Brettanomyces bruxellensis: Overview of the genetic and phenotypic diversity of an anthropized yeast

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Abstract

Human-associated microorganisms are ideal models to study the impact of environmental changes on species evolution and adaptation because of their small genome, short generation time, and their colonization of contrasting and ever-changing ecological niches. The yeast *Brettanomyces bruxellensis* is a good example of organism facing anthropogenic-driven selective pressures. It is associated with fermentation processes in which it can be considered either as a spoiler (*e.g*. winemaking, bioethanol production) or as a beneficial microorganism (*e.g*. production of specific beers, kombucha). Besides its industrial interests, noteworthy parallels and dichotomies with *Saccharomyces cerevisiae* propelled *B. bruxellensis* as a valuable complementary yeast model. In this review, we emphasize that the broad genetic and phenotypic diversity of this species is only beginning to be uncovered. Population genomic studies have revealed the co-existence of auto- and allotriploidization events with different evolutionary outcomes. The different diploid, autotriploid and allotriploid subpopulations are associated with specific fermented processes, suggesting independent adaptation events to anthropized environments. Phenotypically, *B. bruxellensis* is renowned for its ability to metabolize a wide variety of carbon and nitrogen sources, which may explain its ability to colonize already fermented environments showing low-nutrient contents. Several traits of interest could be related to adaptation to human activities (*e.g*. nitrate metabolization in bioethanol production, resistance to sulphite treatments in winemaking). However, phenotypic traits are insufficiently studied in view of the great genomic diversity of the species. Future work will have to take into account strains of varied substrates, geographical origins as well as displaying different ploidy levels to improve our understanding of an anthropized yeast's phenotypic landscape.

Keywords: polyploidy, hybridization, adaptation, fermentation, beer, wine

1. Introduction

Yeasts are eukaryotic species encountered in most, if not all, earth biomes (Starmer & Lachance, 2011). Yeasts are heterotrophic, they are described as primary decomposers of organic matter, and are particularly associated with the early colonization of nutrient-rich substrates. The ability of some yeast species to efficiently perform alcoholic fermentation, *i.e.* to convert sugar into ethanol, made it possible to forge close ties with human beings, leading to a tight co-evolution between yeast and human (Starmer & Lachance, 2011). Their simple life cycle, their small genomes (10 to 20Mbp) which are genetically diverse and their multifaceted metabolisms have propelled yeasts as valuable models to study evolutionary genetics and ecology (Gladieux et al., 2014; Hittinger et al., 2015). Besides the famous yeast model organism *Saccharomyces cerevisiae*, the genus *Brettanomyces* (of which *Dekkera* is a synonym) has received a lot of attention recently, due to its association with various food processes and to its unusual genomic composition. This genus belongs to the *Pichiaceae* family, which is a part of the *Saccharomycotina* subphylum (Kufferath & Van Laer, 1921; Riley et al., 2016). It is composed of six species (*B. nanus*, *B. naardenensis, B. bruxellensis*, *B. anomalus*, *B. custersianus* and *B. acidodurans*), with *Allodekkera sacchari* being the closest non-*Brettanomyces* species known to date (Jutakanoke et al., 2017; Péter et al., 2017) (Figure 1A). Recently, high quality genomes were obtained for five *Brettanomyces* species, revealing distant species separated in two clades with *B. nanus* and *B. naardenensis* on one hand, and *B. bruxellensis*, *B. anomalus* and *B. custersianus* on the other hand (Roach & Borneman, 2020). The calculation of average nucleotide identity along the genomes between each species pairs, ranging between 60.6% and 77.1%, revealed that *Brettanomyces* species are relatively distant of each other: as a comparison, the most distant species within *Saccharomyces* genus show 79.9% nucleotide identity (*S. cerevisiae* and *S. eubayanus*) and 75.2% within *Metschnikowia* genus (*M. hawaiiana* and *M. orientalis*), two yeast genus frequently associated with fermented foods (Lachance, Lee, & Hsiang, 2020; Roach & Borneman, 2020). The five *Brettanomyces* species whose genome is fully sequenced exhibit gene family expansions related to fermentation, such as glucosidase enzymes involved in starch or galactose metabolism, as well as in nitrogen assimilation. In addition,

twelve horizontal gene transfer (HGT) events were detected within *Brettanomyces* genus, and may explain the ability of *B. bruxellensis* and *B. anomalus* to utilize sucrose (Roach & Borneman, 2020). Gene expansions and HGTs are well described markers of domesticated subpopulations of *S. cerevisiae* (Gallone et al., 2018; Giannakou, Cotterrell, & Delneri, 2020; Legras et al., 2018; Peter et al., 2018). The identification of interesting parallels with the evolution of *S. cerevisiae* supported *Brettanomyces* as a valuable yeast model to study adaptation to fermentative environments.

Due to its positive or negative role in different industrial applications, *B. bruxellensis* is the species that has received the most attention. It was first isolated by N. Hjelte Claussen in 1904 as being responsible for the special flavor of British beers, and therefore was the first patented microorganism (Claussen, 1904; Steensels et al., 2015). In brewery, *B. bruxellensis* plays a major role for some special beer types such as the Lambics from Belgium, Flanders Red and Brown ales, or the 'coolship ales' from the USA (Bokulich, Bamforth, & Mills, 2012; Claussen, 1904; De Roos & De Vuyst, 2019). It contributes to the peculiar flavour of spontaneous fermented beers, bringing additional complexity to their aromatic bouquet. In addition, over the past decades, the growing popularity of craft beers and the rise of microbreweries led to an increased interest among brewers who use *B. bruxellensis* alone or in co-culture with other species. For example, *B. bruxellensis* has been described as a good candidate for beers with a spicy phenolic note (Holt, Mukherjee, Lievens, Verstrepen, & Thevelein, 2018). *Brettanomyces bruxellensis'* scientific literature with a focus on brewery is substantial (Capece, Romaniello, Siesto, & Romano, 2018; De Roos & De Vuyst, 2019; Lentz, 2018; Serra Colomer, Funch, & Forster, 2019; Steensels et al., 2015). *Brettanomyces bruxellensis* is also involved in the elaboration of other spontaneous fermented beverages such as kombucha, kefir, etc. in which its role is mostly considered as positive (Lynch, Wilkinson, Daenen, & Arendt, 2021; Tran et al., 2020).

Despite its positive contribution to particular beer flavours and traditional beverages, *B. bruxellensis* is also recognised as a major spoiler for top-selling ale and lager beers due to sensory or turbidity defects (Shimotsu et al., 2015). The same account for wine, where *B. bruxellensis* negative contribution is also

widely described, amplified by its resistance to sulphite, one of the common control method historically used (Monica Agnolucci, Tirelli, Cocolin, & Toffanin, 2017; Avramova et al., 2019; Blomqvist & Passoth, 2015; Malfeito-Ferreira, 2018; Schifferdecker, Dashko, Ishchuk, & Piškur, 2014; Suárez, Suárez-Lepe, Morata, & Calderón, 2007). Its presence in wine is associated with the production of volatile molecules (called volatile phenols), associated to unpleasant aromas described as barnyard, horse sweat or burnt plastic (Chatonnet, Dubourdie, Boidron, & Pons, 1992; Kheir, Salameh, Strehaiano, Brandam, & Lteif, 2013). The 'Brett' taint negatively affects up to 25-30% of red wines in the world (Alston, Arvik, Hart, & Lapsley, 2021; Gerbaux, Jeudy, & Monamy, 2000; A. Romano, Perello, Revel, & Lonvaud-Funel, 2008). *Brettanomyces bruxellensis* is able to penetrate the micropores of the barrels and therefore reused barrels – a frequent practice in oenology – are a recurrent cause of contamination (Cartwright, Glawe, & Edwards, 2018; Fabrizio et al., 2015). In bioethanol production, this species is considered as the main yeast spoiler as it can reduce the ethanol yield and cause large economic losses (Basílio et al., 2008; Bassi, Meneguello, Paraluppi, Sanches, & Ceccato-Antonini, 2018; Blomqvist & Passoth, 2015; A.T. de Souza Liberal et al., 2007; Radecka et al., 2015; Seo, Park, Jung, Ryu, & Kim, 2020). Besides its beneficial-spoiler duality, another oddity of *B. bruxellensis* is its ability to act as a first fermenter (even starter) yeast in some processes (*e.g.* craft beer, kombucha, bioethanol) or as a fermentation 'finisher' (*e.g.* wine, Lambic beers, etc.), the actual fermentation usually being carried out by *S. cerevisiae*. This starter-finisher versatility suggests multiple fermentative adaptations that may have arisen from the complex structure of *B. bruxellensis* genome that includes large chromosomal rearrangements, hybridization events , as well as ploidy level variation (Gounot et al., 2020). In particular, a remarkable characteristic of *B. bruxellensis* species resides in the rare coexistence of diploid and steady triploid isolates (Curtin, Kennedy, & Henschke, 2012; Piškur et al., 2012).

