Biogeographical characterization of *Saccharomyces cerevisiae* wine yeast by molecular methods

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**INTRODUCTION**

One of the most important issues in ecological studies is the determination of microbial biodiversity distribution and thus the understanding of whether microorganisms are cosmopolitan or endemic to a specific area or host (Ramette and Tiedje, 2007). Biogeography is the discipline that studies the distribution of biodiversity over space and time (Martiny et al., 2006). During the 18th century, biologists applied this approach to study the geographic distribution of plant and animal diversity, and only more recently, interest in the geographic distribution of microorganisms has increased. The aim of microbiogeography is to reveal where microorganisms live, their abundance and distribution, and their diversity over different environmental scales. In fact, genetic distance may be correlated with geographic distance and/or environmental characteristics (e.g., salinity, depth, latitude; Schuller et al., 2012). The scope of microbiogeography also encompasses the understanding of the processes generating and maintaining the distribution of microorganisms (Ramette and Tiedje, 2007). Other goals of this field are to propose and evaluate theories regarding the creation and evolution of such diversity patterns in the environment (Ramette and Tiedje, 2007). The first paradigm in microbial biogeography, "*Alles is overal, maar het milieu selecteert*" ("everything is everywhere, but the environment selects") was offered by Baas Becking more than 70 years ago (O’Malley, 2008). This appealing idea was based on the small size and high dispersal potential of microorganisms and their large populations and low presumed extinction rates (Ramette and Tiedje, 2007). However, even if the field of microbial biogeography is not new, the determinism of microbial diversification and distribution has been poorly documented and is not well understood. This may partly be due to the natural properties of microorganisms (e.g., their small size, which makes access within different environmental matrices difficult, their huge diversity, and the complexity of precisely defining their species) and the lack of an adequate sampling strategy. Recently, the development of new molecular tools has partially resolved these limitations; in fact, recent developments have allowed the survey of uncultivated microorganisms in the environment and the characterization of microbial community structure (Christen, 2008). Furthermore, these tools are now generally automated and allow the moderate throughput essential to studies involving the characterization of numerous samples of different origins. In particular, the use of DNA, RNA and protein sequences for the construction of evolutionary trees has allowed a better understanding of the way in which biodiversity was generated. Hence, the application of molecular phylogenetic methods to study natural microbial ecosystems has resulted in the unexpected discovery of many evolutionary lineages (Suzzi, 2011). Moreover, metagenomic and metatranscriptomic approaches will allow not only the dissolution of the species concept issue but will also separate the relationship between the notion of species and their spatial distribution. Weber and Keddy (1993) proposed that a trait-based approach should be the basis of a conceptual model for trait-based community assembly. In particular, traits, not taxon names, are the fundamental units of biodiversity and biogeography. Microorganisms that show similar traits share the same ecological niche. Therefore, the principal challenge of microbiology is to identify...
Yeasts of the Saccharomyces sensu stricto species complex (Figure 1) are able to convert sugar into ethanol and CO₂ via fermentation. They have been used for thousands of years by mankind for the production of fermented beverages and foods. These yeasts show interesting features that are specific and not found in other genera; for example, they are able to survive in the absence of oxygen by using the fermentation process (Sicard and Legras, 2011). The Saccharomyces sensu stricto genus is composed of species showing a level of nucleotide divergence similar to that found between birds and humans (Dujon, 2006). The sensu stricto complex is thought to be young; in fact, some studies have suggested that Saccharomyces cerevisiae diverged from the common ancestor of Saccharomyces paradoxus and Saccharomyces cariocanus approximately 5–10 million years ago (Mya), whereas Saccharomyces kudriavzevii, Saccharomyces bayanus, and Saccharomyces mikatae diverged 10–15, 15–20, and 20 Mya, respectively (reviewed in Replansky et al., 2008).

Recently, Libkind et al. (2011) identified a new species very similar to Saccharomyces bayanus and called it Saccharomyces eubayanus sp. nov., which exists in apparent sympatry in Nethofagus (Southern beech) forests in Patagonia. This species is 99.5% identical to the non-Saccharomyces cerevisiae portion of the Saccharomyces pastorianus genome sequence. Because Saccharomyces pastorianus and Saccharomyces bayanus (a complex hybrid of Saccharomyces eubayanus, Saccharomyces uvarum, and Saccharomyces cerevisiae) are considered to be “a product of the artificial brewing environment with no occurrence in nature,” they may be associated with domestication events and hybrid lineages, whereas Saccharomyces uvarum and Saccharomyces eubayanus may be conserved as descriptors of the species.

Recently, the budding yeast S. cerevisiae has been considered to be an important model for ecological and evolutionary genetics. The ancestor of the sensu stricto complex underwent whole-genome duplication. This event was followed by the loss of approximately 90% of the duplicated genes. In fact, comparison of the S. cerevisiae genome with that of the pre-duplication species Kluyveromyces waltii reveals the presence of approximately 500 paralogs among the 5500 genes (Replansky et al., 2008). These duplications may then be subjected to mutations, which may be related to the evolution of new functions or sequence divergence, and inactivation due to the accumulation of non-sense mutations, which leads to relics (Liti and Louis, 2005). The duplicated genes may evolve at different rates, providing new functions. For example, the abilities to grow anaerobically and to produce ethanol and the low- and high-affinity glucose systems may be a consequence of genome duplication and may have offered a competitive advantage against bacteria and other microorganisms.

