Occurrence of FFZ genes in yeasts and correlation with fructophilic behaviour

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Fructophily has been described in yeasts as the ability to utilize fructose preferentially when fructose and glucose are available in the environment. In Zygosaccharomyces bailii and Zygosaccharomyces rouxii, fructophilic behaviour has been associated with the presence of a particular type of high-capacity and low-affinity fructose transporters designated Ffz. In this study, a PCR screening was performed in several yeasts using degenerate primers suitable to detect FFZ-like genes. In parallel, fructophilic character was evaluated in the same strains by comparing the relative consumption rate of fructose and glucose. For all the strains in which FFZ-like genes were detected, fructophilic behaviour was observed (26 strains). Results show that FFZ genes are ubiquitous in the Zygosaccharomyces and Starmerella clades. Strains of Lachancea fermentati, Torulaspora microellipsoides and Zygotorulaspora florentina were not fructophilic and did not harbour FFZ genes. It is of note that these new species were recently removed by taxonomists from the Zygosaccharomyces clade, supporting the view that the presence of FFZ-like genes is a main characteristic of Zygosaccharomyces. Among the strains tested, only Hanseniaspora guilliermondiiNCYC2380 was an exception, having a preference for fructose in medium with high sugar concentrations, despite no FFZ-like genes being detected in the screening. Furthermore, this study supports the previous idea of the emergence of a new family of hexose transporters (Ffz facilitators) distinct from the Sugar Porter family.

INTRODUCTION

While most yeasts, like Saccharomyces cerevisiae, ferment glucose preferentially over other sugars, it has been shown that some others exhibit a different behaviour: when glucose and fructose are available in the environment, fructose is utilized first. They are fructophilic. The basis of fructophily in yeasts was first investigated in Sauternes yeast by Sols (1956), who showed that phosphorylation of the sugars was not involved, suggesting a role for ‘transferring agents prior to phosphorylation’. Later, Emmerich & Radler (1983) presented the first evidence that the transport step would certainly be involved in fructophily of Zygosaccharomyces bailii, the Sauternes yeast.

The genus Zygosaccharomyces was reported to comprise 11 species, divided into two groups (Kurtzman et al., 2001; Kurtzman & Robnett, 2003). Later, using multigene sequence analysis, Kurtzman (2003) proposed that the Zygosaccharomyces clade should be divided into four phylogenetic clades: (1) the well-supported clade Zygosaccharomyces, comprising Zygosaccharomyces bailii, Zs. bisporus, Zs. kombuchaensis, Zs. lentus, Zs. melli and Zs. rouxii; (2) the Zygotorulaspora clade, consisting of two species, Zygotorulaspora florentinus (florentina) and Zt. mrakii; (3) the Torulaspora clade, to which Torulaspora microellipsoides belongs; and (4) the Lachancea clade, in which Lachancea cidri and L. fermentati were included.

Since then, new Zygosaccharomyces species have been isolated and classified: Zygosaccharomyces machadoi, related...
to Zs. rouxii (Rosa & Lachance, 2005); Zygosaccharomyces gambelii, distinct from but related to Zs. machadoi (Torriani et al., 2011); Zygosaccharomyces siamensis, related to Zs. mells (Saksinchai et al., 2012); and Zygosaccharomyces sapae, closely related to Zs. rouxii and Zs. mells (Solieri et al., 2013). The genus Zygosaccharomyces then comprised ten recognized species: Zs. bailii, Zs. bisporus, Zs. gambelii, Zs. kombuchaensis, Zs. lentus, Zs. machadoi, Zs. mells, Zs. rouxii, Zs. sapae and Zs. siamensis.

Zs. bailii and Zs. rouxii are well known for their ability to spoil food and beverages, being responsible for high economic losses, due to their particular resilience to harsh conditions, such as low pH, low water activity and presence of weak-acid preservatives (Deák, 2007; Martorell et al., 2007). Despite these spoilage problems, some Zygosaccharomyces yeasts are used in industrial production. For example, Zs. rouxii is commonly used in the production of balsamic vinegar and during the alcoholic fermentation of miso paste and soy sauce (Deák, 2007; Solieri & Giudici, 2008; Sá-Correia et al., 2014) and Zs. kombuchaensis is used in the production of kombucha, a fermented tea-based beverage (Kurtzman et al., 2001).