In this review, we first focus on the genetic and genomic composition of *B. bruxellensis* revealed by population genomic surveys. This genetic diversity is reflected at the phenotypic level, with contrasting behaviours regarding metabolic and life-history traits that have ecological significances. We describe

the impact of such diversity on central metabolism characteristics and technological traits, and we discuss how future researches including both genomic and phenotypic approaches may shed lights on the evolutionary history of a human-associated yeast.

2. Genetic and genomic diversity of *Brettanomyces bruxellensis*

2.1. Population structure of Brettanomyces bruxellensis

A wide variety of molecular tools (*e.g.* AFLP, RAPD) were developed to assess the genetic diversity of *B. bruxellensis* species (reviewed in (Renouf, Lonvaud-Funel, & Coulon, 2007)). Most of these studies focused on the intraspecific diversity of small cohorts: between 10 to 100 isolates mainly from the same process (Agnolucci et al., 2009; Conterno, Lucy Joseph, Arvik, Henick-Kling, & Bisson, 2006; Curtin, Bellon, Henschke, Godden, & de Barros Lopes, 2007; Miot-Sertier & Lonvaud-Funel, 2007). Thus, an important step has been taken with the genotyping of more than 1,000 worldwide strains using 12 microsatellite markers (Avramova, Cibrario, et al., 2018). This study provided deep insights into the genetic diversity and ploidy level in several subpopulations of different ecological origin. The population structure was mainly explained by the ploidy level (~47% of the variance), followed by the geographical origin (~5% of the variance) and the fermented product from which the strain had been isolated (~6% of the variance). However, when considering non-wine isolates, the geographical origin explains ~55% of the total variance, indicating that wine isolates are highly disseminated across the world compared to strains isolated from other substrates. Isolates from different origins (such as beer, kombucha, tequila and bioethanol isolates) were mostly clustered into one or two genetic groups, suggesting different adaptation processes to various anthropic environments (Avramova, Cibrario, et al., 2018). This large population study allowed the identification of different genetic subpopulations within *B. bruxellensis*, which were subsequently validated and refined by whole genome sequencing approaches (Eberlein, Abou Saada, Friedrich, Albertin, & Schacherer, 2021; Gounot et al., 2020; Serra Colomer, Chailyan, et al., 2020) (Supplemental Table 1). At least six subgroups can now be considered, either diploid (two groups) or triploid (four groups) (Figure 1B). Among diploid groups, one

subpopulation is composed mainly of strains isolated from wine, and is named the wine 3 group (known as G2N3, CBS2499-like or Wine 2n in various publications, Supplemental Table 1), the other is associated with kombucha, beer and wine isolates, and we named it the kombucha group (known as G2N1, L14165-like, Farmhouse). Regarding the triploid subpopulations, the beer group contains strains from beer and wine (G3N1, AWRI1608-like, Lambic), the two other groups are strongly associated with winemaking and are referred as wine 1 (or G3N2, AWRI1499-like, Wine 3n) and wine 2 groups (or G3N3, L0308-like). The last group, named teq/EtOH group, contains most isolates from bioethanol and tequila process (CBS5212-like, Tequila). Recent genomic data suggested that the teq/EtOH group was not monophyletic, yet more in-depth analyses are necessary to refine precisely the actual number of teq/EtOH groups. Recently still, genome sequencing revealed an autotetraploid strain (Figure 1B), genetically close to a small subpopulation already observed but described as diploid (G2N2, KOM1449-like). More isolates are needed to delineate this subpopulation and its ecological and genomic features. The vast majority (~80%) of wine strains are found in three groups (wine 1 and wine 2 triploid groups, wine 3 diploid group, Figure 1B), yet some wine isolates can be found occasionally in other groups, with variations depending on the wine producing region (Avramova, Cibrario, et al., 2018; Cibrario et al., 2019). Beer isolates are also distributed in different groups, although to a lesser extent (two main groups). Regarding the other isolation niches (such as kombucha, tequila, bioethanol), additional works and more strains are needed to determine whether they fall within one specific subpopulation, or if they are more disseminated as for winemaking. Dissemination via equipment could play an important role in exchanges within and between ecological niches: craft brewers frequently age beer in wood barrel that previously contained red or white wines (Sanna & Pretti, 2015) and similar practice is common for tequila's ageing (Aguilar-Méndez et al., 2017). *Brettanomyces bruxellensis'* population structure seems to be well-defined, but so far, only anthropic isolates of the species have been described, with no wild strains identified so far.

2.2. Genomic landscape of Brettanomyces bruxellensis

Within the *Saccharomycotina* subphylum, *B. bruxellensis* diverged from the model species *S. cerevisiae* between 200 and 300 MY ago, before the whole-genome duplication (WGD) event that appeared in the *Saccharomyces* lineage 100 MY ago (Fisher, Buskirk, Vignogna, Marad, & Lang, 2018; Guo et al., 2016; Wolfe & Shields, 1997). WGD outcomes (e.g. gene duplication, promoter rewiring) were suggested to be involved in the acquisition of the make-accumulate-consume (MAC) strategy in the *Saccharomyces* genus (Thomson et al., 2005). Thus, it seems that both species, *S. cerevisiae* and *B. bruxellensis*, have independently acquired the MAC ability (Rozpędowska et al., 2011). The first partial genome sequence of *B. bruxellensis* strain CBS 2499 showed around 50% of nucleotide identity with *S. cerevisiae* (Woolfit, Rozpędowska, Piškur, & Wolfe, 2007), and more recent studies identified that at least 3,300 orthologous gene families were conserved across both clades (out of 5000 for *B. bruxellensis*) (Cheng et al., 2017).

Over the past decade, several *de novo* assemblies have been published with increasing quality (Table 1), in particular thanks to the combination of long-read sequencing (e.g. Oxford Nanopore sequencing) with short-read sequencing (Fournier et al., 2017). Such high quality assemblies were used as reference genomes for population genomic surveys (Eberlein et al., 2021; Gounot et al., 2020; Serra Colomer, Chailyan, et al., 2020). These population studies revealed that the pangenome is composed of 5,409 ORFs (open reading frames) with 5,106 core and 303 accessory ORFs within the species. Although no significant functional enrichment was found for the set of accessory genes, some of them were shown to be involved in drug and sugar transports (Gounot et al., 2020). Many core genes involved in carbon and nitrogen uptake have been identified, consistent with the ability of *B. bruxellensis* to metabolise a wide range of complex nutriments (*e.g.* xylose, lactose, cellobiose and nitrate).

In *B. bruxellensis*, the nucleotide diversity estimated by the average number of pairwise nucleotide differences, Pi, is high ($\pi = 1.2\times10^{-2}$) compared to *S. cerevisiae* ($\pi = 3\times10^{-3}$) (Gounot et al., 2020). In addition, roughly 50% of the available strains of *B. bruxellensis* (exclusively isolated from anthropic niches) are triploids (Avramova, Cibrario, et al., 2018). Polyploidy in industrial yeasts is known to confer

194 robustness and environmental stress resistance (Albertin & Marullo, 2012; Querol & Bond, 2009;
195 Steensels, Gallone, & Verstrepen, 2021). Interestingly, while triploidy appears to be predominant in
196 the available strains, aneuploidy is rarer in *B. bruxellensis* than in *S. cerevisiae* with 5.6% and 19.1% of
197 aneuploidy isolates, respectively (Gounot et al., 2020; Peter et al., 2018). Genomic studies have shown
198 that the triploid genomes have different genomic architectures arising from intra- and interspecific
199 hybridization events (Borneman, Zeppel, Chambers, & Curtin, 2014; Eberlein et al., 2021; Gounot et
200 al., 2020). In a recent study, the polyploid genomes were reconstructed using different phasing
201 strategies, revealing that each polyploid subpopulation had a unique history (Abou Saada, Tsouris,
202 Eberlein, Friedrich, & Schacherer, 2021). The different triploid genomes (at least four) are composed
203 of a core diploid *B. bruxellensis* genome and an additional haploid one. These additional genomes are
204 either genetically closely related to the diploid one (with a genetic divergence lower than 1%) or
205 genetically divergent (greater than 3%), indicating auto- as well as allopolyploidization events.
206 Interestingly, the three allopolyploidization events have occurred independently with a specific and
207 unique donor for each of the polyploid subpopulations (Table 1). The closest *Brettanomyces* sister
208 species, *B. anomalus*, shows >23% of genetic dissimilarity with the diploid *B. bruxellensis*' genome,
209 excluding the known *Brettanomyces* sister species as possible donors. Large-scale population genomic
210 surveys with a long-read sequencing strategy will help refine precisely the evolutionary history of each
211 subgenome, and any intertwined relationships.