Scannell et al. (2011) resequenced and reassembled the genomes of S. mikatae, S. kudriavzevii, and S. bayanus and compared them with the S. paradoxus genome (Liti et al., 2009) and the reference genome of S. cerevisiae (Godfay et al., 1996). The authors annotated 5261 sets of genes that are orthologous among all five species and identified 123 genes that could be used as targets of positive selection and may play important roles in ecological specialization. Moreover, these authors underlined that whole-genome duplication still influences yeast evolution and contributes to the genomic and phenotypic differences characterizing S. cerevisiae and its related species. In addition, the presence of two possible horizontal gene transfers from bacteria was described (Scannell et al., 2011). The possibility of a horizontal genetic exchange from bacteria was also suggested by Wei et al. (2007). The YIM789 genome (a yeast isolated from the lung of an AIDS patient with pneumonia) highlighted a putative horizontal transfer of YIM-GNAT (an unknown gene belonging to the GNAT superfamily related to antibiotic resistance) from bacteria and a potential introgression of a 12-kb sequence of chromosome I from a closely related yeast (Wei et al., 2007).

Recent analyses have shown that yeast hybrids may be more abundant in both natural and industrial environments than previously thought. Indeed, almost 10% of Saccharomyces strains previously classified as sensu stricto appear to be hybrids of different species (Liti and Louis, 2005). In fact, interspecific hybrid strains, which contain genetic contributions from both S. cerevisiae and other Saccharomyces spp., may have selective advantages deriving from the combination of desirable traits from both parental species. Recently, several strains involved in winemaking were found to be hybrids between S. cerevisiae and S. kudriavzevii (Gonzales et al., 2006; Erny et al., 2012). Initially, this last species was isolated in Japan, and although Sampaio and Goncalves (2008) also found it in Portugal, it has never been isolated from wine fermentation. However, the Portuguese S. kudriavzevii population showed genetic differences compared with the type strain of the species that represents the Japanese population. In wine fermentation, the

![Figure 1](https://example.com/figure1.png)

**FIGURE 1** | Geographical characteristics and phylogenetic relationship among Saccharomyces species based on the combined sequence analysis of the D1/D2 LSU rRNA gene and ITS (modified from Replansky et al., 2008; Kurtzman et al., 2011).
hybrids exhibit the best properties of both parental species, such as the low-temperature fermentation ability of *S. kudriavzevi* and the high ethanol resistance of *S. cerevisiae*. Dunn et al. (2012) analyzed 69 commercial wine yeasts and compared them with other industrial yeasts, wine yeasts, beer yeasts, and fuel ethanol yeasts. An interspecific hybridization between *S. cerevisiae* and *S. kudriavzevi* in four of the 69 commercial wine strains was observed, and *S. paradoxus* and *S. mikatae* introgression events were detected.

It is unknown when humans began to add selected yeast to make fermented beverages and foods. Such human activities caused hybridization between species and variation of ploidy, which contributed to the evolution of domesticated yeasts.

*Saccharomyces cerevisiae* strains are adapted to different niches, so they represent a rich resource for revealing the evolutionary trajectories of a trait because particular molecular profiles may have been selected in specific environments. Moreover, several studies found evidence for a role of geographical isolation in the differentiation of the *S. cerevisiae* population in nature, indicating that *S. cerevisiae* can be used as a model for evolutionary biology and biogeography (Carretto et al., 2008; Replinsky et al., 2009; Ledhary et al., 2009; Liti et al., 2009).

Recent resequencing and phylogeographic characterization of multiple *S. cerevisiae* isolates provided evidence of substantial genetic and phenotypic diversity (Liti et al., 2009).

Wang et al. (2012) performed a population genetics analysis of wild Chinese isolates with different ecological and geographical origins. They identified eight new, distinct wild lineages (coded as CHI-N-VIII) from a set of 99 Chinese isolates. These lineages were characteristic of specific geographical areas and ecological niches. In particular, these results indicate that a genetically isolated source is important for *S. cerevisiae* population differentiation in nature. In fact, this study showed that oak isolates from different regions in northern China clustered into different lineages, and the Chinese oak isolates were clearly separated from those from North America.

Strains of *S. cerevisiae* associated with vineyards and wine production, hereafter referred to as wine strains, often form a genetically differentiated group that is separate from wild strains isolated from soil and oak tree habitats and strains from other fermentation types, such as palm wine, and sake (Fay and Benavides, 2005; Liti et al., 2009; Schacherer et al., 2009). Several authors have explained these differences as a consequence of domestication. These domestication events were followed by human-associated dissemination of these yeasts throughout the world.

