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

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14 Short running title: *B. bruxellensis*' diversity

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19 Abstract

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

40

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

42 1. Introduction

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

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

74 Due to its positive or negative role in different industrial applications, *B. bruxellensis* is the species that
75 has received the most attention. It was first isolated by N. Hjelte Claussen in 1904 as being responsible
76 for the special flavor of British beers, and therefore was the first patented microorganism (Claussen,
77 1904; Steensels et al., 2015). In brewery, *B. bruxellensis* plays a major role for some special beer types
78 such as the Lambics from Belgium, Flanders Red and Brown ales, or the 'coolship ales' from the USA
79 (Bokulich, Bamforth, & Mills, 2012; Claussen, 1904; De Roos & De Vuyst, 2019). It contributes to the
80 peculiar flavour of spontaneous fermented beers, bringing additional complexity to their aromatic
81 bouquet. In addition, over the past decades, the growing popularity of craft beers and the rise of
82 microbreweries led to an increased interest among brewers who use *B. bruxellensis* alone or in co-
83 culture with other species. For example, *B. bruxellensis* has been described as a good candidate for
84 beers with a spicy phenolic note (Holt, Mukherjee, Lievens, Verstrepen, & Thevelein, 2018).
85 *Brettanomyces bruxellensis'* scientific literature with a focus on brewery is substantial (Capece,
86 Romaniello, Siesto, & Romano, 2018; De Roos & De Vuyst, 2019; Lentz, 2018; Serra Colomer, Funch, &
87 Forster, 2019; Steensels et al., 2015). *Brettanomyces bruxellensis* is also involved in the elaboration of
88 other spontaneous fermented beverages such as kombucha, kefir, etc. in which its role is mostly
89 considered as positive (Lynch, Wilkinson, Daenen, & Arendt, 2021; Tran et al., 2020).

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

93 widely described, amplified by its resistance to sulphite, one of the common control method
94 historically used (Monica Agnolucci, Tirelli, Cocolin, & Toffanin, 2017; Avramova et al., 2019; Blomqvist
95 & Passoth, 2015; Malfeito-Ferreira, 2018; Schifferdecker, Dashko, Ishchuk, & Piškur, 2014; Suárez,
96 Suárez-Lepe, Morata, & Calderón, 2007). Its presence in wine is associated with the production of
97 volatile molecules (called volatile phenols), associated to unpleasant aromas described as barnyard,
98 horse sweat or burnt plastic (Chatonnet, Dubourdie, Boidron, & Pons, 1992; Kheir, Salameh,
99 Strehaiano, Brandam, & Lteif, 2013). The 'Brett' taint negatively affects up to 25-30% of red wines in
100 the world (Alston, Arvik, Hart, & Lapsley, 2021; Gerbaux, Jeudy, & Monamy, 2000; A. Romano, Perello,
101 Revel, & Lonvaud-Funel, 2008). *Brettanomyces bruxellensis* is able to penetrate the micropores of the
102 barrels and therefore reused barrels – a frequent practice in oenology – are a recurrent cause of
103 contamination (Cartwright, Glawe, & Edwards, 2018; Fabrizio et al., 2015). In bioethanol production,
104 this species is considered as the main yeast spoiler as it can reduce the ethanol yield and cause large
105 economic losses (Basílio et al., 2008; Bassi, Meneguello, Paraluppi, Sanches, & Ceccato-Antonini, 2018;
106 Blomqvist & Passoth, 2015; A.T. de Souza Liberal et al., 2007; Radecka et al., 2015; Seo, Park, Jung, Ryu,
107 & Kim, 2020). Besides its beneficial-spoiler duality, another oddity of *B. bruxellensis* is its ability to act
108 as a first fermenter (even starter) yeast in some processes (*e.g.* craft beer, kombucha, bioethanol) or
109 as a fermentation 'finisher' (*e.g.* wine, Lambic beers, etc.), the actual fermentation usually being
110 carried out by *S. cerevisiae*. This starter-finisher versatility suggests multiple fermentative adaptations
111 that may have arisen from the complex structure of *B. bruxellensis* genome that includes large
112 chromosomal rearrangements, hybridization events, as well as ploidy level variation (Gounot et al.,
113 2020). In particular, a remarkable characteristic of *B. bruxellensis* species resides in the rare
114 coexistence of diploid and steady triploid isolates (Curtin, Kennedy, & Henschke, 2012; Piškur et al.,
115 2012).

