments in different parts of the north hemisphere. This is not the case of *S. bayanus* and *S. pastorianus* two species that so far have been only isolated from brewing environments (or winery in case of *S. bayanus*).

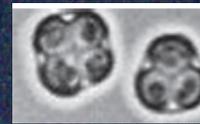
Nowadays Lager brewing strains are mainly referred as *S. pastorianus*. This species is a known hybrid formed by the fusion of a *S. cerevisiae* strain with another *Saccharomyces* psychophilic yeast which contributed to the ability to ferment at low temp (among other things) that characterizes Lager yeasts.

It was believed that *S. bayanus* could be the missing parental species due to some genetic resemblance but results were contradictory.

Tree bark: *Saccharomyces* natural habitat?



Oak's Bark, New Zealand



Sexuality

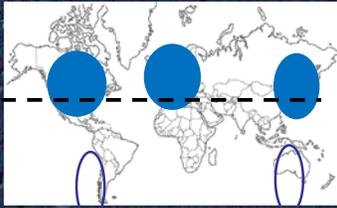
From Zhang et al., 2010

In recent years it has become evident that *Saccharomyces* primary natural habitats are not the grapes or equivalent sugary rich environments. Instead, oak forests, in particular tree bark and soil, became frequent sources of wild *Saccharomyces*. It is difficult to assimilate at first, I know, at least it was for me when I first read about this, but after a few years capturing wild yeasts from natural forest I must say that is true. As proof I can show you this electron micrograph of oak bark showing *Saccharomyces* yeast cells. Also, indicated with the orange arrow, an ascus with 4 ascospores, typical sexual structure of wild *Saccharomyces*. This shows that *Saccharomyces* yeasts not only can thrive in tree bark but are also happy enough to have sex!

Worldwide search for natural *Saccharomyces*



> Known distribution of *saccharomyces* in trees and soil



Caraca, Brasil

> New environments sampled



Brisbane, Australia



Christchurch, NZ



Kyoto, Japon

Nothofagus trees

Several Studies have shown the occurrence of *Saccharomyces* in Canada/USA, Europe and Japan. Five years ago, in cooperation with Dr. Jose Paulo Sampaio from the University of Nova Lisboa in Portugal we started a worldwide search to describe the natural distribution of *Saccharomyces* yeasts. We sampled several geographic regions but focused mainly in the south, visiting Australia and New Zealand, and of course Patagonia. We studied mainly bark and soil from the so called southern beech trees of the genus *Nothofagus*, but also investigated a unique habitat which is the sugary rich fruiting body of the fungus *Cyttaria*.

Worldwide search for natural *Saccharomyces*



> New substrate for *Saccharomyces*:

Cyttaria fungus

Exclusive parasite of Southern Beech (*Nothofagus*)

Endemic to Patagonia (Arg/Chile) and Australia/NZ – Gondwana.

Fruiting bodies are produced on tumors in spring/summer

Rich in sugars (~10%): Glucose, fructose and sucrose



Rich in yeasts

Aboriginal people use them as food source and used to make a fermented beverage: "Chicha"