In addition to Zs. bailii, Zs. rouxii was also characterized as fructophilic. In these yeasts fructose transport is mediated by (1) a specific transport system with high capacity and low affinity for fructose and (2) a transport system with low capacity and high affinity that also transports glucose. The fructophilic behaviour of these yeasts may be explained by their transport kinetics, as fructose competes with glucose for the hexose transporters and promotes their inactivation, more evident at high fructose concentrations (Sousa-Dias et al., 1996; Sousa-Dias, 2000). Both transport systems involve facilitated diffusion mechanisms.

More recently, in Zs. bailii and Zs. rouxii a peculiar type of fructose transporter designated Ffz (Fructose facilitator Zygosaccharomyces) was characterized at the molecular level: a fructose-specific facilitator Ffz1 [ZbFfz1 (Pina et al., 2004) and ZrFfz1 (Leandro et al., 2011)] and a ZrFfz2 that transports fructose and glucose with similar capacity and affinity (Leandro et al., 2011). In the recently sequenced genome of Zs. bailii ISA1307 (Mira et al., 2014) four FFZ-like genes were identified, two being FFZ1-like and two being FFZ2-like. In the genome of Zs. bailii CLIB213T (Galeote et al., 2013) two FFZ2-like genes also seem to be present.

FFz transporters are phylogenetically distinct from all the other previously characterized hexose transporters that belong to the Sugar Porter (SP) family. The FFz proteins belong to a newly described family of sugar transporters that is phylogenetically more similar to drug transporters of the DHA1 family (Leandro et al., 2011). The FFz proteins have highly conserved sequence motifs that differ from conserved motifs of hexose transporters of the SP family (Leandro et al., 2009) and are not conserved in yeast drug transporters of the DHA1 family (Leandro et al., 2011).

Candida magnoliae, Hanseniaspora guilliermondii and Starmerella bacillaris (formerly Candida zemplinina) have also been described as fructophilic yeasts (Ciani & Fatichenti, 1999; Yu et al., 2006; Magyar & Tóth, 2011). In C. magnoliae, an FFz protein, CmFfz1, was recently characterized as a low-affinity high-capacity fructose-specific transporter (Lee et al., 2014).

We have previously reported the key role of Ffz1 (but not Ffz2) from Zs. rouxii CBS732T in the strong fructophilic character of this strain (Leandro et al., 2014). Deletion of FFZ1 leads to simultaneous consumption of fructose and glucose, abolishing the typical fructophilic behaviour of this strain. Furthermore, when the FFZ1 gene was inserted back in a Δffz1 deletion mutant, fructophilic behaviour was recovered. So, at least in this strain, the fructose-specific facilitator ZrFfz1 plays an essential role in its fructophilic behaviour (Leandro et al., 2014).

To date, these new hexose transporters (FFz) have been found only in fructophilic yeasts. Is there a significant correlation between fructophilicity and the presence of FFZ genes in yeasts? This work aims to answer this question. The answer is that whenever FFZ genes are present, yeasts are fructophilic, with the exception of the peculiar fructophilic yeast H. guilliermondii in which no FFZ-like genes were found.

**METHODS**

**Strains and growth media.** Yeast strains used in this study, listed in Table S1 (available in the online Supplementary Material), were obtained from CBS-KNAW Fungal Biodiversity Centre (Centraalbureau voor Schimmelcultures), ISA (Instituto Superior de Agronomia, Lisbon, Portugal), PYCC (Portuguese Yeast Culture Collection, Caparica, Portugal) and E VN (Estação Vitivinícola Nacional, Dois Portos, Portugal). H. guilliermondiiNCYC2380 was kindly provided by H. Albergaria (LNEG, Lisbon). Yeast strains were grown in YPD [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose] medium and incubated at 25°C with orbital shaking (180 r.p.m.) (Environmental Shaker – Incubator ES-20/60; Biosan). The strains were kept on YPD slants.