212 In addition, *B. bruxellensis* genomes show different levels of Loss Of Heterozygosity (LOH). LOH events
213 are a source of genomic rearrangements and can contribute to the rapid onset of phenotypic diversity
214 (Dutta, Dutreux, & Schacherer, 2021; Sampaio et al., 2020; Smukowski Heil et al., 2017). LOH has
215 gained attention for its frequent association with fitness, adaptation, polyploid stabilization, or even
216 pathogenesis in yeasts but also in other organisms such as the oomycete *Phytophthora capsica*, hybrids
217 of the cultivated rice *Oryza* or the *Cobitis* fish species (Beekman & Ene, 2020; Forche et al., 2011; Janko
218 et al., 2021; Lamour et al., 2012; Morales & Dujon, 2012; Todd, Wikoff, Forche, & Selmecki, 2019; R.
219 R.-C. Wang, Li, & Chatterton, 1999). Compared to *S. cerevisiae* where LOH represents 50% of the

genome, *B. bruxellensis* presents a low level of LOH, which is variable across subpopulations (Gounot et al., 2020; Peter et al., 2018). In the diploid isolates (wine 3 and kombucha groups), the LOH regions represent 13% of the entire genome. In the triploid subpopulations, different scenarios were observed. For subpopulations that have undergone an allopolyploidization event, a higher proportion of the genome is under LOH in the beer and wine 1 groups (Figure 1B) with 26.6% and 22.3%, respectively (Eberlein et al., 2021). However, this fraction mainly concerns the acquired haploid genome. By contrast, the genomes of the teq/EtOH group are less impacted by LOH events (10.5%), which mainly involve the core diploid genome from *B. bruxellensis*. For the autotriploid population, LOH was also highlighted and conserved across six sequenced strains, resulting in the presence of only two haplotypes while three were expected (Eberlein et al., 2021). The conservation of a given haplotype over the others may indicate specific selection pressures on the alleles present in such LOH regions. Future studies will have to focus on the gene content of these LOH regions to identify possible genetic signatures of adaptation to anthropized environments.

Regarding the Copy Number Variation (CNV), genes affected by CNV harbour functions related to drug transporters, nitrogen assimilation or ethanol production (Borneman et al., 2014; Sam Crauwels et al., 2014; C. D. Curtin, Borneman, Chambers, & Pretorius, 2012; Gounot et al., 2020). Ploidy level and CNV could play an important role in the adaptation to the ecological niche, where multiple copies of a particular gene or gene family can be beneficial in the new environment. For example, in *S. cerevisiae* yeast, beer isolates have more increased copies of genes involved in maltose uptake and breakdown are amplified (Gallone et al., 2016; Gladieux et al., 2014). In the pathogenic *C. albicans*, CNV are associated to a significant fitness benefit to antifungal drugs (Todd et al., 2019). Within allotriploid isolates, the diploid core genome was more prone to duplication events than the acquired haploid genome (Gounot et al., 2020). Further studies will be needed to determine if CNVs are actually associated with increased adaptation to specific ecological niche, as suggested for nitrate assimilation in bioethanol production process (Galafassi, Capusoni, Moktaduzzaman, & Compagno, 2013). Indeed, genes involved in the nitrogen pathway have been independently lost in several diploid isolates within

different subpopulations, indicating differential selective pressure (Gounot et al., 2020). Genomic rearrangements affecting genes associated with traits of ecological interests have also been reported. For example, genes coding beta-glucosidase activity are lost in beer isolates but conserved in wine isolates (Sam Crauwels et al., 2017). This enzymatic activity could be useful to consume efficiently peculiar carbon sources found in wines aged in oak barrels, due to the liberation of specific wood polysaccharides. These variations were related to different aroma production, indicating technological interest besides ecological significance (Serra Colomer, Funch, Solodovnikova, Hobley, & Förster, 2020). Variations were also observed for maltose-related genes, although it was not possible to associate a phenotype to a specific genotype (S. Crauwels et al., 2015; Serra Colomer, Chailyan, et al., 2020). Concerning Horizontal Gene Transfer (HGT) in *B. bruxellensis*, three events from bacteria were recently highlighted, of which one encompassing an invertase-coding gene (from *Asaia* bacteria). This enzymatic activity is speculated to have conferred the ability to hydrolyse sucrose (Roach & Borneman, 2020). Horizontal gene transfers are drivers of adaptive evolution in eukaryotes and may have contributed to adaptation to high-sugar environments (Gladieux et al., 2014; Schönknecht, Weber, & Lercher, 2014). The prevalence of these HGTs in the different subpopulations has yet to be described, and it will be interesting to assess their possible involvement in niches' adaptation.

Besides the nuclear genome, genetic diversity has also been described for *B. bruxellensis'* mitochondrial genome. The mitogenome is large and variable in size (between 75Kb to 100kb), compact with introns and intergenic sequences (Eberlein et al., 2021; Procházka, Poláková, Piškur, & Sulo, 2010). By contrast to *S. cerevisiae*, the mitochondrial genome contains NADH dehydrogenase subunit genes, which allow the recycling of NAD in presence of oxygen during the fermentation process (Procházka et al., 2010). Across the different genetic subpopulations, the synteny of the mitogenome is well conserved with the exception of the teq/EtOH group for which a large inversion event and increased size (up to 100 kb) due to high intron content in the *COB* and *COX1* genes were evidenced (Eberlein et al., 2021). The intron content is known to be variable within *Saccharomyces* genus, especially for *COX1* and *COB* genes and was shown to be a marker of hybridization events (especially

COX1 introns) (De Chiara et al., 2020; Prasai, Robinson, Scott, Tatchell, & Harrison, 2017). In terms of nucleotide diversity, the genetic subgroups are highly homogeneous. The teq/EtOH group is more distant, with a genetic variation of 2% to 3% with the reference mitogenome. In *Ascomycota* species, the mitochondria inheritance is biparental, implying initial heteroplasmy in case of hybridization events. The outcome of the different mitotypes can be variable: (i) one or the other might be retained stochastically, (ii) one or the other might be retained due to selective pressures (*e.g.* to purge nucleo-cytoplasmic incompatibility or because of higher fitness of one mitotype), (iii) a chimeric mitotype can emerge from the two parental ones due to recombination events (Albertin et al., 2013; De Chiara et al., 2020; Lee et al., 2008). Here, the well-conserved mitotypes across the populations of diploid and polyploidy isolates argue in favour of the selection hypothesis without exchange. By contrast, the teq/EtOH group might have acquired a chimeric mitochondrial DNA, or the mitotype from the donor of the acquired haploid genome. Further studies will be needed to understand the possible impact of these mitochondrial genomes on the phenotypic diversity.

The exploration of the diversity of *B. bruxellensis* genomes has just started. Population genomic surveys unveil a complex genome architecture, with a strong involvement of polyploidy and hybridization events and mechanisms generating intra-specific variation (*e.g.* CNVs, LOH). LOH events appear to have radically shaped the genomic landscape of *B. bruxellensis* polyploids. This phenomenon is widely observed in polyploid yeasts and is an essential source of interindividual variation in predominantly asexual species (Peter et al., 2018; Steensels et al., 2021). Subsequent large-scale whole-genome sequencing will help to identify and understand the forces that shape the evolution of *B. bruxellensis* genomes, especially in the context of ecological divergence and industrial adaptation. However, genomic approaches alone will not be sufficient to formally demonstrate the relationship between the genomic and the phenotypic diversification of the species. Largescale phenotyping approaches are therefore necessary to clarify the link between genetic diversity and adaptation to anthropic fermentation environments.

3. Phenotypic diversity of *Brettanomyces bruxellensis*

Although molecular tools bring priceless information to understand the origin of the species, their formation and diversification in ever-changing environments, the physiological abilities and metabolic features of the organisms can also be used to seek a deeper comprehension of their niche space use.