In a recent study, Legras et al. (2007) investigated the possible effects of human history on spreading and selecting this yeast. In particular, they analyzed 651 yeast strains with 56 different origins (beer, bread, palm wine, wine, and rice wine) from five continents. All wine yeasts grouped together and were well separated from the yeast strains of other technological origins. For “non-wine strains,” a relationship between genotype and the isolation source was found. In particular, three Asian groups of strains were identified: the first included the sake yeast group, and the other two contained rice wine and Chinese distillery strains. Regarding the African yeast populations, a Nigerian palm wine group was identified, which also included an Ivory Coast strain. Ghana sorghum beer strains were distinct from palm wine, Burundi cassava and banana strains, suggesting genetic differentiation among African yeast populations (Legras et al., 2007). The wine yeast group contains strains from ancient vine areas (Lebanon, Europe) as well as “new world” recent vineyards, which suggests a migration of wine yeast all over the world. In addition to the historical human transport across the Mediterranean Sea, the phylogenetic analysis obtained clearly supports the hypothesis of a migration pathway along the Danube valley. The way in which wine strains are naturally propagated is still poorly understood: for yeasts, which grow almost continuously on the surface of wine during the sherry wine process, are likely an example of domestication; in fact, they may present specific features and mutations (Fidalgo et al., 2006). However, for other types of wine strains, we cannot infer such a continuous human control of their culture.

Fay and Benavides (2005) investigated the genetic differences among strains that were of wine origin and those that were not. The population structure of *S. cerevisiae* in nature remains obscure. The different *S. cerevisiae* isolates are characterized by large genetic and phenotypic variations, providing a powerful tool for quantitative genetic studies (Liti et al., 2009). This apparent variation is likely because some studies were performed on laboratory strains, which are highly adapted to artificial conditions and do not represent the true ecological diversity of the species (Steinemetz et al., 2002; Ehrenreich et al., 2010). Recently, it was demonstrated that environmental factors and the interactions between each organism and its environment influence genomic rearrangements and the evolution of phenotypes (Camarasa et al., 2011; Warringer et al., 2011). In particular, Camarasa et al. (2011) related the metabolic traits of *S. cerevisiae* strains with their origins. These strains were isolated from seven different niches (baker, clinical, fermentation processes, laboratory, vineyard, natural, and commercial wine yeasts). The relationships were established using a statistical approach that allowed the identification of specific features common to all strains belonging to the same niche. Some metabolic differences of strains with different origins are shown in Table 1. Phenotypic variation in *Saccharomyces* strains collected from diverse natural habitats, used in industrial processes, and associated with human illness was observed (Kvitk et al., 2008). Phenotypic variation in stress sensitivity and gene expression was also observed. Vineyard isolates survived better in the presence of different stress conditions due to their ability to thrive in more variable natural environments, which facilitated their dispersal into new environments in a manner associated with human interactions (Kvitk et al., 2008). The main approach used to establish a relationship between genotypic variation and phenotypes is mapping of quantitative trait loci (QTL). This technique allowed to map the loci responsible for brewing characteristics in a sake strain, ethanol resistance, xylose utilization for application in the bioethanol industry, acetic acid production, and fermentation performance in wine strains (Borneman et al., 2013).
Table 1 | Sequenced genomes of wine Saccharomyces cerevisiae strains (modified from Borneman et al., 2013).

| S. cerevisiae strain | Project | Origin | Reference |
|----------------------|---------|--------|-----------|
| RM11-1a              | Assembly| Vineyard-USA | Wei et al. (2007) |
| YPS163               | Low coverage assembly| Vineyard-Italy | Doniger et al. (2008) |
| AAW1631              | Assembly| Wine | Borneman et al. (2008) |
| EC1118               | Assembly| Commercial wine yeast | Novo et al. (2009) |
| AAW1936              | Assembly| Commercial wine yeast | Borneman et al. (2011) |
| Lalvin QA23          | Assembly| Commercial wine yeast | - |
| Vin3                 | Assembly| Commercial wine yeast | - |
| YAK269               | Assembly| Wine grapes | - |
| T73                  | Assembly| Wine-Spain | - |
| Y55                  | Low coverage assembly| Grape-France | Liti et al. (2009) |
| Li1528               | Low coverage assembly| Wine-Chile | - |
| BC1187               | Low coverage assembly| Wine-USA | - |
| DBY10106             | Low coverage assembly| Grape-Australia | - |
| Ycl17_E5             | Low coverage assembly| Wine-France | - |
| Y12                  | Low coverage assembly| Palm wine-Africa | - |
| DBYPQ5044            | Low coverage assembly| Bili wine-Africa | - |
| WE372                | Raw data only| Wine-South Africa | - |
| Y12                  | Raw data only| Palm wine-Africa | - |

*Haploid derivative of original isolate; †Haploid sequence representation of diploid strain.

It is well known that the wine yeast, *S. cerevisiae*, plays a major role in the fermentation of grape musts; in fact, it is well adapted to this process (Martini and Vaughan-Martini, 1990; Blondin et al., 2011). In particular, this yeast is adapted to the harsh conditions in grape musts and grapes (high sugar concentration, increasing alcohol concentration, acidity, presence of sulfites, anaerobiosis, and progressive depletion of essential nutrients, such as nitrogen, vitamins, and lipids), and its genome has been modeled, so the understanding of the adaptation phenomenon to the wine environment is a key element in wine yeast genome research (Blondin et al., 2011).