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

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

122 2. Genetic and genomic diversity of *Brettanomyces bruxellensis*

123 2.1. Population structure of *Brettanomyces bruxellensis*

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

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

168 2.2. Genomic landscape of *Brettanomyces bruxellensis*

169 Within the *Saccharomycotina* subphylum, *B. bruxellensis* diverged from the model species *S. cerevisiae*
170 between 200 and 300 MY ago, before the whole-genome duplication (WGD) event that appeared in
171 the *Saccharomyces* lineage 100 MY ago (Fisher, Buskirk, Vignogna, Marad, & Lang, 2018; Guo et al.,
172 2016; Wolfe & Shields, 1997). WGD outcomes (e.g. gene duplication, promoter rewiring) were
173 suggested to be involved in the acquisition of the make-accumulate-consume (MAC) strategy in the
174 *Saccharomyces* genus (Thomson et al., 2005). Thus, it seems that both species, *S. cerevisiae* and
175 *B. bruxellensis*, have independently acquired the MAC ability (Rozpędowska et al., 2011). The first
176 partial genome sequence of *B. bruxellensis* strain CBS 2499 showed around 50% of nucleotide identity
177 with *S. cerevisiae* (Woolfit, Rozpędowska, Piškur, & Wolfe, 2007), and more recent studies identified
178 that at least 3,300 orthologous gene families were conserved across both clades (out of 5000 for
179 *B. bruxellensis*) (Cheng et al., 2017).

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

190 In *B. bruxellensis*, the nucleotide diversity estimated by the average number of pairwise nucleotide
191 differences, π , is high ($\pi = 1.2 \times 10^{-2}$) compared to *S. cerevisiae* ($\pi = 3 \times 10^{-3}$) (Gounot et al., 2020). In
192 addition, roughly 50% of the available strains of *B. bruxellensis* (exclusively isolated from anthropic
193 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

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

233 Regarding the Copy Number Variation (CNV), genes affected by CNV harbour functions related to drug
234 transporters, nitrogen assimilation or ethanol production (Borneman et al., 2014; Sam Crauwels et al.,
235 2014; C. D. Curtin, Borneman, Chambers, & Pretorius, 2012; Gounot et al., 2020). Ploidy level and CNV
236 could play an important role in the adaptation to the ecological niche, where multiple copies of a
237 particular gene or gene family can be beneficial in the new environment. For example, in *S. cerevisiae*
238 yeast, beer isolates have more increased copies of genes involved in maltose uptake and breakdown
239 are amplified (Gallone et al., 2016; Gladieux et al., 2014). In the pathogenic *C. albicans*, CNV are
240 associated to a significant fitness benefit to antifungal drugs (Todd et al., 2019). Within allotriploid
241 isolates, the diploid core genome was more prone to duplication events than the acquired haploid
242 genome (Gounot et al., 2020). Further studies will be needed to determine if CNVs are actually
243 associated with increased adaptation to specific ecological niche, as suggested for nitrate assimilation
244 in bioethanol production process (Galafassi, Capusoni, Muktaduzzaman, & Compagno, 2013). Indeed,
245 genes involved in the nitrogen pathway have been independently lost in several diploid isolates within

246 different subpopulations, indicating differential selective pressure (Gounot et al., 2020). Genomic
247 rearrangements affecting genes associated with traits of ecological interests have also been reported.
248 For example, genes coding beta-glucosidase activity are lost in beer isolates but conserved in wine
249 isolates (Sam Crauwels et al., 2017). This enzymatic activity could be useful to consume efficiently
250 peculiar carbon sources found in wines aged in oak barrels, due to the liberation of specific wood
251 polysaccharides. These variations were related to different aroma production, indicating technological
252 interest besides ecological significance (Serra Colomer, Funch, Solodovnikova, Hopley, & Förster,
253 2020). Variations were also observed for maltose-related genes, although it was not possible to
254 associate a phenotype to a specific genotype (S. Crauwels et al., 2015; Serra Colomer, Chailyan, et al.,
255 2020). Concerning Horizontal Gene Transfer (HGT) in *B. bruxellensis*, three events from bacteria were
256 recently highlighted, of which one encompassing an invertase-coding gene (from *Asaia* bacteria). This
257 enzymatic activity is speculated to have conferred the ability to hydrolyse sucrose (Roach & Borneman,
258 2020). Horizontal gene transfers are drivers of adaptive evolution in eukaryotes and may have
259 contributed to adaptation to high-sugar environments (Gladieux et al., 2014; Schönknecht, Weber, &
260 Lercher, 2014). The prevalence of these HGTs in the different subpopulations has yet to be described,
261 and it will be interesting to assess their possible involvement in niches' adaptation.