**DNA manipulations.** Genomic DNA from yeast cells was isolated as described by Hoffman & Winston (1987). Primers ITS5 (5’-GGAA-TGAAAAGTCGTAACAAGG-3’) and LR6 (5’-GGGCGGAGGTCTTTGCTTGC-3’) were used to amplify the D1/D2 region of the large ribosomal subunit with NZYProof DNA polymerase (NZYTech) (5 min at 95°C; 40 cycles of 30 s at 95°C, 1 min at 52°C and 1 min at 72°C; 7 min at 72°C) to confirm the integrity of the genomic DNA isolated and strain identity. Sequencing was performed with the reverse NL1 primer (5’-GTATTAGCCAAGTCTCTTGC-3’) at STAB VIDA (Caparica, Portugal).

Degenerate primers were designed based on conserved protein motifs of FFz proteins from Zs. bailii and Zs. rouxii (ZbFfz1, ZrFfz1, ZrFfz2) and from several putative FFz proteins from filamentous fungi found in public databases. The conserved motif LAPFCEL, located between transmembrane domains 2 and 3, was used to design the forward primer FFZ_FW (5’-TNYTTGGCCICCTTYGYGA-3’), and the conserved motif GLPXIXL, located in transmembrane domain 8, was used to design the reverse primer FFZ_REV (5’-ARISMIATRTAIG-GIARRCC-3’). These primers were used to amplify FFZ-like genes.
from the yeasts under study with NZYLong DNA polymerase (NZYTech) (4 min at 94 °C; 30 cycles of 1 min at 94 °C, 1 min at 40–50 °C and 1 min at 72 °C; 7 min at 72 °C). Sequencing was performed in both forward and reverse directions with the same primers used for FFZ amplification. Sequence similarity searches were performed using the BLAST network service on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

**Evaluation of fructophilic behaviour by fructose and glucose relative consumption rates.** Strains were pre-inoculated overnight in YNB (yeast nitrogen base; Difco) with 10 g fructose l−1+10 g glucose l−1, or in YP [1% (w/v) yeast extract and 2% (w/v) peptone] with 100 g fructose l−1+100 g glucose l−1 or in YP with 150 g fructose l−1+150 g glucose l−1. Cells were transferred to the same medium (initial OD600 of 0.1) in Erlenmeyer flasks with a volume ratio flask/medium: 5:1 and incubated at 25 °C with orbital shaking (180 r.p.m.). Zs. kombuchaensis CBS8849T and Zs. lentus PYCC5838T were not grown without a sugar, because their growth was poor under shaking conditions, probably due to their sensitivity to reactive oxygen species (Steel et al., 1999, 2002). OD600 was measured in an Ultraspec 2100pro (Amersham Biosciences) spectrophotometer, in cuvettes with 1 cm path length, to follow growth. To monitor sugar consumption, fructose and glucose concentrations were measured by enzyme assays (kit ref. 10 139 106 035; Boehringer Mannheim/Roche/r-biopharm) in supernatants collected along the growth curve after removing the cells by filtration through a 0.2 μm cellulose acetate filter (Advantec).

**Hexose transport assays.** H. guilliermondii NCYC2380 was grown in YP containing 100 g fructose l−1+100 g glucose l−1, with orbital shaking at 25 °C. Cells were harvested by centrifugation at an OD600 of 0.8–1.2, washed twice with ice-cold water and resuspended in ice-cold water to a final concentration of 80 mg dry weight ml−1. Sugar transport assays were performed as described by Loureiro-Dias & Peinado (1984) and Leandro et al. (2011). Radioactivity was measured in a Beckman LS3801 liquid scintillation analyser. Each assay was performed in triplicate. Dry weight was determined by submitting 100 μl of the cell suspension to 80 °C for 24 h in pre-weighed aluminium foil cups (in triplicate).

**Reproducibility of results.** Assays were performed at least in duplicate. Results of representative experiments are shown.

**RESULTS**

**Screening for the presence of FFZ-like genes**

The alignments necessary to design the primers were performed in the BioEdit sequence alignment editor (Hall, 1999) using the CLUSTAL W algorithm (Thompson et al., 1994), with ZbFz1, ZrFz1 and ZrFz2 sequences, taking into account sequences of hexose transporters of the SP family and drug transporters of the DHA1 family, from several yeast species and filamentous fungi, to clearly identify highly conserved regions from transporters similar to the Ffzs and that were Fz-specific.