3.1. Intraspecific diversity of central metabolism and nutrient requirements

The nutritional requirements of *B. bruxellensis* are less described than those of model species such as *S. cerevisiae*. Nevertheless, *B. bruxellensis* exhibits atypical characteristics related to the metabolism of carbon, oxygen, nitrogen and other nutrients, that may explain its ability to colonise harsh environments described as 'apocalyptic' (Smith & Divol, 2016). The main phenotypic characteristics of *B. bruxellensis* have already been reviewed (Blomqvist & Passoth, 2015; de Barros Pita et al., 2019; Schifferdecker et al., 2014; Serra Colomer et al., 2019; Smith & Divol, 2016; Steensels et al., 2015). However, phenotypic traits have been little studied in the light of genetic diversification. *Brettanomyces bruxellensis* is characterized by growth variability which is partly related to ecological origin and/or to the genetic group (da Silva et al., 2019; Louw, du Toit, Alexandre, & Divol, 2016). As an example, bioethanol isolates grow faster compared to wine isolates on seven carbon sources (sucrose, cellobiose, maltose, lactose, glucose, fructose and galactose) (da Silva et al., 2019) and the variability of growth was highlighted for other genetic groups as well (Figure 2).

Generally, central regulatory mechanisms like Glucose Catabolite Repression GCR (da Silva et al., 2019; Leite et al., 2012) and Nitrogen Catabolite Repression NCR (Cajueiro, Parente, Leite, de Morais Junior, & de Barros Pita, 2017; de Barros Pita, Leite, de Souza Liberal, Simões, & de Morais, 2011; de Barros Pita & Tiukova, 2013; Galafassi, Capusoni, et al., 2013) seem to be less strict in *B. bruxellensis* compared to *S. cerevisiae* (*i.e.* non-glucose and non-ammonium sources can be metabolized even in presence of high glucose/ammonium concentration). Diversity was observed in GCR, with *B. bruxellensis*' bioethanol isolates being less susceptible to GCR than wine isolates (Blomqvist, Eberhard, Schnürer, & Passoth, 2010; da Silva et al., 2019). There is no data available to our knowledge for isolates from other

substrates, yet this feature needs more research as it may have significant ecological and technological implications: the simultaneous use of different carbon and nitrogen sources could increase the efficiency of absorption in low-nutrient media and be associated with higher fitness in specific environments.

The most striking characteristic of *B. bruxellensis* is its ability to use a wide range of carbon sources as shown by large-scale phenotypic analyses (Cibrario, Miot-Sertier, et al., 2020; Conterno et al., 2006; Crauwels et al., 2015; da Silva et al., 2019; Galafassi et al., 2011; Smith & Divol, 2018). However, the carbon utilisation varies among isolates: a survey on the assimilation of 190 different carbon sources on seven strains clearly showed a variability between isolates, that could be related to different processes (Crauwels et al., 2015). By contrast to wine isolates, most beer strains were not able to consume galactose, some β-disaccharides (cellobiose and gentiobiose) or some β-substituted monosaccharides (arbutin and β-methyl-D-glucoside) (Crauwels et al., 2015; Serra Colomer, Funch, Solodovnikova, Hobley, & Förster, 2020). This observation could be explained by a variation of beta-glucosidase and alpha-glucosidase activity between the genetic groups (Serra Colomer, Funch, et al., 2020). Different environmental selective pressures could be responsible for this feature (Serra Colomer, Funch, et al., 2020). However, further work involving more than seven isolates is needed, including strains representative of all described subpopulations to properly assess the relationship between carbon assimilation and process.

Like *S. cerevisiae*, *B. bruxellensis* is able to ferment sugars into ethanol even in the presence of oxygen, a phenomenon called the Crabtree effect, which supports the 'Make-Accumulate-Consume' (MAC) strategy (Rhind et al., 2011; Smith & Divol, 2016). The evolutionary and metabolic significances of the Crabtree effect remain highly discussed, although a simplistic viewpoint suggests that metabolic shifts (from respiration to fermentation and back again) are energetically costly and time-consuming (Pfeiffer & Morley, 2014). Furthermore, *B. bruxellensis* is an acetogenic yeast, which produces large amounts of acetic acid in addition to ethanol under aerobic conditions. This production varies a lot

between strains (up to 10-fold) (Freer, 2002; Freer, Dien, & Matsuda, 2003) and could have ecological implication since acetic acid release in the environments is described as a useful feature to outcompete other microorganisms (Rozpędowska et al., 2011) or to lure/deter flies involved in yeast dissemination (Dzialo, Park, Steensels, Lievens, & Verstrepen, 2017). Regarding oxygen impact, conflicting results have been highlighted, with some studies connecting oxygen input to increased or decreased growth (Aguilar Uscanga, Délia, & Strehaiano, 2003; da Silva et al., 2019; Smith & Divol, 2018). The conservation of the NADH dehydrogenase subunit genes in the mitogenome suggests specific oxygen's need during *B. bruxellensis*' fermentation (Procházka et al., 2010). The oxygen requirement is barely deciphered in *B. bruxellensis* and should be the subject of deeper studies in the near future, particularly to determine its possible impact on niche colonization.

In addition to peculiarities regarding carbon and oxygen metabolism, *B. bruxellensis* has atypical nitrogen and vitamins requirements, which may explain its ability to colonize certain niches. In general, *B. bruxellensis* seems to have higher nitrogen needs than *S. cerevisiae* (Leite et al., 2012), and *B. bruxellensis* is able to grow on nitrate as sole nitrogen sources (Borneman et al., 2014; Conterno et al., 2006). This could be advantageous in wine or in bioethanol environment where nitrate is present and not consumed by the first *S. cerevisiae* fermenter (Cajueiro et al., 2017; de Barros Pita et al., 2011). Pena-Moreno et al. even reported that the presence of nitrate boosted ethanol production and growth for some bioethanol strains (Morales-de la Peña, Welti-Chanes, & Martín-Belloso, 2019). Nitrate consumption capacity varies depending on the ecological origin of isolation (Borneman et al., 2014; Crauwels et al., 2015; da Silva et al., 2019). The nitrate assimilation cluster (NIT cluster) was found to be probably involved in the observed phenotypic variation: it encompasses genes encoding nitrate transporter, nitrate reductase, nitrite reductase and two related transcription factors. It has evolved differently between strains and ecological groups. While NIT cluster was duplicated in a wine diploid isolate capable of assimilating nitrate (belonging to the wine 3 subgroup) and it was partially deleted or subjected to gene conversion in beer isolates unable to assimilate nitrate (belonging to the beer subpopulation) (Borneman et al., 2014; Gounot et al., 2020). In a wine triploid isolate able to assimilate

nitrate (from the wine 1 group), three haplotypes are present. To sum up, selective pressures in different ecological niches may have allowed different evolutionary trajectories, although researches involving more representative strains are needed to have the full picture. Regarding vitamins requirements, *B. bruxellensis* was initially defined as an auxo-autotroph (Peynaud and Domercq, 1956) but recent results indicate that biotin is the only vitamin which strongly affects the biotic capacity of *B. bruxellensis* in the long term (von Cosmos & Edwards, 2016). Thiamine requirements appear to depend on the strain and on the presence of ethanol in the growth medium (von Cosmos & Edwards, 2016).

3.2. Traits of technological interest

Another notable characteristic of *B. bruxellensis* species is its ability to convert hydroxycinnamic acids (HCAs) into volatile phenols. HCA metabolism may have an important impact from an ecological viewpoint: HCA have antimicrobial properties, and the ability to convert HAC into less toxic compounds could promote yeast growth (Richard, Viljanen, & Penttilä, 2015). Besides, HAC are important dietary antioxidants for flies. However, flies are not able to detect directly HCA and use ethyl phenols as an indirect indicator of their presence (Dweck, Ebrahim, Farhan, Hansson, & Stensmyr, 2015). Flies interaction with yeasts have consequences on their dissemination and the possibility to colonize new environments, and volatile phenols could be a key factor mediating yeast-insect interactions (Stefanini, 2018). Besides their ecological importance, volatile phenols are considered as off-flavours in oenology (for review (Suárez et al., 2007; Wedral, Shewfelt, & Frank, 2010)). The term 'volatile phenols' generally includes 4-ethylphenol (4-EP), 4-ethylguaiacol (4-EG) and 4-ethylcatechol (4-EC) and their vinyl forms, 4-vinylphenol (4-VP), 4-vinylguaiacol (4-VG), 4-vinylcatechol (4-VC). These are produced through the conversion of hydroxycinnamic acids (HCAs): p-coumaric acid, ferulic acid and caffeic acid respectively. The production of volatile phenols by *B. bruxellensis* strains is variable and was extensively described in oenological conditions (M. Agnolucci et al., 2009; Monica Agnolucci et al., 2010; Conterno et al., 2006; Di Toro et al., 2015; Hixson et al., 2012; Madsen et al., 2017; D. Romano et al., 2017; Vigentini