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

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

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

297 3. Phenotypic diversity of *Brettanomyces bruxellensis*

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

301 3.1. Intraspecific diversity of central metabolism and nutrient requirements

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

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

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

326 The most striking characteristic of *B. bruxellensis* is its ability to use a wide range of carbon sources as
327 shown by large-scale phenotypic analyses (Cibrario, Miot-Sertier, et al., 2020; Conterno et al., 2006;
328 Crauwels et al., 2015; da Silva et al., 2019; Galafassi et al., 2011; Smith & Divol, 2018). However, the
329 carbon utilisation varies among isolates: a survey on the assimilation of 190 different carbon sources
330 on seven strains clearly showed a variability between isolates, that could be related to different
331 processes (Crauwels et al., 2015). By contrast to wine isolates, most beer strains were not able to
332 consume galactose, some β -disaccharides (cellobiose and gentiobiose) or some β -substituted
333 monosaccharides (arbutin and β -methyl-D-glucoside) (Crauwels et al., 2015; Serra Colomer, Funch,
334 Solodovnikova, Hopley, & Förster, 2020). This observation could be explained by a variation of beta-
335 glucosidase and alpha-glucosidase activity between the genetic groups (Serra Colomer, Funch, et al.,
336 2020). Different environmental selective pressures could be responsible for this feature (Serra
337 Colomer, Funch, et al., 2020). However, further work involving more than seven isolates is needed,
338 including strains representative of all described subpopulations to properly assess the relationship
339 between carbon assimilation and process.

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

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

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

373 nitrate (from the wine 1 group), three haplotypes are present. To sum up, selective pressures in
374 different ecological niches may have allowed different evolutionary trajectories, although researches
375 involving more representative strains are needed to have the full picture. Regarding vitamins
376 requirements, *B. bruxellensis* was initially defined as an auxo-autotroph (Peynaud and Domercq, 1956)
377 but recent results indicate that biotin is the only vitamin which strongly affects the biotic capacity of
378 *B. bruxellensis* in the long term (von Cosmos & Edwards, 2016). Thiamine requirements appear to
379 depend on the strain and on the presence of ethanol in the growth medium (von Cosmos & Edwards,
380 2016).

381 3.2. Traits of technological interest

382 Another notable characteristic of *B. bruxellensis* species is its ability to convert hydroxycinnamic acids
383 (HCAs) into volatile phenols. HCA metabolism may have an important impact from an ecological
384 viewpoint: HCA have antimicrobial properties, and the ability to convert HAC into less toxic compounds
385 could promote yeast growth (Richard, Viljanen, & Penttilä, 2015). Besides, HAC are important dietary
386 antioxidants for flies. However, flies are not able to detect directly HCA and use ethyl phenols as an
387 indirect indicator of their presence (Dweck, Ebrahim, Farhan, Hansson, & Stensmyr, 2015). Flies
388 interaction with yeasts have consequences on their dissemination and the possibility to colonize new
389 environments, and volatile phenols could be a key factor mediating yeast-insect interactions (Stefanini,
390 2018). Besides their ecological importance, volatile phenols are considered as off-flavours in oenology
391 (for review (Suárez et al., 2007; Wedral, Shewfelt, & Frank, 2010)). The term 'volatile phenols' generally
392 includes 4-ethylphenol (4-EP), 4-ethylguaiacol (4-EG) and 4-ethylcatechol (4-EC) and their vinyl forms,
393 4-vinylphenol (4-VP), 4-vinylguaiacol (4-VG), 4-vinylcatechol (4-VC). These are produced through the
394 conversion of hydroxycinnamic acids (HCAs): p-coumaric acid, ferulic acid and caffeic acid respectively.
395 The production of volatile phenols by *B. bruxellensis* strains is variable and was extensively described
396 in oenological conditions (M. Agnolucci et al., 2009; Monica Agnolucci et al., 2010; Conterno et al.,
397 2006; Di Toro et al., 2015; Hixson et al., 2012; Madsen et al., 2017; D. Romano et al., 2017; Vigentini

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

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

424 rejection by the consumers. The metabolic pathway is not fully understood, but the amino-acid
425 content and the oxygen availability in fermented beverages are key parameters for their production
426 (Grbin & Henschke, 2000; Grbin, Herderich, Markides, Lee, & Henschke, 2007). The state of art is
427 somewhat inconsistent, which may be explained by the difficulty to properly measure such compounds
428 and their seemingly random occurrence in fermented beverages. From an ecological aspect, all these
429 aromatic compounds could play key roles in natural environments, including signalling and
430 communication with other organisms, and/or attractants for animals, particularly insects (Dzialo et al.,
431 2017).

