

Saccharomyces eubayanus—a tale of endless mysteries

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The discovery in 2011 of *Saccharomyces eubayanus* (Libkind et al. 2011) provided the answer to a question that had occupied yeast researchers for decades. Its finding in pristine *Nothofagus* (Southern beech) forests in the Patagonian Andes settled the debate on the progenitors of lager beer yeasts, whose current taxonomic designation is *S. pastorianus* (synonym: *S. carlsbergensis*). Contrary to ale yeasts that are “pure” *S. cerevisiae* lines, the hybrid nature of lager strains was evident since the early 1980s (Martini and Martini 1987, Nilsson-Tillgren et al. 1981, Vaughan-Martini and Kurtzman 1985). Intriguingly, however, although one of the progenitors of lager strains was identified as an *S. cerevisiae* ale yeast (Dunn and Sherlock 2008, Nakao et al. 2009), the non-*cerevisiae* parent remained elusive. *Saccharomyces bayanus*, a species described much earlier in 1895, was thought to represent the source of lager-specific alleles (Bond 2009, Montrocher et al. 1998, Nakao et al. 2009, Rainieri et al. 2006, Turakainen et al. 1994, Vaughan-Martini and Kurtzman 1985) despite some contradictory observations indicating that *S. bayanus* contained also *S. cerevisiae* alleles. We now recognize that the reason for these inconsistent observations derives from the fact that different studies analyzed different genes of what we currently know to be a very heterogeneous hybrid genome with contributions from *S. cerevisiae*, *S. eubayanus*, and *S. uvarum* (Libkind et al. 2011).

The study of the Patagonian isolates coincided with the emergence of whole genome analyses and included a comparison of genomes of *S. eubayanus*, *S. uvarum*, *S. bayanus*, and *S. pastorianus*. The genomes of *S. eubayanus* and the non-*cerevisiae* subgenome of *S. pastorianus* had 99.56% identity, which demonstrated that *S. eubayanus* was the elusive non-*cerevisiae* progenitor of lager yeasts. That study also showed that *S. uvarum* was the sister taxon of *S. eubayanus* and, importantly, was not a synonym of *S. bayanus*, thus confirming earlier reports (e.g. Nguyen and Gaillardin 2005). In fact, whereas *S. uvarum* is a natural nonhybrid species found in nature, *S. bayanus* is exclusively found in anthropic environments and, as indicated above, represents a complex hybrid. This confusion prevailed in the literature until recently and resulted in the inaccurate use of the name *S. bayanus* or *S. bayanus* variety *uvarum* to refer to *S. uvarum* (e.g. Caudy et al. 2013, Cliften et al. 2003, Kellis et al. 2003). This was one of the reasons why the Patagonian isolates were formally named as the “true” (*eu* in Latin) “*bayanus*,” thus confining *S. bayanus* to its unique hybrid nature. Nowadays, both *S. uvarum* and *S. eubayanus* are seen as natural species that are the closest relatives of each other and, thus share features that are relevant for understanding the evolution of the genus *Saccharomyces*. These two sibling species are the most distant rel-

atives of *S. cerevisiae* and it is likely that they speciated from their common ancestor in temperate forests of the Andean Patagonia in South America because it is there that their diversity is the highest. Moreover, they share a similar niche, the trees of the genus *Nothofagus*—although not the same *Nothofagus* species (Eizaguirre et al. 2018, Libkind et al. 2011), and a preference for low to moderate temperatures, a likely reminiscence of their native habitats in the Southern Hemisphere. This last attribute explains why the hybridization of *S. cerevisiae* and *S. eubayanus* was favored in the low temperature environments where Bavarian monks developed and improved the brewing of lager beer since the XV century (Corran 1975, Hutzler et al. 2022, Meusdoerffer 2009).