et al., 2008; Zepeda-Mendoza et al., 2018). Most studies involved end-point analyses, which makes it difficult to distinguish between the intrinsic capacities of the strains and their modulation by external parameters. Furthermore, the respective importance of the different factors governing such variability is poorly described: a few authors showed that volatile phenol production was both strain-dependent and wine matrix-dependent (Chandra, Madeira, Coutinho, Albergaria, & Malfeito-Ferreira, 2016; Cibrario, Miot-Sertier, et al., 2020; Dias, Pereira-da-Silva, Tavares, Malfeito-Ferreira, & Loureiro, 2003; Zhu, Zhang, & Lu, 2012). The niche of isolation could also play a role and brewing isolates have a more efficient metabolism toward ferulic acid (leading to 4-EG) than p-coumaric acid compared to wine isolates (Lentz & Harris, 2015). Some wine and soft drink isolates possess a duplication of the Vinyl Phenol Reductase gene, which is absent in beer isolates (Crauwels et al., 2017). However, while all these studies confirmed the existence of intraspecific variation, their impact on the production of volatile phenols and their possible ecological significance remains to be properly assessed.

Brettanomyces bruxellensis show important intra-specific variability regarding the production of other aroma-active molecules such as esters, fatty acids, terpenes, phenolic compounds, N-heterocycle, (see *Brettanomyces* aroma wheel, (Lucy Joseph, Albino, & Bisson, 2017; Serra Colomer, Funch, et al., 2020; Tyrawa, Preiss, Armstrong, & van der Merwe, 2019)). Although some environmental factors may impact ester production (*e.g.* ethanol and p-coumaric acid concentrations (Conterno, Aprea, Franceschi, Viola, & Vrhovsek, 2013; Lucy Joseph, Kumar, Su, & Bisson, 2007)), esterase activity is also strain-dependent (Holt et al., 2018; Spaepen & Verachtert, 1982; Steensels et al., 2015; Verstrepen et al., 2003). The intraspecific variation in the production of esters is mirrored at the aromatic level and impacts fruitiness, which is particularly important for Lambic beer style (Van Oevelen, Spaepen, Timmermans, & Verachtert, 1977). The activity of some alpha-glucosidase and beta-glucosidase enzymes may be involved in the release of aromatic compounds like terpenes (Crauwels et al., 2014; Daenen et al., 2007; Serra Colomer, Funch, et al., 2020; Vervoort et al., 2016). N-heterocycles can be produced by *B. bruxellensis* in beverages (mostly in wine or beer) (for review see (Snowdon, Bowyer, Grbin, & Bowyer, 2006)). These molecules are responsible for the 'mousy' off-flavor, which causes

rejection by the consumers. The metabolic pathway is not fully understood, but the amino-acid content and the oxygen availability in fermented beverages are key parameters for their production (Grbin & Henschke, 2000; Grbin, Herderich, Markides, Lee, & Henschke, 2007). The state of art is somewhat inconsistent, which may be explained by the difficulty to properly measure such compounds and their seemingly random occurrence in fermented beverages. From an ecological aspect, all these aromatic compounds could play key roles in natural environments, including signalling and communication with other organisms, and/or attractants for animals, particularly insects (Dzialo et al., 2017).

Brettanomyces bruxellensis shows an incredible ability to persist over long time in some industrial environments (Cibrario, Miot-Sertier, et al., 2020). Such capacity of persistence could be linked to the various physiological states of the cells, beside the classical free-living (planktonic) state. Indeed, *B. bruxellensis* is able to form biofilm, pseudo-mycelium, chlamydospore-like structure and VBNC (Viable But Non Culturable) cells. While many aspects remains unknown or contradictory, a physiological change between these forms could be induced by stress conditions related to the presence of some chemical compounds and/or to nutrient limitation (Uscanga, Delia, & Strehaiano, 2000). Intraspecific diversity was observed in the ability to enter or exit the VBNC state (Capozzi et al., 2016; Longin et al., 2016) as well as to form pseudo-hyphae (Martyniak, Bolton, Kuksin, Shahin, & Chan, 2017) or biofilm (Lucy Joseph et al., 2007). Recently, two concomitant studies (Dimopoulou, Renault, et al., 2019; Lebleux et al., 2020) have suggested that the ability to produce biofilms depends on the genetic groups. Although only a limited number of strains were tested, the beer group showed robustness regarding biofilm formation in glucose-rich or glucose-limited media. For other genetic groups, biofilm's production was more dependent on environmental characteristics, suggesting different regulatory mechanisms (Dimopoulou, Renault, et al., 2019). These differences were also partly mirrored by the biochemical and physicochemical properties of the surface of the strains (Dimopoulou, Renault, et al., 2019). Finally, whether *B. bruxellensis* is actually able to sporulate and to undergo a sexual cycle remains a mystery. Early description of ascospore-like forms by van der Walt

(Van der Walt, 1964) prompted the definition of a teleomorphic stage (ie the sexual reproductive stage of a fungal species in mycology). The teleomorph was named *Dekkera bruxellensis* (*B. bruxellensis* being the anamorph counterpart, that reproduces asexually). Since then, ascospores formation was scarcely described (A.T. de Souza Liberal et al., 2007), and most authors consider *B. bruxellensis* as lacking an effective sexual cycle (Hellborg & Piškur, 2009). In particular, the existence of a meiosis should be associated with triploid instability, which is not evidenced at the genomic level. The impact of polyploidy and hybridization on the ability to switch from one form to another (planktonic, sessile, biofilm, pseudo-mycelium) remains to be elucidated. From an ecological viewpoint, the possibility to adopt various lifestyles and switch from one to another cellular morphologies may facilitate niches adaptation and drastic environment changes, as it the case for dimorphic fungi whose yeast-hyphae switch is recognized as an essential adaptation for host colonization and pathogenicity (Boyce & Andrianopoulos, 2015).

As one of the main worldwide wine spoilers, winemakers try to prevent and/or control *Brettanomyces* contamination through several methods such as the use of filtration, application of ozone, high pressure, ultrasound, ultraviolet irradiation, pulsed electric fields, chitosan or the addition of sulphite (Supplemental Table 2). So far, most studies evaluating the efficiency of these different applications have included a very small subset of strains (Supplemental Table 2) with questionable representativeness, especially for wine isolates that are found into different genetic groups. As a result, conflicting conclusions have been recorded: for example, chitosan treatments have sometimes been described as very effective (Bağder Elmacı et al., 2015) or moderately effective against *B. bruxellensis* (Petrova, Cartwright, & Edwards, 2016). This incongruity has been partly resolved, a strain-dependent sensitivity to chitosan was demonstrated (Paulin et al., 2020). In future work, it could be interesting to define a standardized method to evaluate the efficiency of anti-microbial treatments against *B. bruxellensis* including a panel of isolates representative of the genetic diversity of the species.

In winemaking, the most common treatment is the addition of sulphur dioxide (Barata et al., 2008). High intraspecific diversity in sulphite tolerance was repeatedly demonstrated in several studies (Avramova, Cibrario, et al., 2018; Dimopoulou, Hatzikamari, Masneuf-Pomarede, & Albertin, 2019; Galafassi, Toscano, Vigentini, Piškur, & Compagno, 2013). On a cohort of 100 isolates from winemaking, three phenotypic groups were defined: sensitive (slowed growth), tolerant (delayed growth) and resistant (no impact on growth) (Vigentini, Lucy Joseph, Picozzi, Foschino, & Bisson, 2013). A recent study was able to connect sulphur sensitivity to the genetic groups, with two triploid groups (wine 1 and wine 2) containing most tolerant/resistant isolates (Avramova, Vallet-Courbin, Maupeu, Masneuf-Pomarède, & Albertin, 2018). Competition experiments between sensitive/tolerant isolates under increasing concentration of sulphite showed specific adaptation of isolates from wine 1 allotriploid group to high SO_2 environments (Avramova et al., 2019). Different *SSU1* haplotypes (*SSU1* encoding sulphite efflux pump) related to variable SO_2 tolerance were characterized (Valdetara et al., 2020; C Varela, Bartel, Roach, Borneman, & Curtin, 2019; Cristian Varela, Bartel, Onetto, & Borneman, 2020). It revealed that gene dosage effect (the number of *SSU1* haplotype) as well as the *SSU1* regulation could be at least partially involved in SO_2 tolerance (Varela et al., 2019). In a recent experiment, Bartel et al conducted a laboratory experimental evolution in presence of sulfur dioxide concentrations, and evidenced adaptive evolution in different genetic backgrounds targeting partly SSU1 (Bartel et al., 2021). A genotyping study showed that the two genetic groups (wine 1 and wine 2) containing the most tolerant/resistant isolates of *B. bruxellensis* were scarcely isolated before 1990 and that their proportion had increased steadily since then, possibly with the increase of sulphur use in oenology (Cibrario et al., 2019). Sulphur tolerance/resistance in *B. bruxellensis* could thus be the result of anthropization and adaptation to winemaking environments as for wine strains of *S. cerevisiae* (Gallone et al., 2016; García-Ríos & Guillamón, 2019; Kaewkod, Bovonsombut, & Tragoolpua, 2019). The fact that sulphite tolerant/resistant phenotypes are present in two different genetic cluster (one autotriploids, one allotriploid) may indicate independent acquisition and possibly

different adaptive mechanisms. How much and how the change in ploidy contributes to the resistant/tolerant phenotype has to be resolved in future studies.