432 *Brettanomyces bruxellensis* shows an incredible ability to persist over long time in some industrial
433 environments (Cibrario, Miot-Sertier, et al., 2020). Such capacity of persistence could be linked to the
434 various physiological states of the cells, beside the classical free-living (planktonic) state. Indeed,
435 *B. bruxellensis* is able to form biofilm, pseudo-mycelium, chlamydospore-like structure and VBNC
436 (Viable But Non Culturable) cells. While many aspects remains unknown or contradictory, a
437 physiological change between these forms could be induced by stress conditions related to the
438 presence of some chemical compounds and/or to nutrient limitation (Uscanga, Delia, & Strehaiano,
439 2000). Intraspecific diversity was observed in the ability to enter or exit the VBNC state (Capozzi et al.,
440 2016; Longin et al., 2016) as well as to form pseudo-hyphae (Martyniak, Bolton, Kuksin, Shahin, & Chan,
441 2017) or biofilm (Lucy Joseph et al., 2007). Recently, two concomitant studies (Dimopoulou, Renault,
442 et al., 2019; Lebleux et al., 2020) have suggested that the ability to produce biofilms depends on the
443 genetic groups. Although only a limited number of strains were tested, the beer group showed
444 robustness regarding biofilm formation in glucose-rich or glucose-limited media. For other genetic
445 groups, biofilm's production was more dependent on environmental characteristics, suggesting
446 different regulatory mechanisms (Dimopoulou, Renault, et al., 2019). These differences were also
447 partly mirrored by the biochemical and physicochemical properties of the surface of the strains
448 (Dimopoulou, Renault, et al., 2019). Finally, whether *B. bruxellensis* is actually able to sporulate and to
449 undergo a sexual cycle remains a mystery. Early description of ascospore-like forms by van der Walt

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

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

474 In winemaking, the most common treatment is the addition of sulphur dioxide (Barata et al., 2008).
475 High intraspecific diversity in sulphite tolerance was repeatedly demonstrated in several studies
476 (Avramova, Cibrario, et al., 2018; Dimopoulou, Hatzikamari, Masneuf-Pomarede, & Albertin, 2019;
477 Galafassi, Toscano, Vigentini, Piškur, & Compagno, 2013). On a cohort of 100 isolates from
478 winemaking, three phenotypic groups were defined: sensitive (slowed growth), tolerant (delayed
479 growth) and resistant (no impact on growth) (Vigentini, Lucy Joseph, Picozzi, Foschino, & Bisson, 2013).
480 A recent study was able to connect sulphur sensitivity to the genetic groups, with two triploid groups
481 (wine 1 and wine 2) containing most tolerant/resistant isolates (Avramova, Vallet-Courbin, Maupeu,
482 Masneuf-Pomarède, & Albertin, 2018). Competition experiments between sensitive/tolerant isolates
483 under increasing concentration of sulphite showed specific adaptation of isolates from wine 1
484 allotriploid group to high SO₂ environments (Avramova et al., 2019). Different *SSU1* haplotypes (*SSU1*
485 encoding sulphite efflux pump) related to variable SO₂ tolerance were characterized (Valdetara et al.,
486 2020; C Varela, Bartel, Roach, Borneman, & Curtin, 2019; Cristian Varela, Bartel, Onetto, & Borneman,
487 2020). It revealed that gene dosage effect (the number of *SSU1* haplotype) as well as the *SSU1*
488 regulation could be at least partially involved in SO₂ tolerance (Varela et al., 2019). In a recent
489 experiment, Bartel et al conducted a laboratory experimental evolution in presence of sulfur dioxide
490 concentrations, and evidenced adaptive evolution in different genetic backgrounds targeting partly
491 *SSU1* (Bartel et al., 2021). A genotyping study showed that the two genetic groups (wine 1 and wine 2)
492 containing the most tolerant/resistant isolates of *B. bruxellensis* were scarcely isolated before 1990
493 and that their proportion had increased steadily since then, possibly with the increase of sulphur use
494 in oenology (Cibrario et al., 2019). Sulphur tolerance/resistance in *B. bruxellensis* could thus be the
495 result of anthropization and adaptation to winemaking environments as for wine strains of
496 *S. cerevisiae* (Gallone et al., 2016; García-Ríos & Guillamón, 2019; Kaewkod, Bovonsombut, &
497 Tragoolpua, 2019). The fact that sulphite tolerant/resistant phenotypes are present in two different
498 genetic cluster (one autotriploids, one allotriploid) may indicate independent acquisition and possibly