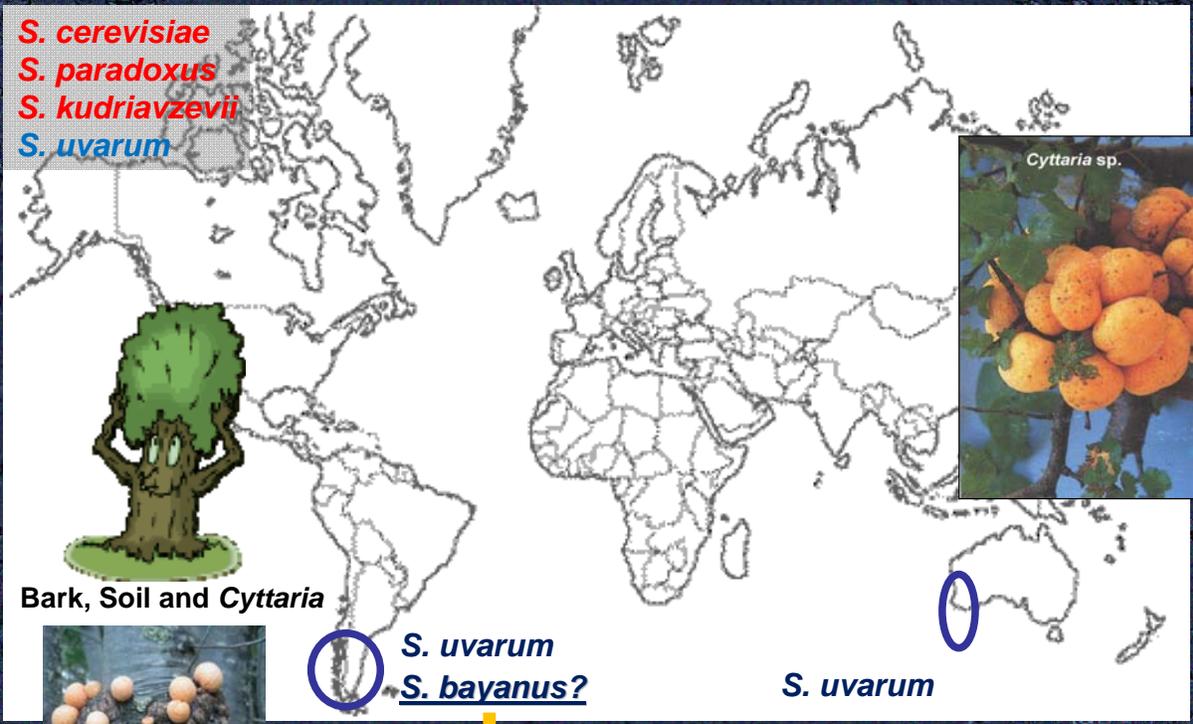


This fungus is an exclusive parasite of *Nothofagus* trees which are endemic of Patagonia, Australia and New Zealand. Trees infected with this fungus produce tumors from which *Cyttaria* fruiting bodies appear in late spring and summer. These galls contain many simple sugars and therefore when mature go through a spontaneous natural fermentation process mediated by yeasts. In fact, local Indians used to elaborate a fermented beverage from this fungus. Unfortunately, this unique habit of producing and alcoholic drink by fermenting a fungus with another fungus (yeast) has been lost. Probably due to the replacement of *Cyttaria* with better tasting fruits like apples (brought by the Spanish).

Natural *Saccharomyces* in South Hemisphere



S. cerevisiae
S. paradoxus
S. kudriavzevii
S. uvarum



Bark, Soil and *Cyttaria*



S. uvarum
S. bayanus?

S. uvarum

Patagonian *S. "bayanus"*

From our worldwide survey we obtained thousands of wild *Saccharomyces* strains of different species. We also found that *Nothofagus* forests in the south are occupied mostly by the cold tolerant *Saccharomyces uvarum*, although we saw that in Patagonian forests it was not alone. In certain species of *Nothofagus* we were able to isolate a second species, also adapted to cold, which based on preliminary genetic analysis seemed similar to *S. bayanus*. We will name it for now Patagonian *S. bayanus*, given it was only recovered from such geographic region.

Identifying Patagonian *S. bayanus*



S. uvarum (*S. bayanus* var. *uvarum*)



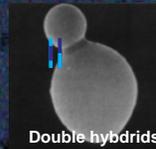
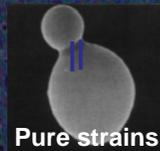
S. bayanus (*S. bayanus* var. *bayanus*) } LAGER



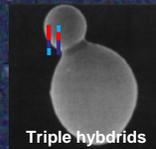
Hybrid *S. pastorianus* (*S. bayanus* var. *carlsbergensis*)



Patagonian *S. "bayanus"* vs *S. bayanus* (*S. bayanus* var. *S. bayanus*)



S. uvarum
= Patagonian *S. "bayanus"*



S. uvarum
= Patagonian *S. "bayanus"*
S. cerevisiae

Given that there were no records of wild *S. bayanus* strains we decided to compare it with a group representative collection strains of *S. bayanus*.