In conclusion, although a high number of surveys investigated various *B. bruxellensis* traits with fundamental and applied interest, many of these published studies used a small subset of strains usually not representative of the genetic diversity of the species (Figure 3, Supplemental Table 3). *Brettanomyces bruxellensis* literature is scattered with incongruences that could be directly related to intraspecific diversity and the lack of representability of the isolates tested. It highlights the necessity, for future research, to properly take into consideration strains from different ploidy levels, with varied hybridization backgrounds (auto- and allotriploids), isolated from various substrates and geographical origins and distributed within the different genetic populations described.

4. Discussion and perspectives

4.1. Brettanomyces bruxellensis, the fermentation finisher

Brettanomyces bruxellensis has attracted increasing attention recently due to its involvement in industrial processes and its unusual genomic composition. *Saccharomyces cerevisiae* is one of the most intensively studied eukaryotic models, at the fundamental level in molecular, cellular and ecology biology, but also from an applied point of view as it is widely used as a fermentation starter for several human processes (Boone, 2014; Chambers & Pretorius, 2010; Goddard & Greig, 2015; Peter & Schacherer, 2016). Interestingly, while *B. bruxellensis* species is associated with similar anthropic processes (*e.g.* oenology, brewery), it is particularly recognized for its competence to colonize already fermented environments (such as wine or beer rather than grape must or wort) (Schifferdecker et al., 2014). *Brettanomyces bruxellensis* is thus a perfect model of fermentation finisher, a complementary counterpoint to *S. cerevisiae* model of fermentation starter, sharing the same industrial niches, but not with the same temporality. Interestingly, no isolates of *B. bruxellensis* were identified outside human related processes so far. Several reasons may explain the non-detection of wild isolates yet. First, *B. bruxellensis* is a slow-growing yeast species, so it may be easily outcompeted by other yeast species

whenever culturable methods are used (Agnolucci et al., 2017). Another possibility is that *B. bruxellensis* could be part of biofilm communities in natural environments – albeit at low-abundance and/or in Viable But Not Cultivable (VBNC) state preventing its isolation. Indeed, 40-80% of cells on Earth live and persist in multispecies communities (bacteria, archaea, eukaryotes, etc.) forming biofilms, *e.g.* aggregated structures frequently enclosed into matrixes of extracellular polymeric substances (Flemming et al., 2016; Flemming & Wuertz, 2019). Growth in polymicrobial biofilms is relatively protected from environmental variations, and could improve survival in hostile environments (Flemming & Wuertz, 2019). The identification of low-abundance or VBNC species within complex communities remains a challenge, prompting the use of metagenomics, culturomics or reverse genomics approaches (Martellacci et al., 2019; Ryu et al., 2021). *Brettanomyces bruxellensis* has abilities to form VBNC or to be involved in microbial consortia like kombucha's SCOBY (Symbiotic Community of Bacteria and Yeast) (Savary et al., 2021). These characteristics make it plausible the hypothesis of natural biofilms as one of their wild environments, although no definite evidences are described so far. Besides biofilms, natural fermentations are another putative example of ecosystems that could foster the growth of wild *B. bruxellensis*. Many insects, mammals or birds store foods (fruits, grains, meat, etc.) for times of less-plentiful sustenance. Food storage is prone to microbial growth or spoilage, and natural fermentations occurred long before humans, and long before human-directed fermentations (Carrigan et al., 2015; Post & Urban, 1993; Ruxton, Wilkinson, Schaefer, & Sherratt, 2014; Wiens et al., 2008). These naturally-occurring fermentations are poorly described, but they probably display similar characteristics to *B. bruxellensis*' anthropic environments: presence of ethanol and organic acids, wide variety of ever-evolving sugar, nitrogen and other nutrients contents, oxygen availability, succession of complex microbial communities, etc. In such natural fermentations, *B. bruxellensis* could play second fiddle and colonize the environments after more efficiently-growing and fermenting microorganisms. In natural environments also, the role of wild-fermentation finisher would be congruent with *B. bruxellensis'* scavenging abilities (Smith & Divol, 2016). However, this hypothesis remains purely speculative: the literature is scarce regarding the microbial communities

associated with food caching or hoarding , and so are the available isolates from these niches (Herrera, Kramer, & Reichman, 1997; Post & Urban, 1993). Obtaining and studying natural isolates would be an ideal option, but even in the absence of data on their wild counterparts, future works both at the genomic and phenotypic levels will help outlining the characteristics of *B. bruxellensis*' natural reservoirs. In particular, study of the central metabolism and the molecular mechanisms (HGT, LOH, CNV, etc) driving their evolution will help to determine which metabolic functions are conserved, lost or gained and their possible involvement into adaptations to anthropic environments. For example, determining whether maltose metabolization or beta-glucosidase activity were acquired before *B. bruxellensis*' anthropic associations may give clues concerning its possible natural niches (*i.e.* crops- or wood-related). Future works should explore more thoroughly all these aspects (central metabolism, VBNC and biofilm abilities, etc.) to gain more insights into a fermentation-related microorganism and to improve our possibilities of controlling the species in various processes.

4.2. Exploring the relationships between polyploidy/hybridization and adaptation to anthropized environments

Genomic analyses unveiled a genetic diversity of *B. bruxellensis* related to specific substrates. Diploid isolates coexist with triploid ones of hybrid origins, and several subpopulations of diploid and triploid individuals are described. Some of these groups are related to specific substrate origins (wine, beer, kombucha, tequila) and display adaptive traits related to their ecological niche (*e.g.* sulphite tolerance/resistance in winemaking, ability to metabolize nitrate in bioethanol production, maltose utilization in beer process). In industrial processes, where stress is omnipresent, polyploidy and aneuploidy are recurrent events in domesticated populations of *S. cerevisiae* (Peter et al., 2018; Querol & Bond, 2009; Steensels et al., 2021). In *B. bruxellensis*, the level of aneuploidy (roughly 5%) is surprisingly low considering: 1- the proportion of aneuploidy in other yeast species (19% in *S. cerevisiae*, 15% and 33% in the pathogenic yeasts *Cryptococcus neoformans* and *Candida albicans*, respectively (Gounot et al., 2020; Peter et al., 2018; Rhodes et al., 2017; Scopel, Hose, Bensasson, & Gasch, 2021; Selmecki, Forche, & Berman, 2006)); and 2- the proportion of triploids in *B. bruxellensis*

(>50%) which are known to evolve quickly toward aneuploidy and diploidy in *Saccharomyces* species (Avramova, Cibrario, et al., 2018; Gerstein, McBride, & Otto, 2008; Todd, Forche, & Selmecki, 2017). The phylogeny and evolutionary history of *B. bruxellensis* species are particularly complex to reconstruct because of the intertwined events of polyploidization and hybridization (Linder, Moret, & Nakhleh, 2003). New approaches will be needed to unravel its complex genetic architecture and to elucidate the precise relationship between genomic evolution and the actual adaptation to anthropized environments. The evolution of many species (plant, animal, microorganism) is strongly shaped by human activities. When anthropogenic-driven transformation is purposefully associated with improved attributes, the species are labelled as domesticated, as in *S. cerevisiae* for which several independent domestication events were evidenced (Gallone et al., 2018; Giannakou et al., 2020; Steensels, Gallone, Voordeckers, & Verstrepen, 2019). In the case of *B. bruxellensis*, the situation seems to be more complicated: the fact that some beer isolates are used by brewers for their improved ability to metabolize specific sugars may correspond to domestication. Conversely, adaptation to winemaking environments through acquisition of sulphite resistance is probably an unintentional consequence of anthropogenic influence. Thus, for wine groups, the term 'domestication' does not apply to what seems to be an adaptive evolution at the expense of humans. A few works already provided evidence of various beneficial or detrimental traits associated with the different anthropized environments colonized by *B. bruxellensis*. The underlying molecular mechanisms remain to be explored more thoroughly, in particular the role played by LOH, HGT, CNV and other mechanisms in the evolution and adaptation of the species. How polyploidization and hybridization actually impacted the evolutionary routes of *B. bruxellensis* is still an open question. Future directions should take advantage of this model yeast to examine closely the influence of anthropogenic activities on the species and their genomic, phenotypic and adaptive consequences, whether positive or negative for humans.