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

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

509 4. Discussion and perspectives

510 4.1. *Brettanomyces bruxellensis*, the fermentation finisher

511 *Brettanomyces bruxellensis* has attracted increasing attention recently due to its involvement in
512 industrial processes and its unusual genomic composition. *Saccharomyces cerevisiae* is one of the most
513 intensively studied eukaryotic models, at the fundamental level in molecular, cellular and ecology
514 biology, but also from an applied point of view as it is widely used as a fermentation starter for several
515 human processes (Boone, 2014; Chambers & Pretorius, 2010; Goddard & Greig, 2015; Peter &
516 Schacherer, 2016). Interestingly, while *B. bruxellensis* species is associated with similar anthropic
517 processes (*e.g.* oenology, brewery), it is particularly recognized for its competence to colonize already
518 fermented environments (such as wine or beer rather than grape must or wort) (Schifferdecker et al.,
519 2014). *Brettanomyces bruxellensis* is thus a perfect model of fermentation finisher, a complementary
520 counterpoint to *S. cerevisiae* model of fermentation starter, sharing the same industrial niches, but not
521 with the same temporality. Interestingly, no isolates of *B. bruxellensis* were identified outside human
522 related processes so far. Several reasons may explain the non-detection of wild isolates yet. First,
523 *B. bruxellensis* is a slow-growing yeast species, so it may be easily outcompeted by other yeast species

524 whenever culturable methods are used (Agnolucci et al., 2017). Another possibility is that
525 *B. bruxellensis* could be part of biofilm communities in natural environments – albeit at low-abundance
526 and/or in Viable But Not Cultivable (VBNC) state preventing its isolation. Indeed, 40-80% of cells on
527 Earth live and persist in multispecies communities (bacteria, archaea, eukaryotes, etc.) forming
528 biofilms, e.g. aggregated structures frequently enclosed into matrixes of extracellular polymeric
529 substances (Flemming et al., 2016; Flemming & Wuertz, 2019). Growth in polymicrobial biofilms is
530 relatively protected from environmental variations, and could improve survival in hostile environments
531 (Flemming & Wuertz, 2019). The identification of low-abundance or VBNC species within complex
532 communities remains a challenge, prompting the use of metagenomics, culturomics or reverse
533 genomics approaches (Martellacci et al., 2019; Ryu et al., 2021). *Brettanomyces bruxellensis* has
534 abilities to form VBNC or to be involved in microbial consortia like kombucha’s SCOBY (Symbiotic
535 Community of Bacteria and Yeast) (Savary et al., 2021). These characteristics make it plausible the
536 hypothesis of natural biofilms as one of their wild environments, although no definite evidences are
537 described so far. Besides biofilms, natural fermentations are another putative example of ecosystems
538 that could foster the growth of wild *B. bruxellensis*. Many insects, mammals or birds store foods (fruits,
539 grains, meat, etc.) for times of less-plentiful sustenance. Food storage is prone to microbial growth or
540 spoilage, and natural fermentations occurred long before humans, and long before human-directed
541 fermentations (Carrigan et al., 2015; Post & Urban, 1993; Ruxton, Wilkinson, Schaefer, & Sherratt,
542 2014; Wiens et al., 2008). These naturally-occurring fermentations are poorly described, but they
543 probably display similar characteristics to *B. bruxellensis*’ anthropic environments: presence of ethanol
544 and organic acids, wide variety of ever-evolving sugar, nitrogen and other nutrients contents, oxygen
545 availability, succession of complex microbial communities, etc. In such natural fermentations,
546 *B. bruxellensis* could play second fiddle and colonize the environments after more efficiently-growing
547 and fermenting microorganisms. In natural environments also, the role of wild-fermentation finisher
548 would be congruent with *B. bruxellensis*’ scavenging abilities (Smith & Divol, 2016). However, this
549 hypothesis remains purely speculative: the literature is scarce regarding the microbial communities