**Saccharomyces cerevisiae** **WINE YEAST**

*Saccharomyces cerevisiae* is one of the best model systems used for understanding microbial ecology and evolutionary genetics. Many functional analysis projects have been dedicated to the investigation of its molecular biology since its genome was first sequenced more than 10 years ago. In fact, a large amount of genomic data for *S. cerevisiae* strains is available (Wei et al., 2007; Novo et al., 2009; Borneman et al., 2011, 2013): there are 28 assembled genome sequences (mainly in draft format), and 19 are available as unassembled sequencing reads. Moreover, 35 sequences are available through project-specific websites (Borneman et al., 2013). In Table 2, wine *S. cerevisiae* sequenced strains are reported.

Recently, Borneman et al. (2012) described the genome sequence of the thiol-releasing commercial wine yeast hybrid VIN7. They showed that VIN7 is an almost complete allotetraploid interspecific hybrid of *S. cerevisiae* and *S. kudriavzevii* that contains a heterozygous diploid *S. cerevisiae* genome and a haploid *S. kudriavzevii* genome. Both parental strains showed a European origin; in particular, the *S. cerevisiae* portion of the VIN7 genome was closely related to wine yeast but distant from the commercial wine yeasts QA23 and EC1118 (Borneman et al., 2012). The genomes of *S. cerevisiae/S. kudriavzevii* hybrid strains display a mosaic structure that likely resulted from selective pressures experienced over time (Querol and Bond, 2009).

A comparative genome analysis between *S. cerevisiae* industrial and laboratory strains highlighted how the environment influences genomic structure and helped to identify genomic loci involved in the regulation of industrial phenotypes. In particular, substantial conservation throughout a core set of genes was observed, whereas many other regions displayed nucleotide substitutions likely involved in diversification and specialization events (Borneman et al., 2008).

Gene transfer is an important aspect of yeast diversification and may play a major role in adaptation to the wine fermentation ecosystem. Novo et al. (2009) sequenced the complete genome of the diploid commercial wine yeast EC118. They identified 34 ORFs encoding proteins potentially involved in carbon and nitrogen metabolism, cellular transport, and the stress response that were absent from S288c. BLASTP analysis suggested that these genes specific for EC1118 were acquired from non-*S. cerevisiae* donors. In fact, the closest relatives to EC1118 were found to be in species belonging to two clades. The first contained the *Lachancea*, *Zygosaccharomyces*, *Kluyveromyces*, Saccharomyces, and *Eremothecium* genera, and the second species belonged to a large,
recently reassessed clade containing Debaryomyces, some Pichia, and a number of medically important Candida species.

The yeast genome is quite small at only 12 Mb but is highly packed, with approximately 6000 genes distributed over 16 chromosomes. Additionally, it contains two small, cytoplasmatic chromosomes. Furthermore, it contains two small, cytoplasmatic chromosomes. Moreover, data indicate that yeast populations on wine berries are highly diverse. Thus, the occurrence of specific natural strains likely depends on numerous factors such as climate conditions, the geographical location of the vineyard, the ripeness of the grapes, the age of the vineyard, the soil type, the grape variety, the application of antifungal agents, and the technique used to harvest (Combina et al., 2005; Valero et al., 2005, 2007; Rasper et al., 2006; Nisiotou and Nychas, 2007; Chavan et al., 2009; Li et al., 2010; Cordero-Busso et al., 2011). In fact, another study found a relationship between specific natural strains and a particular terroir (Frezier and Dubourdieu, 1992; Sabate et al., 1998; Lopes et al., 2002; Schuller et al., 2005; Valero et al., 2007). Thus, the following definition of vitivinicultural "terroir" was provided: "Vitivinicultural "terroir" is a concept which refers to an area in which collective knowledge of the interactions between the identifiable physical and biological environment and applied vitivinicultural practices develops, providing distinctive characteristics for the products originating from this area" (Resolution OIV/Viti 333/2010).

However, insufficient quantitative data are available to establish general conclusions on the influence of these factors on the evolution of the fermentative biota of a given viticultural region, and extensive biogeographical surveys over many years are necessary (Schuller and Casal, 2007). Discrimination at the strain level thus becomes a strategic activity for the wine industry because it may link territory, environment, and final products for wine valorisation.

The species present on intact, undamaged berries have been reported to mainly belong to the group of oxidative basidiomyceteous yeasts such as Hanseniaspora uvarum, Cryptococcus spp., Rhodotorula spp., Sporobolomyces spp., and Filobasidium spp. as well as to the dimorphic ascomyceteous black yeast Aureobasidium pullulans (Prakitchaiwattana et al., 2004; Barata et al., 2008, Barata et al., 2012). In contrast, the most relevant fermentative wine yeast, S. cerevisiae, only occurs at concentrations less than 10–100 cfu/g of berry (Fleet, 2003). This yeast is present in nature at very low concentrations. On the surface of undamaged berries, its concentration is lower than 0.1%, although it is easily found on berries damaged by birds or insects (24%), which represent approximately 1 in 1000 grapes. In any case, some authors (Mortimer and Polisnelli, 1999) have shown that a population of yeast that is the primary source of natural yeast in wine production exists on grapes. Moreover, data indicate that yeast populations on wine grapes increase from 10^6–10^7 cfu/g on immature berries to 10^9–10^10 cfu/g on mature berries. Insects and birds are important agents for the dispersal of yeasts in different habitats. Regarding the role of insects as a vector for S. cerevisiae cells, Mortimer and Polisnelli (1999) demonstrated the presence of a flow of S. cerevisiae cells between the natural environment and cellars; because this yeast is not an airborne, it needs a vector to move. In particular, Francesca
et al. (2012) also highlighted that migratory birds may act as vector for S. cerevisiae cells, but they are not a “reservoir” because the yeast cells survive in the gut for only 12 h. Stefani et al. (2012) isolated yeast strains from wasps, grapes, and fermentations from the same vineyard over a span of different months and years. The results obtained showed that these strains were more similar to each other than strains derived from other environmental and geographical locations. Wasps therefore may play a role in maintaining ecological diversity.