The description of both auto- and allotriploid groups raises the question of their origin. The recent genomic approaches, and particularly phasing methods, allowed the precise description of the

genomic content of these triploids that harbour a core diploid genome added with an additional haploid one of various intra- and inter-specific origins (Abou Saada et al., 2021). Triploid formation in *B. bruxellensis* happened at least four times, suggesting the successive occurrence of a not-so-rare event of polyploid formation, followed by a not-so-rare establishment mechanism (selection or random genetic drift). Regarding the possible mechanisms of polyploid formation, accidental miss-repartition of chromosomes during mitosis can lead to unbalanced number of chromosomes in daughter cells (Todd et al., 2017; Wertheimer, Stone, & Berman, 2016). However, this pathway would account only for the formation of autotriploids (not allotriploids), furthermore without heterozygosity increase, which is described in *B. bruxellensis* wine 2 autotriploid group. In addition, the presence of a complete haploid set of chromosomes tends to invalidate the hypothesis of mitosis mishaps, which would more frequently lead to aneuploids rather than polyploids. Autopolyploidy can also occur via endoreduplication – replication of the whole nuclear genome in absence of mitosis – a phenomenon frequently described in plants (Harari, Ram, Rappoport, Hadany, & Kupiec, 2018; Sugimoto-Shirasu & Roberts, 2003). However, endoreduplication accounts only for even ploidy levels, not for odd (triploid) ones, and is not associated with increased in heterozygosity levels. The production of unreduced gametes, followed by intra- or interspecific hybridization, is another route of polyploid formation frequently described in plants and animals (Otto & Whitton, 2000). The absence of evidence of sexual cycle in *B. bruxellensis* makes this mechanism less likely. Protoplast (spheroplast) fusion could be an interesting hypothesis: protoplast formation could occur after cell wall digestion in insects' guts and subsequent protoplasts fusion could allow intra- and inter-specific hybridization of non-sexual species (Steensels et al., 2014). To date, protoplast fusion is the most likely hypothesis of auto- and allotriploid formation, yet the literature lacks of formal evidence and future genomic and ecological analyses may shed lights on the possible routes of polyploid formation in *B. bruxellensis*. In addition to polyploidization/hybridization mechanism, the prominence of *B. bruxellensis*' triploids indicates that triploid formation was followed by the successful establishment of these lineages, through either neutral genetic drift or natural/artificial selection. It seems highly unlikely that random genetic drift

could account alone for the presence of triploid lineages in *B. bruxellensis*: firstly, polyploidy is related to energetic and resource costs that, far from being neutral, should lead to its counter-selection (Comai, 2005; Neiman, Kay, & Krist, 2013). Secondly, at least four auto- and allotriploid events have led to the independent establishment of triploid lineages, a high number for a purportedly stochastic phenomenon. Thirdly, some of these triploid lineages show (or are suspected to show) higher fitness in their environments of predilection, such as sulphite resistance for the wine 1 and wine 2 groups, the possible metabolization of maltose and other complex sugars for the beer group, or increased growth and the ability to metabolize nitrate for teq/EtOH group (Avramova, Vallet-Courbin, et al., 2018; S. Crauwels et al., 2015; Galafassi, Capusoni, et al., 2013; Serra Colomer, Chailyan, et al., 2020). All these elements suggest that polyploidization in *B. bruxellensis* is not neutral from an evolutionary viewpoint, although more genomic and phenotypic studies are needed to deepen our understanding of the evolutionary fates of these polyploids compared to their diploid counterparts.

Finally, the large genetic differences (up to 3%) recorded between subpopulations raise the question of the actual number of species within this clade (Eberlein et al., 2021). No sexual cycle was formally recorded to date and no gene flow between *B. bruxellensis* subpopulations was ever described, suggesting independent evolution within each clade, and speciation in progress. Future large-scale genomic and phenotypic analyses will help determine whether we should still consider *B. bruxellensis* as a single but complex species with diverse subpopulations, or whether we should redefine *B. bruxellensis* as a complex of single species.

4.3 Brettanomyces bruxellensis, a yeast model to study the relationship between polyploidy, hybridization and adaptation to human-related environments

Polyploidy and hybridization, two frequently associated events, have long been described as key evolutionary mechanisms underlying radiation and adaptation in many clades of plants, animals or fungi (Gregory & Mable, 2005; Otto & Whitton, 2000; Todd et al., 2017; Van De Peer, Mizrachi, & Marchal, 2017). In flowering plants, where the impact of polyploidy is the most studied, polyploidy and hybridization are frequently associated with phenotypic diversification, trait innovation, increased

fitness, invasiveness abilities, adaptation to harsh environments and domestication (Ainouche & Wendel, 2014; Soltis & Soltis, 2009). Historically less studied in microorganisms, polyploidy and hybridization are now well established as driving evolutionary forces in yeast, Chromalveolata, but also in prokaryotes, including Bacteria and Archaea (Albertin & Marullo, 2012; Marcet-Houben & Gabaldón, 2015; Soppa, 2022). Yeast usually enables the development of systems biology approaches, and *B. bruxellensis* triploids may allow to investigate the fates of genes duplicated by whole genome duplication and their impact on phenotypic traits. In addition, the close association between various human processes and different ploidy/hybridization status makes of *B. bruxellensis* a valuable model to decipher the evolutionary mechanisms involved in the adaptation to anthropized niches. The study of *B. bruxellensis'* diploid and triploid populations may provide valuable insights into the ecological and evolutionary significance of natural polyploidy.

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Data Accessibility

Phenotypic data files (figure 2): Avramova et al. (2018) DOI: 10.3389/fmicb.2018.01260

Genomic data (figure 1B): Eberlein et al. (2021) DOI: 10.1101/gr.275380.121

Author contribution

JH, CE, JS and WA conceived the original outline of the review, with substantial inputs from all authors. All authors screened the literature and selected the subset of publications to be included. JH, CE, JS and WA drafted a first version. MDL helped more specifically drafting the central metabolism section, IMP and CMS the aroma/biofilm/technological part, and PB helped with the aroma section. JH, CE and WA contributed to the figures. All authors extensively revised the manuscript.

Tables and Figures

Figure 1. Genetic position and population structure of *Brettanomyces bruxellensis*.

1A. Neighbor-joining tree of the *Brettanomyces* genus.

The distance tree comprises the six *Brettanomyces* species, *Allodekkera sacchari* (the closest sister species to *Brettanomyces* clade), *Ogataea polymorpha, Saccharomyces cerevisiae* and *Saccharomyces bayanus* as outgroups. 26S ribosomal RNA gene sequences were used. A global alignment with free end gaps was prepared using Geneious (Prime 2020.2) and the default setting for multiple alignments. The Neighbor-joining tree was built with the Tamura-Nei Model of genetic distances. The final tree represents a consensus of 1,000 resampled trees obtained with an extended majority rules method. The consensus supports of the nodes are given in %. Sequences data was downloaded from NCBI.

1B. Genetic diversity within *Brettanomyces bruxellensis* species.

Brettanomyces bruxellensis subpopulations are represented by different colors and named from previous reports (Avramova, Cibrario, et al., 2018; Gounot et al., 2020). The tree was built from whole genome Illumina short-read sequencing of 71 *B. bruxellensis* isolates aligned to the reference genome *B. bruxellensis* (Fournier et al., 2017) and 24,313 genetic variants evenly distributed across the genome (Eberlein et al., 2021). The ploidy level of each population was schematized, two chromosomes represent diploid groups, three chromosomes represent triploids ones. Light grey, dark gray and white chromosomes represent the independent haploid acquired genomes from unknown sister species.