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

562 4.2. Exploring the relationships between polyploidy/hybridization and adaptation to anthropized
563 environments

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

576 (>50%) which are known to evolve quickly toward aneuploidy and diploidy in *Saccharomyces* species
577 (Avramova, Cibrario, et al., 2018; Gerstein, McBride, & Otto, 2008; Todd, Forche, & Selmecki, 2017).
578 The phylogeny and evolutionary history of *B. bruxellensis* species are particularly complex to
579 reconstruct because of the intertwined events of polyploidization and hybridization (Linder, Moret, &
580 Nakhleh, 2003). New approaches will be needed to unravel its complex genetic architecture and to
581 elucidate the precise relationship between genomic evolution and the actual adaptation to
582 anthropized environments. The evolution of many species (plant, animal, microorganism) is strongly
583 shaped by human activities. When anthropogenic-driven transformation is purposefully associated
584 with improved attributes, the species are labelled as domesticated, as in *S. cerevisiae* for which several
585 independent domestication events were evidenced (Gallone et al., 2018; Giannakou et al., 2020;
586 Steensels, Gallone, Voordeckers, & Verstrepen, 2019). In the case of *B. bruxellensis*, the situation
587 seems to be more complicated: the fact that some beer isolates are used by brewers for their improved
588 ability to metabolize specific sugars may correspond to domestication. Conversely, adaptation to
589 winemaking environments through acquisition of sulphite resistance is probably an unintentional
590 consequence of anthropogenic influence. Thus, for wine groups, the term 'domestication' does not
591 apply to what seems to be an adaptive evolution at the expense of humans. A few works already
592 provided evidence of various beneficial or detrimental traits associated with the different anthropized
593 environments colonized by *B. bruxellensis*. The underlying molecular mechanisms remain to be
594 explored more thoroughly, in particular the role played by LOH, HGT, CNV and other mechanisms in
595 the evolution and adaptation of the species. How polyploidization and hybridization actually impacted
596 the evolutionary routes of *B. bruxellensis* is still an open question. Future directions should take
597 advantage of this model yeast to examine closely the influence of anthropogenic activities on the
598 species and their genomic, phenotypic and adaptive consequences, whether positive or negative for
599 humans.

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

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

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

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

647 4.3 *Brettanomyces bruxellensis*, a yeast model to study the relationship between polyploidy,
648 hybridization and adaptation to human-related environments

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

654 fitness, invasiveness abilities, adaptation to harsh environments and domestication (Ainouche &
655 Wendel, 2014; Soltis & Soltis, 2009). Historically less studied in microorganisms, polyploidy and
656 hybridization are now well established as driving evolutionary forces in yeast, Chromalveolata, but also
657 in prokaryotes, including Bacteria and Archaea (Albertin & Marullo, 2012; Marcet-Houben & Gabaldón,
658 2015; Soppa, 2022). Yeast usually enables the development of systems biology approaches, and
659 *B. bruxellensis* triploids may allow to investigate the fates of genes duplicated by whole genome
660 duplication and their impact on phenotypic traits. In addition, the close association between various
661 human processes and different ploidy/hybridization status makes of *B. bruxellensis* a valuable model
662 to decipher the evolutionary mechanisms involved in the adaptation to anthropized niches. The study
663 of *B. bruxellensis*' diploid and triploid populations may provide valuable insights into the ecological and
664 evolutionary significance of natural polyploidy.

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1578 [Data Accessibility](#)

1579 Phenotypic data files (figure 2): Avramova et al. (2018) DOI: 10.3389/fmicb.2018.01260

1580 Genomic data (figure 1B): Eberlein et al. (2021) DOI: 10.1101/gr.275380.121

1581 [Author contribution](#)

1582 JH, CE, JS and WA conceived the original outline of the review, with substantial inputs from all
1583 authors. All authors screened the literature and selected the subset of publications to be included.

1584 JH, CE, JS and WA drafted a first version. MDL helped more specifically drafting the central
1585 metabolism section, IMP and CMS the aroma/biofilm/technological part, and PB helped with the

1586 aroma section. JH, CE and WA contributed to the figures. All authors extensively revised the

1587 manuscript.