However, whether these strains participate in alcohol fermentation in the cellar is still controversial: some authors (Cani et al., 2004) observed that only cellar strains were responsible for alcohol fermentation in vats, whereas others showed that grapevine strains’ may be partially responsible for alcohol fermentation (Constanti et al., 1997; Gutiérrez et al., 1999; Le Jeune et al., 2006).

Molecular Methods

By using the techniques developed by molecular geneticists, new phylogenetic relationships were recognized, the number of separate species groups was reduced, and the diversity within the groups was increased (McCallough et al., 1998). Moreover, molecular methods revised the study of biogeography and positively impacted the final interpretation of biogeographic patterns (Ramet et al. and Tisdal, 2007). In particular, a better knowledge of the microbial ecology of local ecosystems is essential to understand the winemaking process and to generate products with a local character, thereby allowing the development of modern winemaking practices and the diversification of wine products. Grapevine cultivation and wine production spread throughout the Mediterranean Sea toward Greece (5000 B.C.), Italy (900 B.C.), France (800 B.C.), northern Europe (100 AD) and much later, to the Americas (1500 AD). There are approximately 7.5 million hectares of vineyards across the world, mainly concentrated within the earth’s temperate zones, and two million are located in Europe (OIV, Statistical Report on World Vitiviniculture, 2012).

In particular, many molecular methods allow the identification of S. cerevisiae at the strain level, and they are required not only to investigate the diversity of this species but also to select strains for use as pure cultures, a widespread practice in winemaking industries where strains contribute to a specific characteristic of the final product (Dequin, 2001; Suzuki et al., 2012).

Sipiczki (2011) highlighted that S. cerevisiae wine strains are polyclonal and that the clones can differ significantly in oenological performance and genotype. The genomes of yeasts are subjected to duplications, deletions, and rearrangements that may cause the acquisition of new functions and gene specialization (Caballes et al., 2011). Some authors (Guillo et al., 1997; Nadal et al., 1999) highlighted that aneuploidy may be a method of yeast adaptation through the modification of the expression of some genes involved in this process (Legras et al., 2007). In any case, aneuploids (Intante et al., 2003; Bradbury et al., 2005; Legras et al., 2007; Lopandic et al., 2007), triploids (Cummins and Fogel, 1978; Takahashi, 1978; Thornton, 1986), polyploids (e.g., Takahashi, 1978; Bakalkinsky and Snow, 1990; Gujo et al., 1997; Naumov et al., 2000, 2002) and rarely haploids (Lopandic et al., 2007) may be present in the natural yeast biota of fermenting wine (Sipiczki, 2011).

Some studies have shown that genomic variability depends on telomeric recombination, which is important for adaptation to new environments and different metabolic sources and to overcome environmental stress, and on the insertion of transposable elements. Transposable elements comprise ~3% of the total sequenced genome of S. cerevisiae S288c (Carreto et al., 2008).

Carreto et al. (2008) showed that wine strains differed dramatically from the reference laboratory strain in Ty element composition, whereas clinical strains were similar to S288C in Ty element composition. Thus, it is likely that clinical strains and S288C had a common ancestor, and the differences found in wine strains may be due to the selective pressures that affect particular regions of the genome in response to adaptation to the environment. In particular, the variable genes were involved in metabolic functions related to cellular homeostasis or transport of different solutes such as ions, sugars, and metals. To better understand the population structure of wine S. cerevisiae strains, ecological studies using a polyphasic approach in order to define the biogeographical patterns have been carried out: a strict collaboration between phylogeneticists and ecologists and the development of new statistical tools provide a more comprehensive understanding of the factors controlling the S. cerevisiae biodiversity and biogeochelmistry. The main molecular methods used for biogeographical studies are reported in Table 3.

CGH Array-Based Comparative Genomic Hybridization

Comparative genomic hybridization (CGH) is capable of detecting loss, gain and amplification of copy number at the chromosome level. Detection of amplifications is known to be sensitive down to less than 1 Mb. Therefore, one must take into consideration that although CGH is sensitive to specific types of copy number gains, its resolution for regional deletions is more limited. The use of array CGH overcomes this limitation, with improvements in resolution and dynamic range, in addition to the ability to directly map aberrations to the genome sequence and improved throughput (Weiss et al., 1999). This approach has been recently applied to investigate the evolutionary importance of genome size in S. cerevisiae (Edwards-Ingram et al., 2004; Dunn et al., 2005, 2012; Gerstein et al., 2006). Dunn et al. (2012) used this technique to study copy number variations (CNVs) across subtelomeric regions, non-S288C genomic regions, retroposons, and the non-nuclear mtDNA and 2-mm plasmids of 83 S. cerevisiae strains isolated from different industrial and natural environments. The obtained clusters for the different types of features showed that most of the CNVs occurred either in subtelomeric regions or among the classes of transposable elements and that there were no commercial wine strains that appeared to be absolutely identical to each other. Thus, these CNVs did not produce any clear phylogeny, so it is likely that an active interchange of these regions occurred rather than separate lineages descending from isolated ancestors, suggesting that most of these strains are the result of interbreeding between industrial and wild strains.