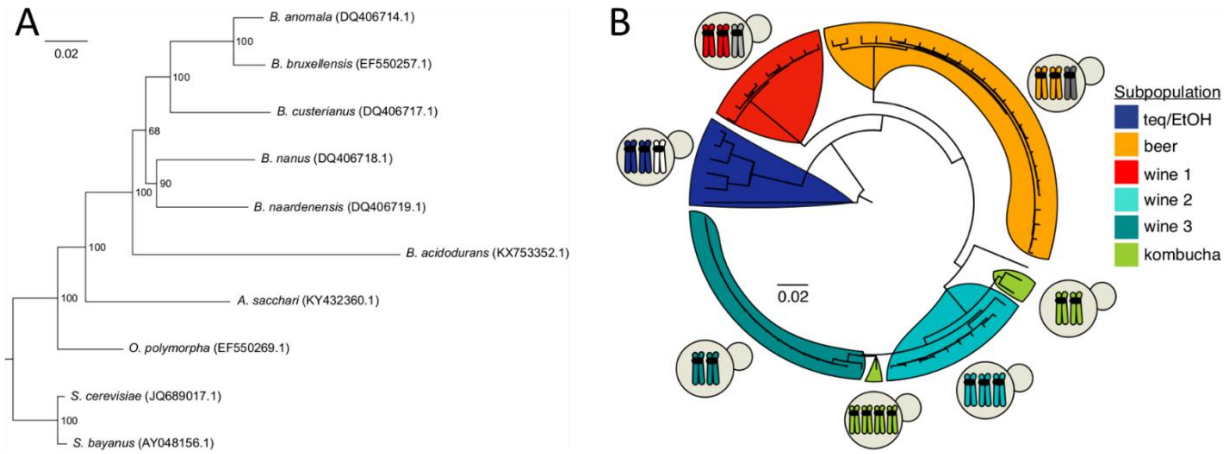


Figure 2. Growth parameters for different subpopulations of *B. bruxellensis*.

Genetic groups as described by previous reports (Avramova, Cibrario, et al., 2018; Gounot et al., 2020). Growth data was taken from Avramova et *al.* (Avramova, Vallet-Courbin, et al., 2018). The growth parameters correspond to the lag phase (hour), maximum OD (600 nm) without or with sulphite (0.6 $mg.L^{-1}$ of molecular SO_2). For each genetic group, mean values are represented by a circle, and error bars correspond to standard error. Top letters represent significance groups as defined by Kruskal-Wallis test when significant (p-value < 0.05). P-values were 0.0054, 0.11 and $5.4\ 10^{-10}$ for lag phase, maximum OD without or with sulphite respectively.

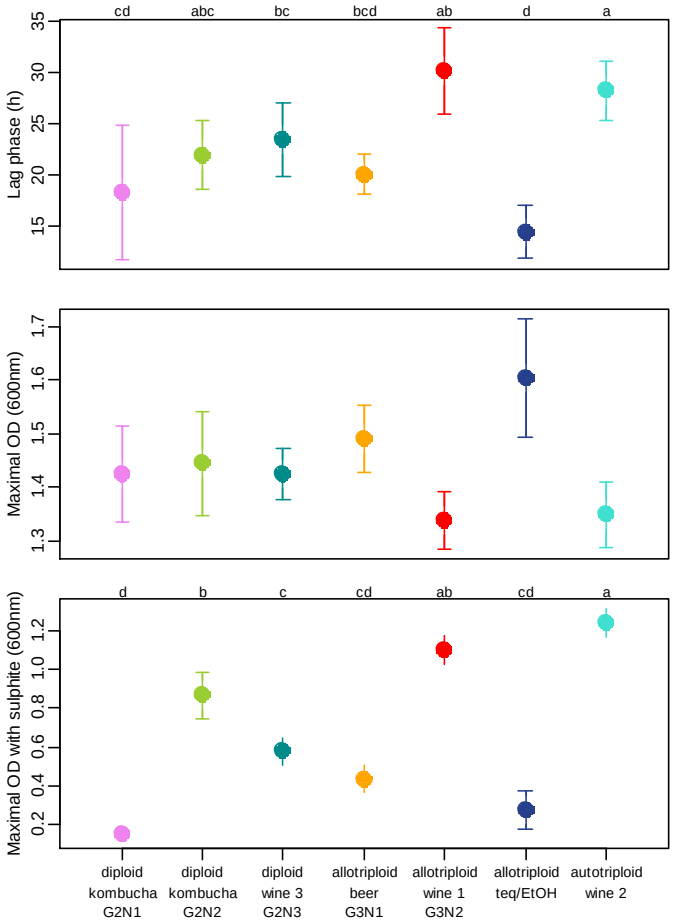
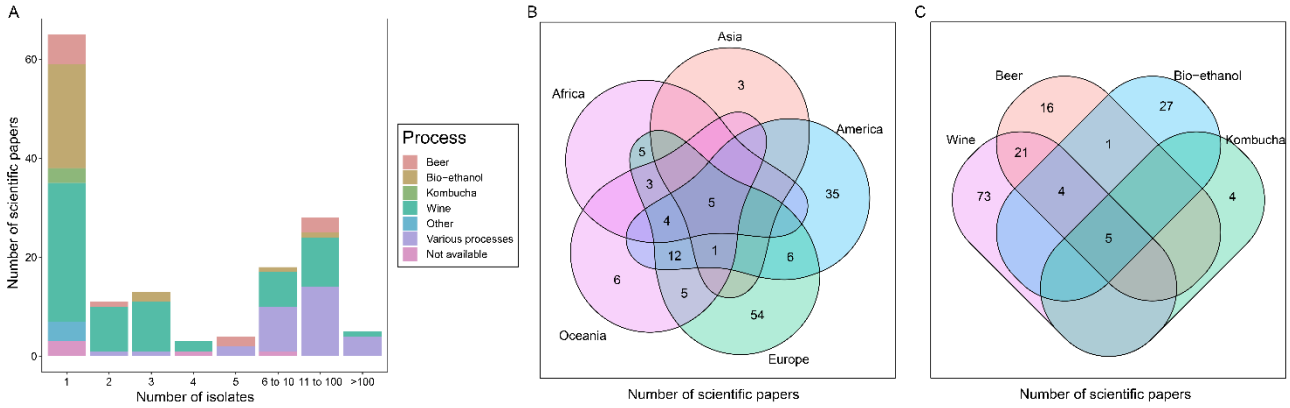


Figure 3. Representativity and diversity of *Brettanomyces bruxellensis* collections studied in the literature.

3A: Histogram of the number of isolates of *B. bruxellensis* used in the studies referenced in supplemental Table 3, with their process/substrate of isolation.

3B: Venn diagram of the number of studies including isolates of *B. bruxellensis* from various geographical origins.

3C: Venn diagram of the number of studies including isolates of *B. bruxellensis* from various process origins.



1629 Table 1. Whole-genome sequencing of *B. bruxellensis* isolates.

1630 [1]ND stands for 'not determined' by the corresponding publication.

Publication	**Isolates ID (or number of isolates)**	**Niche; origin of isolation**	**Country/region of isolation**	**Ploidy[1]**
Woolfit et al. (2007)	CBS 2499	Wine	France	ND
Piškur et al. (2012)	CBS 2499	Wine	France	Diploid
Curtin et al. (2012)	AWRI 1499	Wine	Australia	Triploid
Crauwels et al. (2014)	ST05.12/22 = VIB X9085	Beer	Belgium	Diploid
Valdes et al. (2014)	LAMAP 2480	Wine	Chile	ND
Borneman et al. (2014)	AWRI 1608	Wine	Australia	Triploid
	AWRI 1613	Wine	Australia	Diploid
Crauwels et al. (2015)	ST05.12/26 = MUCL 49865	Beer	Belgium	Diploid
	ST05.12/48	Beer	Belgium	Diploid
	ST05.12/53	Beer	Belgium	Triploid
	ST05.12/59 = CBS 6055	Dry ginger ale	United States of America	Triploid
Olsen et al. (2015), Jiang, et al. (2019)	CBS 11270	Bioethanol	Sweden	Diploid
	CBS 2796	Wine	Germany	ND
Fournier et al. (2017)	9 strains	Wine, beer, bioethanol	Europe, Oceania, Africa	Diploid, triploid
	53 strains	Wine, beer, bioethanol, soft drink	Europe, Oceania, Africa, America	Diploid, triploid
Colomer et al. (2020)	64 strains	Beer, bioethanol, wine, kombucha, tequila	Europe, Oceania, Africa, America	Diploid, triploid
Eberlein et al. (2021)	71 strains	Beer, bioethanol, wine, kombucha, tequila	Europe, Oceania, Africa, America	Diploid, triploid

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