1588 Tables and Figures

1589 Figure 1. Genetic position and population structure of *Brettanomyces bruxellensis*.

1590 1A. Neighbor-joining tree of the *Brettanomyces* genus.

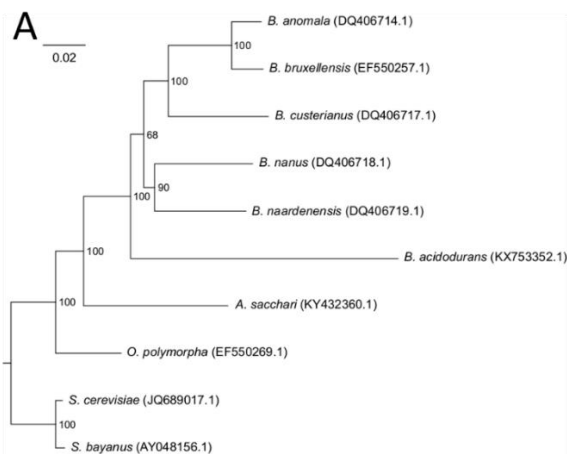
1591 The distance tree comprises the six *Brettanomyces* species, *Allodekкера sacchari* (the closest sister
 1592 species to *Brettanomyces* clade), *Ogataea polymorpha*, *Saccharomyces cerevisiae* and
 1593 *Saccharomyces bayanus* as outgroups. 26S ribosomal RNA gene sequences were used. A global
 1594 alignment with free end gaps was prepared using Geneious (Prime 2020.2) and the default setting for
 1595 multiple alignments. The Neighbor-joining tree was built with the Tamura-Nei Model of genetic
 1596 distances. The final tree represents a consensus of 1,000 resampled trees obtained with an extended
 1597 majority rules method. The consensus supports of the nodes are given in %. Sequences data was
 1598 downloaded from NCBI.

1599 1B. Genetic diversity within *Brettanomyces bruxellensis* species.

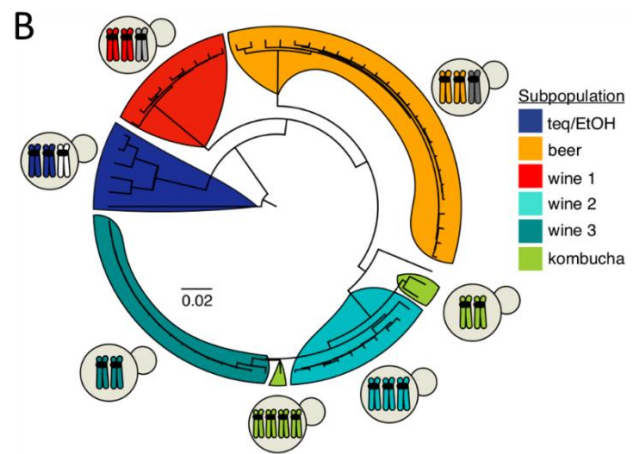
1600 *Brettanomyces bruxellensis* subpopulations are represented by different colors and named from
 1601 previous reports (Avramova, Cibrario, et al., 2018; Gounot et al., 2020). The tree was built from whole
 1602 genome Illumina short-read sequencing of 71 *B. bruxellensis* isolates aligned to the reference genome
 1603 *B. bruxellensis* (Fournier et al., 2017) and 24,313 genetic variants evenly distributed across the genome
 1604 (Eberlein et al., 2021). The ploidy level of each population was schematized, two chromosomes
 1605 represent diploid groups, three chromosomes represent triploids ones. Light grey, dark grey and white
 1606 chromosomes represent the independent haploid acquired genomes from unknown sister species.