Genome Sequence and Functional Annotation

The genetic diversity of Saccharomyces strains can also be assessed using genome sequencing and functional genomic analysis of transcript profiles. These approaches are useful to aid in the
Table 3 | Molecular approaches used for S. cerevisiae biogeographical studies.

| Molecular methods | Origin | Reference |
|-------------------|--------|-----------|
| aCGH              | Brazil, Italy, USA | Weiss et al. (1999); Edwards-Ingram et al. (2004); Dunn et al. (2005), (2012); and Gerstein et al. (2006) |
| Genome sequence and functional annotation | USA, Japan, France, Italy, Germany | Kintak et al. (2008); Cavalieri (2008); Muller and McCusker (2009a, 2011); Rolland et al. (2009); Bullard et al. (2010); Lelandais et al. (2011); and Scannell et al. (2011) |
| PFGE              | Spain, Japan, UK, USA, France, South Africa, Ivory coast, Switzerland, West Africa, Russia, Portugal, Germany, China | Schwartz and Cantor (1984); Johnston and Mortimer (1986); Vezinhet et al. (1990, 1992); Bidon et al. (1995); Prezzi and Dubourdieu (1992); Birones et al. (1996); Egli et al. (1998); Goto-Yamamoto et al. (1998); Mesa et al. (1999); Rohde et al. (2001); Sipiccki et al. (2001, 2004); Sipiccki (2011); Perez-Oritz et al. (2002); Carro et al. (2003); Schuller et al. (2004); Antunovic et al. (2005); Dunn et al. (2005); Au et al. (2006); and Wang et al. (2012) |
| mtDNA-RFLP       | France, Italy, Portugal | Vezinhet et al. (1990, 1992); Querol et al. (1994); Vezinhet et al. (1995); Schuller et al. (2000); and Di Maio et al. (2012) |
| RAPD-PCR         | Spain, Chile, Peru, Uruguay, France, Italy | Quesada and Ceria (1995); Cavalieri et al. (1996); Martinez et al. (2007); and Toftalo et al. (2007) |
| Microsatellites analyses | New Zealand, Vietnam, France, Belgium, Russia, Czech Republic, Spain, The Netherlands, China, Taiwan, Japan, Costa Rica, Australia, Portugal, Austria, Germany, Brazil, Spain, Ghana, Nigeria, Lebanon | Ness et al. (1993); Versaveud et al. (1995); Gallego et al. (1998); Hennique et al. (2001); Bradbury et al. (2005); Lagrue et al. (2005); Schuller et al. (2005, 2007); Ayoub et al. (2006); Schuller and Cavai (2007); Li et al. (2008); Muller and McCusker (2009b); and Richards et al. (2009) |
| k sequences      | Lebanon, China, Vietnam, Japan, Taiwan, USA, The Netherlands, Italy, France, Portugal | Ness et al. (1993); Lagrue and Karst (2003); Schuller et al. (2004, 2012); Lagrue et al. (2005); and Franco-Quartier et al. (2011) |
| MLST             | Lebanon, China, Vietnam, Japan, Taiwan, USA, Italy, France, Germany, Indonesia, Chile, Uruguay, South Africa, New Zealand | Ben-Ari et al. (2005); Fay and Bernardes (2005); Au et al. (2006); Ayoub et al. (2006); and Vigentini et al. (2009) |

Understanding of speciation, life history variation, conditional fitness trade-offs and the long-term maintenance of complex genomic variation (Scannell et al., 2011). Genome sequencing provides the most complete understanding of the genomic structure of an organism and allows wide comparisons to be made between related species. Scannell et al. (2011) improved the genome sequences of three species belonging to the Saccharomyces sensu stricto complex (S. bayanus var. uvarum, S. kudriavzevii, and S. mikatae) and compared them with the genomes of S. cerevisiae and S. paradoxus. They identified 5261 annotated protein coding orthologs across all of the studied species. Moreover, they found genes that had been lost in one or more lineages. Generally, the lost genes were derived from yeast genome duplications, suggesting that this phenomenon still influences yeasts and contributes to phenotypic differentiation. These authors also detected lineage-specific gains and found, in particular, two horizontal gene transfers from bacteria. These genes differentiated the analyzed species, indicating their involvement in speciation and adaptation. Other authors such as Rolland et al. (2009) have also identified the presence of horizontal gene transfers from bacteria, confirming that this phenomenon plays important functional and evolutionary roles. To characterize the genomes of large numbers of individuals, microarray-based methods provide an alternative to DNA sequencing. This method allows the identification of conserved and non-conserved regions across microbial populations. The use of tiling arrays followed by analysis of the DNA region via polymerase chain reaction (PCR) is useful to determine whether the absence of hybridization is due to deletion of a chromosomal region or due to areas of large sequence polymorphism (LSP). Muller and McCusker (2011) characterized the genome-wide distribution of LSPs in 88 S. cerevisiae strains of diverse geographical origins and source substrates using high-density tiling arrays. They showed that LSPs occurred in the subtelomeric regions of chromosomes, where they did not disrupt essential gene expression. Moreover, this study revealed the presence of introgressions. In particular, clinical strains contained S. paradoxus DNA fragments. In another study, Muller and
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Wine strains generally have a large diversity in the number and fragments in the genomes of three different *S. cerevisiae* isolates (K vitek et al., 2008; Cavalieri, 2009; Bullard et al., 2010). Conservation of a transcriptional response indicates functional relatedness of the organisms under investigation (Lelandais et al., 2011). In fact, genome rearrangements can modify gene expression and alter phenotypes. Koteck et al. (2008) measured whole-genome expression in 52 strains collected from different niches (industrial processes and human illnesses) in the presence of different stress conditions. Wine strains were able to grow in the majority of the tested conditions; for example, copper resistance was predominant in wine strains, suggesting that the use of copper in the vineyard strongly selected against strains that were copper sensitive (Koteck et al., 2008). This evidence confirmed that the process of fermentation imposes a strong selective pressure and therefore is a powerful evolutionary force in the generation of diversity (Koteck et al., 2008; Cabillos et al., 2011; Sipiczki, 2011).