What we first saw is that all the strains regarded as pure *S. bayanus* were actually hybrids, either double hybrids with contributions of *S. uvarum* and patagonian bayanus, or triple hybrids with genetic contributions of a third species: *S. cerevisiae*. We also checked that patagonian bayanus was a clean lineage, a pure strain that had no contributions from other *Saccharomyces* species.

Taxonomic conclusions

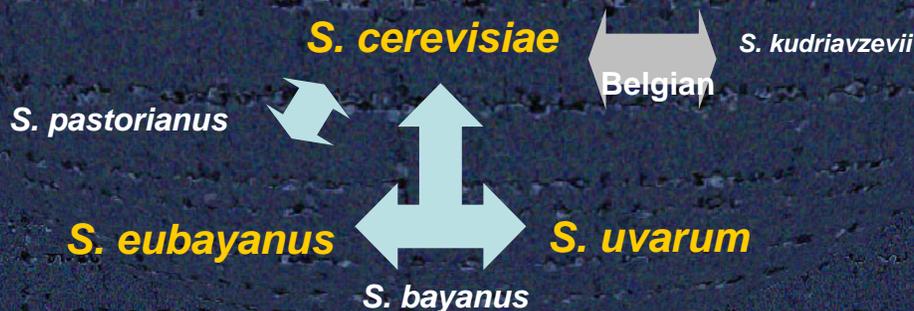


- > *S. bayanus* is not a valid species (a group of double or triple hybrid strains)
- > *S. uvarum* is a valid species.
- > Patagonian *S. "bayanus"*, is a valid species different from *S. uvarum*



***S. eubayanus* (= the real bayanus)**

Genetic constitution of brewing strains:



We found that *S. bayanus* is not a valid species, but a group of hybrids. That *S. uvarum* is a real species distinct of the patagonian *S. bayanus* strains, and that these constitute a real species too which we named *S. eubayanus*, meaning the true or real bayanus.

NEXT.

As a summary we can say that brewing strains are constituted mainly from genetic contributions of three biological species: *S. cerevisiae*, *S. uvarum* and the recently described *S. eubayanus*. *S. pastorianus* and *S. bayanus* are hybrids of different combinations of these species or of all of them, as is the case of several *S. bayanus* strains.

NEXT.

Sorry to complicate things further but you should know that hybrids between *cerevisiae* and a fourth species named *kudrivazevii* have been detected in Belgian beers.

LAGER YEAST



S. cerevisiae ⇒ ALE



S. uvarum



S. eubayanus

Natural species



S. bayanus (Suva + Seub + Scer)

Hybrid strains



S. pastorianus (Seub?? + Scer) ⇒ LAGER



Is *S. eubayanus* the missing progenitor of the Lager yeast?

Then, to reinforce this, we can say that “*S. uvarum*” and “*S. eubayanus*” should be used as descriptors of biologically meaningful species, whereas “*S. bayanus*” and “*S. pastorianus*” should be restricted to the domesticated and hybrid strains.

The next question to answer was whether *S. eubayanus* could be the progenitor of Lager yeast as suggested by our previous findings, and how this could happen.

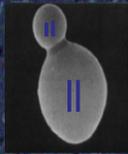


> Is *S. eubayanus* the progenitor of the Lager Yeasts?

Complete genome sequencing using Next Generation Sequencing Technology



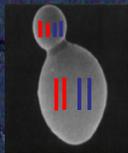
S. eubayanus



...ATGCCTGATTCTTTAATTGGGCCTAATCATC...