Therefore, the finding of *S. eubayanus* in South America solved one mystery but created another—the Atlantic Ocean and 12 000 km separated the region where *S. eubayanus* was found from the region where, plausibly, the formation of the hybrid *S. pastorianus* took place. Understandably, that incongruence and the relevance of those findings for brewing motivated multiple researchers to investigate in more detail the distribution of *S. eubayanus*. The next breakthrough occurred in 2014 when *S. eubayanus* was found in bark and rotten wood of oak and other deciduous trees in four provinces of West and Northwest China (Bing et al. 2014). Although the authors argued for a Far East Asian origin of lager beer yeast and, by so doing, removed an ocean from the equation, 7000 km still separated their findings in China from the original lager brewing region. Nevertheless, the genome of a Tibetan strain and the non-ale subgenome of *S. pastorianus* had a sequence identity of 99.82%, which was higher than the corresponding Patagonian comparison. As the interest of brewers and yeast researchers on the cryotolerant wild ancestor of lager beer was growing, and various publications on this topic emerged (e.g. Hebly et al. 2015, Krogerus et al. 2015), so was the mystery of *S. eubayanus* distribution deepening. Europe is arguably the best sampled territory for wild and industrial yeasts and has a long history of studies involving yeast isolation. These investigations date back more than a century to pioneers of yeast biology like Emil Christian Hansen in Denmark, the first to obtain a pure yeast culture in 1883, Paul Lindner in Germany, or Alexandre Guillaumond in France. Tangible evidence of these endeavors are the various strains of *Saccharomyces* spp. available in culture collections since the end of the XIX century. However, none fit the description of *S. eubayanus*. Moreover, the hypothesis of an *S. cerevisiae* × *S. eubayanus* hybridization in the wild, far from the brewing environment, followed by a posterior colonization of the brewery appears unpalatable. As our ability to dissect genomes increased it become

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clear that the occurrence of hybrid strains of *S. pastorianus* was restricted to the brewing environment and none had been found in natural environments, thus indicating that it was a direct product of domestication.

By this time, rare strains of *S. eubayanus* were also isolated in North America (Peris et al. 2014) and 2 years later in New Zealand (Gayevskiy and Goddard 2016). Gradually, more *S. eubayanus* strains encapsulating the genetic and geographic diversity of the species were analyzed with whole genome data and a global population and phylogeographic perspective emerged (Eizaguirre et al. 2018, Langdon et al. 2020, Nespolo et al. 2020, Peris et al. 2016). In summary, it was found that: (i) *S. eubayanus* is most abundant and diverse in Andean Patagonia; (ii) globally, there are two major populations (PA and PB), further divided into six subpopulations (PA-1, PA-2, PB-1, PB-2, PB-3, and Holarctic); (iii) isolates from South America belong to all subpopulations except the Holarctic one, and isolates from outside South America belong to the PB-1 or Holarctic subpopulations (or are admixtures between PA and PB); (iv) the Holarctic-subpopulation includes isolates found in North America and Tibet, which are the closest known wild relatives of the *S. eubayanus* subgenome of *S. pastorianus*, thus suggesting that the parent genome is Holarctic and not Patagonian.

In spite of these advances, the elephant remained in the room—conclusive proof that *S. eubayanus* is present in Europe was missing. In spite of the lack of European cultures, some observations hinted at this possibility. Almeida et al. (2014) detected conspicuous introgressions from *S. eubayanus* in *S. uvarum* wine and cider strains, although introgressions were rare in strains isolated from natural environments in Europe or elsewhere. Then, in 2019, Alsammar et al. (2019) claimed the detection of *S. eubayanus* at low altitudes in the Italian Alps, using metabarcoding. Environmental sequences of the ITS1 region matched those of *S. eubayanus*, which constituted the first detection, albeit indirect, of the species in Europe. However, caution is recommended in the interpretation of these results. First, there is a single nucleotide polymorphism in the ITS1 region between *S. eubayanus* and *S. uvarum*, a species, i.e. found in Europe in natural environments. Second, it is known that a hybrid strain can exhibit the same result as the rRNA locus can fix one allele from an original two-allele hybrid and, third, the dataset contained very few reads that mapped to *S. eubayanus* (Langdon et al. 2020). Nevertheless, climate modeling based on the known locations of isolation of *S. eubayanus* predicted its occurrence in some regions in Europe, especially the northern part of the Iberian Peninsula to the British isles, France and Germany (Langdon et al. 2020).