**PULSED-FIELD GEL ELECTROPHORESIS**

Wine strains generally have a large diversity in the number and size of chromosomes that can be observed by pulsed-field gel electrophoresis (PFGE) analysis, which separates chromosome-sized DNA molecules. This method was first described by Schwartz and Cantor (1984) and is still one of the most powerful tools to investigate the biogeography and speciation of this yeast in nature. Analysis of the chromosomes of wine yeast strains by PFGE demonstrated the presence of chromosome-length polymorphisms, which are derived from chromosomal rearrangements such as translocations and deletions (Carro et al., 2003). Carro et al. (2003) suggested that the subtelomeric plasticity of chromosome I, which contains several membrane-associated genes, may induce rapid adaptive changes of the yeast strains in response to specific environmental cues (substrates). The reciprocal translocation between chromosomes VII and XVI generated the SSU1-R allele, which confers sulfite resistance to yeast cells and was described as the first case of adaptive evolution, likely occurring as a consequence of the use of sulfites as a preservative in wine production (Goto-Yamamoto et al., 1998; Perez-Ortin et al., 2002). Many authors have showed that karyotyping is more discriminative than other approaches for yeast typing because it is able to highlight polymorphisms in electrophoretic chromosome profiles in natural *S. cerevisiae* populations from almost all wine-growing regions of the world (Johnston and Mortimer, 1986; Vezinhet et al., 1990, 1992; Bidonne et al., 1992; Frezier and Dubourdieu, 1992; Briones et al., 1996; Eglit et al., 1998; Prowhe et al., 2001; Schuller et al., 2004; Sipiczki et al., 2004; Antunovics et al., 2005; Au et al., 2006; Wang et al., 2012).

This approach showed that strains isolated from the same fermentation generally differ in chromosome length (Eglit et al., 1998; Mesa et al., 1999; Sipiczki et al., 2001, 2004; Antunovics et al., 2005), indicating that clones with different sets of chromosomes propagate at the same time and in succession during fermentation (Sipiczki, 2011). Wang et al. (2012) applied this technique to type *S. cerevisiae* strains with different ecological and geographical origins to better understand the ecology of *S. cerevisiae*. The obtained results showed that a wide divergence of populations of wild *S. cerevisiae* exist and that this divergence is only marginally affected by human activity. Dutu et al. (2003) revealed the existence of a set of deleted or amplified genes common to wine and other industrial yeasts, and certain genes have been identified as a possible wine yeast signature, particularly genes encoding membrane transporters.

**mtDNA-RFLP**

*Saccharomyces cerevisiae* mtDNA is characterized by an elevated mutation rate. In particular, base-substitution mutations and length polymorphisms can be highlighted by restriction fingerprinting of mtDNA using endonucleases with different target sites (e.g., *Dde I*, *Hinf I*, and *Rsa I*). The reliability and discrimination power of this fingerprinting technique are similar to those of PFGE.

The use of mtDNA-restriction fragment length polymorphism (RFLP) revealed a wide range of polymorphisms in mitochondrial genomes and mitochondrial genes (Vezinhet et al., 1996; Querol et al., 1994; Versavaud et al., 1995; Lopez et al., 2003). This technique was used together with PFGE by Vezinhet et al. (1992) to study the evolution of *S. cerevisiae* strains isolated from different wine regions over 6 years. The study demonstrated that some strains were widely distributed in the studied areas and present over several years, indicating that they are endemic to that region. More recently, Di Maio et al. (2012) used this method to investigate the biodiversity of wine yeast populations isolated over several years from Sicilian wineries where commercial yeast strains have never been used. mtDNA-RFLP allowed the differentiation of 209 of 918 yeast strains. Schuller et al. (2005) performed a large-scale biogeographical survey on the genetic diversity of *S. cerevisiae* strains isolated from spontaneous fermentations and identified 297 different genetic patterns among 1620 strains isolated from 54 small-scale fermentations of grapes from three vineyards located in the Vinho Verde region (Portugal) during a 3 year period. Almost all of the obtained patterns were unique, showing the large biodiversity of *S. cerevisiae* in that region.