S. eubayanus

99,5 % homology



S. pastorianus
Lager yeast

...ATGCCTGATGTCTTTAATTGTGCCTATTTCATC...
...AGGGCCTTGATATGTGATTGTGTTTATTTCATC...

Non-cerevisiae genome

S. cerevisiae

Confirmed: *S. eubayanus* is the missing progenitor of Lager yeast

For the previous experiments we studied only portions of DNA that we knew were informative. For this we decided to completely sequence the genome of several strains including of course *S. eubayanus* and *S. pastorianus* representatives. We saw that the non-cerevisiae (or non-ALE) portion of *S. pastorianus* genome was almost identical to the one of *S. eubayanus*.

But, Is it 99,5% close enough? you might be asking yourself. Well For example, wild strains that belong to the same species from different populations (like *S. cerevisiae* or even *S. uvarum*) can differ in their complete genome in up to 2 %. Here, we see only 0,5% difference. Thus we conclude that *S. eubayanus* is the missing progenitor of Lager Yeast.



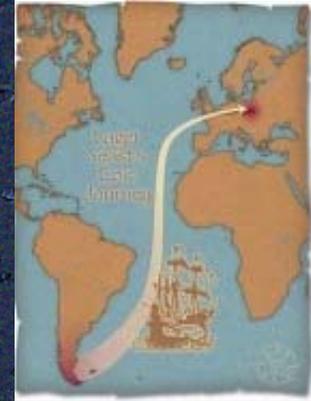
¿How did the hybrid originated?

1) *S. eubayanus* already existed in Europa and then moved to Patagonia, it become extint in Europe or has narrow distribution.

- Comprehensive fungal biodiversity studies consistently fail to find it.
- Based on the high genetic similarity between Seub and Spas, the Patagonian populations should be considered a result of secondary colonization.
- However, these is not in agreement with the high abundance and frequency of occurrence of Seub in Patagonian native forests.
- Also, one might expect to find it in other environments like North America, Oceania.

2) *S. eubayanus* travelled from Patagonia and hybridized.

- Wild yeasts produce highly resistant spores
- Yeasts can be vectored by insects and humans
- Timber, locally fermented beverages and foods, exportation of native plants, are examples of additional dispersal mechanisms.
- 200 years window for such event to happen (1600-1800)