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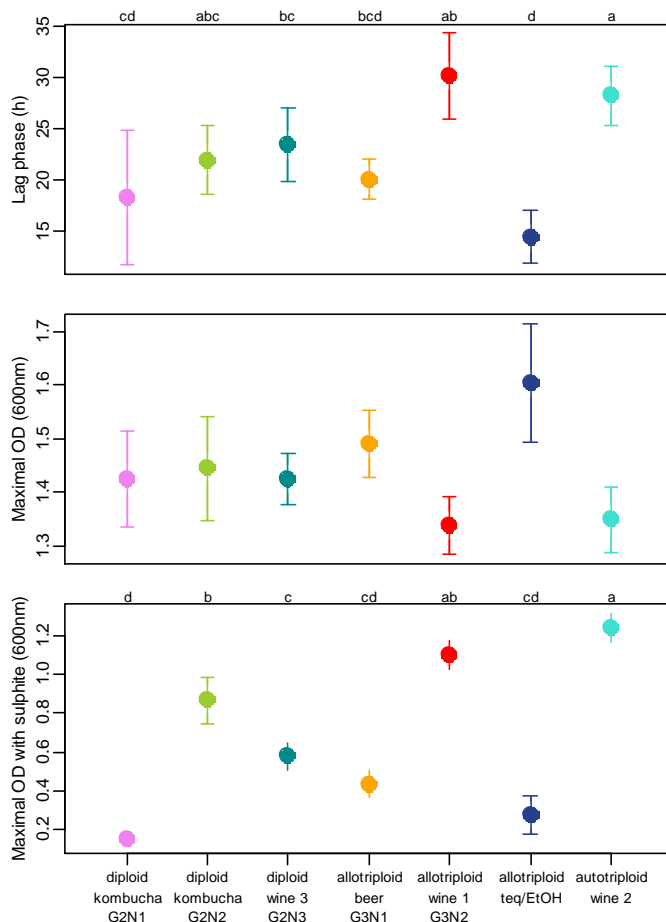
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1611 Figure 2. Growth parameters for different subpopulations of *B. bruxellensis*.

1612 Genetic groups as described by previous reports (Avramova, Cibrario, et al., 2018; Gounot et al., 2020).
1613 Growth data was taken from Avramova et al. (Avramova, Vallet-Courbin, et al., 2018). The growth
1614 parameters correspond to the lag phase (hour), maximum OD (600 nm) without or with sulphite (0.6
1615 mg.L⁻¹ of molecular SO₂). For each genetic group, mean values are represented by a circle, and error
1616 bars correspond to standard error. Top letters represent significance groups as defined by Kruskal-
1617 Wallis test when significant (p-value < 0.05). P-values were 0.0054, 0.11 and 5.4 10⁻¹⁰ for lag phase,
1618 maximum OD without or with sulphite respectively.



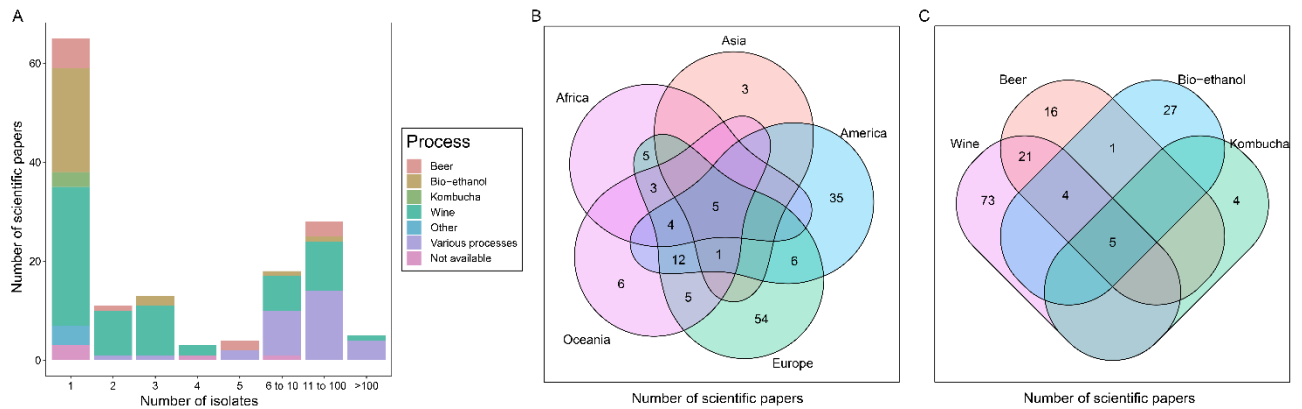
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1620 Figure 3. Representativity and diversity of *Brettanomyces bruxellensis* collections studied in the
 1621 literature.

1622 3A: Histogram of the number of isolates of *B. bruxellensis* used in the studies referenced in
 1623 supplemental Table 3, with their process/substrate of isolation.

1624 3B: Venn diagram of the number of studies including isolates of *B. bruxellensis* from various
 1625 geographical origins.

1626 3C: Venn diagram of the number of studies including isolates of *B. bruxellensis* from various process
 1627 origins.



1628

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

1630 ¹ND 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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