**RAPD-PCR**

This technique is based on the use of a single short primer (8–12 nucleotides) that amplifies “anonymous” DNA sequences and represents a powerful typing method for many yeast and bacterial species (Querajas and Cenis, 1995; Martinez et al., 2007; Tofalo et al., 2007). In fact, the annealing of the primer at several points allows the user to obtain a complex banding pattern that is specific for each strain (Ivey and Phister, 2011). This method was used by Cavalieri et al. (1998) to differentiate 166 *S. cerevisiae* strains...
isolated from Tuscany and Sicily, two Italian regions. In this case, random amplified polymorphic DNA (RAPD)-PCR allowed the recognition of 16 patterns, and only 10 were strain specific. Tofalo et al. (2007) used this approach to recognize genetically different S. cerevisiae strains, which were clustered in subgroups related to the four different wine-producing areas of the Apulia region (Italy). The obtained results showed that the genetic differences reflect the phenotypic biodiversity.

**MICROSATELLITE ANALYSES**

Microsatellites, also known as simple sequence repeats (SSRs) or short tandem repeats (STRs), are repeating sequences of 1–6 base pairs of DNA that are characterized by a high level of polymorphism. They occur within many open reading frames but are even more frequent in non-coding regulatory regions. In S. cerevisiae, microsatellites have been described as abundant and highly polymorphic in length (Richards et al., 2009), and for this reason, they are used as a reproducible and portable typing method (Gallego et al., 1998; Hennequin et al., 2001; Schuller et al., 2004; Bradbury et al., 2005; Legras et al., 2005). Recently, an increasing number of microsatellites have been described for S. cerevisiae, with the aim of identifying the most polymorphic loci with a high allelic diversity that can be used for both strain identification and the establishment of strain geographical or technological origin. Several studies used this approach to type S. cerevisiae strains of different geographical origins (Ness et al., 1993; Versavaw et al., 1995; Gallego et al., 1998; Hennequin et al., 2001; Bradbury et al., 2005; Legras et al., 2005; Schuller et al., 2005, 2007; Muller and McCusker, 2009b). For example, Schuller and Casal (2007) analyzed six polymorphic microsatellite loci in 361 strains isolated from the Vinho Verde region in Portugal during the 2001–2003 harvest seasons. Fifty-two new alleles were identified in addition to the 41 alleles previously described (ScAT1–ScAT6). Recently, a database of 246 genotypes has been compiled that includes 78 commercial strains, 14 wine isolates, and three laboratory strains by screening for single-nucleotide polymorphisms (SNPs) in loci on genes involved in wine production. In particular, they focused on the identification structure and evolution (Fay and Benavides, 2005; Aa et al., 2006). Strains are characterized using the DNA sequences of internal fragments of multiple housekeeping genes where variation accumulates relatively slowly and tends to be selectively neutral. It is highly reliable and highly discriminatory at the strain level, and because it is based on nucleotide sequencing, the results are easily comparable between laboratories.

Recently, this technique was applied to study S. cerevisiae populations in different vineyards (Fay and Benavides, 2005; Aa et al., 2006). Ayoub et al. (2006) tested a set of seven loci of 84 S. cerevisiae strains of different origins. 65 strains were isolated from traditional wineries in Lebanon, and the others were commercial wine strains and Asian isolates. MLST profiling allowed the differentiation of the Asian group of strains from the Lebanese and European commercial strains that appear closely related, suggesting the introduction of genetic material from Asian strains into Lebanon. Vigentini et al. (2009) studied the genetic biodiversity of an S. cerevisiae collection including 33 commercial strains, 14 wine isolates, and three laboratory strains by screening for single-nucleotide polymorphisms (SNPs) in loci on genes involved in wine production. In particular, they focused on the identification of SNPs as new genetic markers. Several studies report the efficacy of this analysis for studying the evolution of a microbial population (Ben-Ari et al., 2005; Aa et al., 2006). The obtained results showed that the collection was characterized by a low polymorphism rate and degree of heterozygosity and that the gene coding for the trehalose-6-phosphate synthase enzyme, which is involved in ethanol resistance, could be used as a molecular target. In fact, this gene showed a sequence diversity of 1.42% with seven different nucleotide substitutions.


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CONCLUSION
Biogeographical studies revealed that S. cerevisiae species consists of both "domesticated" and "wild" lineages which are phylo-
genetically distinct. These populations probably derives from the
whole-genome duplication of a common ancestor strain. In par-
ticular, for S. cerevisiae wine yeast a clear geographical origin was
established and it suggests that many of the differ-
et strains evolved independently for long time. They modified
from other yeasts (strains differ not only for their origin but also for genetic transfers
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bacteria. The main difference have been found in genes encoding
cell wall proteins and associated with aminoacid uptake which are
important for the production of sensorially relevant aroma com-
ponents. So, the genomic tools are crucial to better understand
 genetic and molecular basis of yeast evolution and the "art" of wine
making, so characterization of other yeast environmental isolates
will be useful to develop tailor strains to meet consumer demand.

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