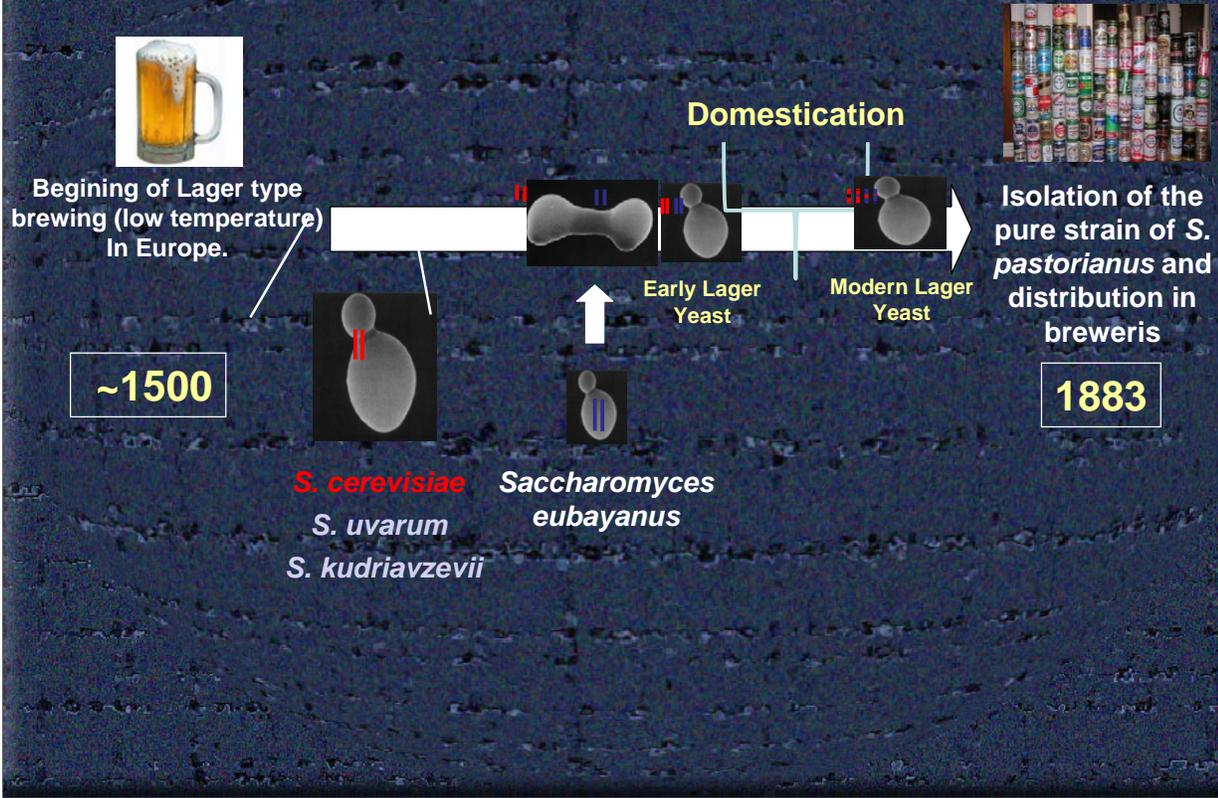
We worked on two hypotheses. The first one meant that the parental strain of *S. eubayanus* naturally lived in European forests that now have disappeared, or that no one, despite the large number of surveys that have been done, could yet find it. The hypothetical scenario implies that *S. eubayanus* strains from Patagonia arrived there from Europe and that they are not native. This is because of the high genetic similarities we see between both. But, this contradicts with the high frequency and abundance of *S. eubayanus* in Patagonian forests, higher than any other wild *Saccharomyces* that has been found in other parts of the world. Also, if it has colonized South America, why it has not colonized other regions like North America, Australia and so on.

NEXT.

Our second hypothesis implies an epic journey of a Patagonian strain of *S. eubayanus* to Europe. Such a journey might not represent a difficulty for wild yeasts because they produce highly resistant spores. These spores can be carried around by insects and humans. When transatlantic trade started, spores or cells could have taken a ride in timber, fermented beverages or food, native plants and so on.

We detect an approximately 200 years window when this could have happened, initiating when Patagonia was definitely colonized and reasonable time before the first Lager yeast was isolated by Hansen in late 19th century.

Origin of LAGER YEAST



We speculate that when Lager production started in Europe in 15th century fermentation was carried out by *S. cerevisiae* which can still ferment at low temperatures, although we know it can do it better at higher temperatures; or as an alternative, by cold tolerant European species like *S. uvarum* or *S. kudriavzevii*. Or a combination, in those years mixed fermentations were common. When *S. eubayanus* appeared and hybridized with a *S. cerevisiae* already adapted to brewing, it become an early Lager yeast. This hybrid was then further domesticated and became the modern Lager yeast that we use today.

Evidence of Domestication



Maltose metabolism

Isomaltose (gene IMA1): involved in cleavage of maltose.

Duplication and insertion of *S. cerevisiae* gene into *S. eubayanus* portion of DNA

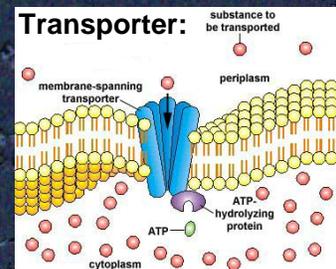
↑
Improved Maltose Fermentation

Sulfite metabolism

Sulfate transporters (genes SUL1 and SUL2): involved in transporting sulfate, the precursor of sulfite, inside the cell.