In this issue, Bergin et al. (2022) put an end to the enduring mystery of the absence of *S. eubayanus* in Europe and report its isolation in Ireland. However, their findings fit well in the gallery of baffling mysteries that *S. eubayanus* keeps offering us. Indeed, Geraldine Butler and her team from the University College Dublin (UCD) neither traveled to the wilderness nor used a sophisticated enrichment protocol. Instead, the first isolation of *S. eubayanus* in Europe was embedded in an undergraduate research program and did not use the selective *Saccharomyces* isolation protocol or low temperature incubations (Sampaio and Gonçalves 2008). Nevertheless, this remarkable breakthrough was not achieved without hard work. Since 2018, Butler's team and their undergraduate students have reported the isolation and genome sequencing of several yeast species like, e.g. *Metschnikowia pulcherrima* (Venkatesh et al. 2018) or *Ogataea degrootiae* (Ó Cinnéide et al. 2021), mostly from soil samples using an isolation protocol similar to the one employed in their isolation of *S. eubayanus*. By surveying soil collected

from a wooded area in the UCD campus containing oak (*Quercus robur*), beech (*Fagus sylvatica*), and sycamore (*Acer pseudoplatanus*), the authors isolated two cultures of *S. eubayanus* among approximately 100 yeast cultures. Interestingly, this rare frequency of isolation of *S. eubayanus* differs from what was originally observed in Andean Patagonia but follows all other isolation records outside Patagonia.

When Bergin et al. (2022) analyzed the genomes of the novel Irish *S. eubayanus* strains, they concluded that they belonged to the Holarctic population, which fits well in our current understanding of the global population structure of the species. Then the authors compared the *S. eubayanus* portion of *S. pastorianus*, using representatives of the two known lager yeast lineages, Saaz and Froberg (Dunn and Sherlock 2008, Walther et al. 2014), with Holarctic strains representing the known geographic range of this transcontinental population, i.e. North America, East Asia (Tibet), and Europe. By mapping nucleotide polymorphisms shared between the domesticated lager genomes and only one of the three wild candidate ancestors, they defined blocks of shared ancestry to investigate another *S. eubayanus* mystery—which wild strain is more similar to *S. pastorianus*, thus being the closest “living ancestor” to the non-*cerevisiae* subgenome of lager yeasts?

As it has become customary in the *S. eubayanus* tale, solving one mystery invariably leads to the emergence of a new one. Although the Irish isolates are separated from the region in Europe where lager hybrids were probably formed by just 1300 km, the proportion of the *S. pastorianus* genome that has shared ancestry with them varies between 42.3% and 37.7% (comparisons with Saaz and Froberg genomes, respectively), whereas the Tibetan strains have higher shared ancestry values of 51.9%–56.1% (5.8%–6.2% for the North American strains). Therefore, the Irish isolates cannot claim the title of being the closest relatives of the non-*cerevisiae* subgenome of *S. pastorianus*. Moreover, they grow poorly on maltose and are not expected to grow on maltotriose, two important sugars in beer wort. North American and Tibetan strains have the genetic machinery for transporting and metabolizing these sugars, albeit utilization of maltotriose was not observed in any *S. eubayanus* strain and growth on maltose is variable (Baker and Hittinger 2019, Brouwers et al. 2019).

To resolve the conflict between geography and ancestry, two hypotheses remain to be explored in future studies. As was earlier proposed by Peris et al. (2016), the complex ancestries seen in the Holarctic population, with different parts of the genome in different geographical isolates revealing distinct phylogenetic signals, might be the outcome of a turbulent hybridization onset of lager yeasts. Those authors postulated that no one isolate of *S. eubayanus* is the direct ancestor of the parent of the lager yeasts due, possibly, to multiple hybridization events and incomplete lineage sorting. Clearly the results of Bergin et al. (2022) fit well in this scenario. Evidently, the alternative hypothesis that isolates that share more similarities with *S. pastorianus* have yet to be found in Europe cannot be dismissed.

It, thus appears that despite the exciting findings of Bergin et al. (2022), *S. eubayanus* has not yet surrendered all its mysteries. Now that we know that it lives in Europe, what is its distribution? Does the distribution of *S. uvarum*, a species relatively frequent in continental Europe, correlate negatively with the European distribution of *S. eubayanus*? What is its natural ecology in the Old World? Is there a single European lineage, i.e. the direct ancestor of the parent of the lager yeasts? And did the out-of-Patagonia migration involve more than one route and recurrent events involving trans-Atlantic and trans-Pacific dispersion? It seems that in spite of the extraordinary discovery of Irish isolates, the tale of *S. eu-*

bayanus has not come to an end and exciting new questions are bound to capture our curiosity in the near future. There is though one important lesson that this remarkable yeast has just taught us. Despite of all the impressive advances at the molecular, cellular, and evolutionary level, our understanding of yeast ecology is so rudimentary that a simple program of yeast isolation on our own backyard can yield extraordinary and unforeseen results.

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