Inactivation of genes SUL1, only SUL2 active (more efficient in brewing).

↑
Enhanced Sulfite Production



Some of the genetic changes we detected when comparing the Patagonian eubayanus genome with that of the non-Ale portion of Lager yeast gave us some idea of the modifications that occur during the domestication process.

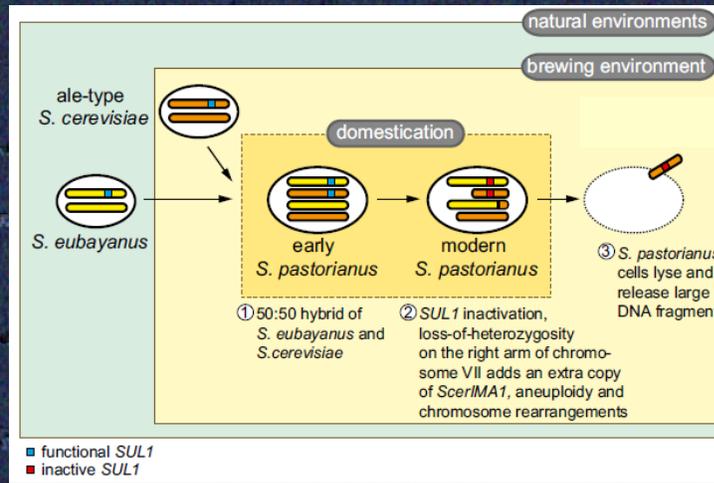
For example we found that the *S. cerevisiae* genes related to a better utilization of maltose were integrated into the *S. eubayanus* portion of Lager yeasts, suggesting strong selection for optimal sugar utilization during brewing.

We all know that Sulfite formation is also important in lager-brewing because sulfite is an antioxidant and flavor stabilizer. There are two types of transporters that get sulfate inside *Saccharomyces* cells, and they are genetically encoded by *SUL1* and *SUL2* genes. Both *S. cerevisiae* and *S. eubayanus* DNA portions of Lager yeast have both versions, but both *SUL1* genes are inactivated due to mutations which make *SUL2* the only active sulfate transporter. Coincidentally this is the most efficient transporter of the two in brewing conditions. These are only a few examples of the genetic changes that occurred during the domestication of Lager yeast, and help us to understand better the genetics of fermentation and the genes important for brewing.

Evidence of Domestication

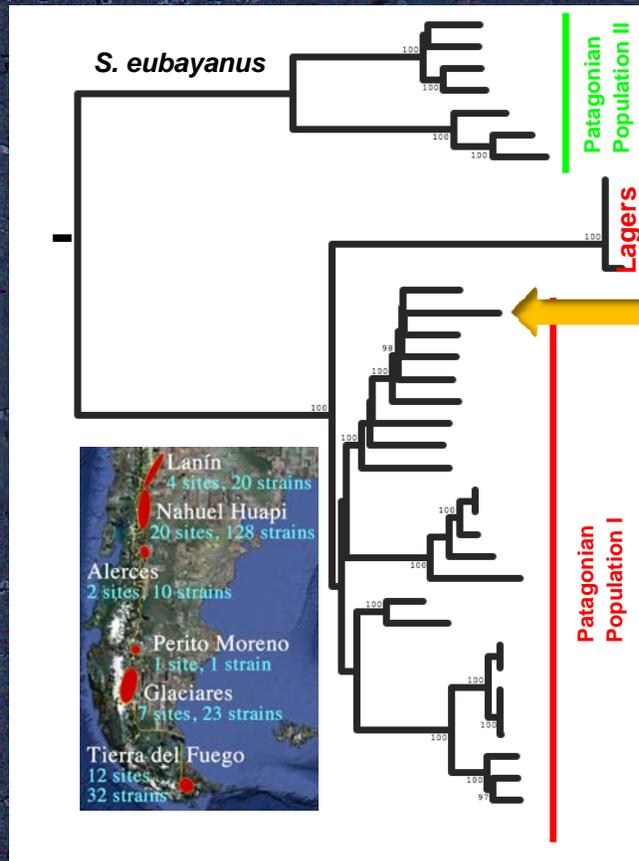


Hypothetical origin of *S. pastorianus*



Just to make it clearer, or maybe not, we speculate that a wild *S. eubayanus* from Patagonia hybridized with a ale-type yeast (already domesticated) within the brewing environment and formed a 50:50 hybrid, which suffered a domestication process involving the genetic inactivation of less efficient sulfate transporters, the addition of extra copies of cerevisiae genes related the assimilation of maltose, and various chromosome rearrangements including the loss of several ones.

New strains and unpublished data



S. eubayanus is quite diverse in Patagonia, so it is highly improbable that it arrived from Europe.

Type strain

All lager yeasts have very similar *S. eubayanus* DNA suggesting the hybridization event occurred once or at least twice with very similar *Seub* strains (natural population not yet found).

A large set of novel cold tolerant strains are now available for Lager brewing experimentation.

PATAGONIAN LAGER COMING SOON?

To end, I will like to share with you some new data that resulted from a new survey we did in Patagonia that in this case covered all the distribution of the *Nothofagus* trees, the hosts of *Cyttaria* fungus and *S. eubayanus*. Below you can see a map of Patagonia with the sampled area. *Nothofagus* forests are only located along the Andean mountains, in the limit with Chile. We wanted to know if there were distinct *eubayanus* populations in Patagonia. As you can see, we obtained hundreds of new isolates. We perform again genetic analyses with the new isolates and included additional LAGER strains typically used for brewing. What you see in the figure is a phylogenetic tree, and shows the diversity of *S. eubayanus*. The end of each branch correspond to a different strain, if the branch ends with vertical line (like a T rotated to the right), well this means there are several strains which genetically identical. The branch distance between two strains gives a measure of the genetic differences they have.

Ok, so what is important to get from this is that there are two main populations of *S. eubayanus* in Patagonia, at least that we know. Population I in red and Population II in green. Within each population there is plenty of variability. For a species to diverge so much needs at least thousands of years, so the idea that this species came from Europe is not well supported. While wild Patagonian *S. eubayanus* show large heterogeneity, the *eubayanus* portion of the LAGER strains are very similar among each other. This suggest that the hybridization event occurred only once or if more, it occurred with a similar *S. eubayanus* strain. This strain is related to the population II of the patagonian *S. eubayanus*, but you can see still have not found the exact natural population from which this strain came from. I say still because we keep looking for it I'm positive that we will find it soon. Nevertheless, I must highlight the importance of the huge set of novel cold tolerant strains of *S. eubayanus* that has been collected, which make an excellent resource for brewing experimentation, and who knows, maybe the rise of a new era for LAGERS.

Acknowledgements



-C.T. Hittinger, J. Dover, M. Johnston

Dpt. Biochemistry and Molecular Genetics, Univ. Colorado, Aurora, USA.



-E. Valerio, C. Gonçalves, P. Gonçalves & J.P. Sampaio

Centro de Recursos Microbiológicos, Departamento de Ciências da Vida Universidade Nova de Lisboa, Caparica, Portugal.



- Maria Rosa Giraudo de van Broock

- **Institutions:** INIBIOMA, CONICET, Universidad Nacional del COMAHUE, Universidade Nova de Lisboa, Colorado University, Administración Parques Nacionales.

- **Funding sources:** UNComahue, CONICET, ANPCyT, Fundacao da Ciencia e Tecnologia (Portugal), NIH (USA).



Email: libkindfd@comahue-conicet.gov.ar / diego.libkind@gmail.com



1st Latinoamerican Microbreweries Conference
1st Patagonian Homebrew Competition



23rd-26th of November 2012
Bariloche, PATAGONIA

www.beerart.com.ar