Screening for the presence of FFZ-like genes was performed with (1) several strains of the genus Zygosaccharomyces, (2) yeasts that had in the past belonged to this genus but are now reassigned to other genera such as Lachancea, Torulaspora and Zygotorulaspora, these clades being close to the Zygosaccharomyces clade, (3) yeasts previously described as fructophilic [e.g. C. magnoliae (Yu et al., 2006)] and (4) other yeasts phylogenetically related to fructophilic yeasts (e.g. Starmerella bombicola).

During the selection of yeast strains, the origin of the isolate was taken into account (Table S1). As FFZ genes were first described in osmophilic spoilage yeasts, Zs. bailii and Zs. rouxii, we chose, whenever possible, strains isolated from food and drinks and/or environments with high sugar content.

The fructophilic yeasts Zs. bailii ISA1307 and Zs. rouxii CBS732T, the genomes of which are available (Mira et al., 2014; Genolevures database, http://cbi.labri.fr/Genolevures/), were used as positive controls in screening for the presence of FFZ-like genes. The glucophilic yeasts Sa. cerevisiae and Debaryomyces hansenii were used as negative controls as they do not have FFZ-like genes in their genomes [Saccharomyces Genome database (www.yeastgenome.org); Genolevures database (http://cbi.labri.fr/Genolevures/)]. With the degenerate primers designed for amplification of the FFZ genes, a fragment with the expected length (about 700 bp) was obtained for the positive controls (Zs. rouxii CBS732T and Zs. bailii ISA1307), demonstrating the suitability of the designed pair of primers to amplify the genes of interest.

Amplification of a DNA fragment of a similar size was also obtained for all yeast strains tested from the genera Zygosaccharomyces and Starmerella, and for C. magnoliae (Table 1). Confirmation that the fragments correspond to FFZ-like genes was obtained by sequencing.

As expected, no amplification was obtained for the negative controls Sa. cerevisiae PYCC3507 or De. hansenii CBS767T. No amplification was also obtained for other yeasts tested, such as H. guilliermondii NCYC2380, L. fermentati ISA1888T, T. microcliopsis PYCC5189, Zt. florentina PYCC4169T or Zt. mrakii CBS4218T (Table 1).

Although the primers were designed to amplify both FFZ1 and FFZ2, we observed that per strain only one of the genes was preferentially amplified, even in Zs. rouxii CBS732T in which both were previously characterized (Leandro et al., 2011) and in Zs. bailii ISA1307 in the genome of which both genes are present (Mira et al., 2014).

**Analysis of the fructophilic/glucophilic behaviour of the strains**

Fructophilic/glucophilic behaviour was evaluated based on the relative rates of consumption of fructose and glucose in minimal and rich medium with equimolar concentrations of these hexoses. For lower sugar concentrations, minimal synthetic medium was used so as to define its sugar content (10 g fructose l−1+10 g glucose l−1). For high sugar concentrations, YP rich medium with 100 g fructose l−1+100 g glucose l−1 or 150 g fructose l−1+150 g glucose l−1 was used instead of YNB to prevent exhaustion of other nutrients affecting sugar uptake.
Table 1. Screening for the presence of FFZ-like genes by PCR and for strain fructophilic/glucophilic behaviour

<table>
<thead>
<tr>
<th>Strain</th>
<th>FFZ screening</th>
<th>YNB + 10 g fructose l⁻¹ + 10 g glucose l⁻¹</th>
<th>YP + 100 g fructose l⁻¹ + 100 g glucose l⁻¹</th>
<th>YP 150 g fructose l⁻¹ + 150 g glucose l⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candida magnoliae PYCC2903ᵀ</td>
<td>FFZ1</td>
<td>Glucophilic</td>
<td>Fructophilic</td>
<td>–</td>
</tr>
<tr>
<td>C. magnoliae PYCC3191</td>
<td>FFZ1</td>
<td>Simultaneous</td>
<td>Fructophilic</td>
<td>–</td>
</tr>
<tr>
<td>Hanseniaspora guillermondii NCYC2380</td>
<td>Negative</td>
<td>Simultaneous</td>
<td>Fructophilic</td>
<td>–</td>
</tr>
<tr>
<td>Lachancea fermentati ISA1888ᵀ</td>
<td>Negative</td>
<td>Simultaneous</td>
<td>Fructophilic</td>
<td>Glucophilic</td>
</tr>
<tr>
<td>Starmerella bacillaris PYCC3044</td>
<td>FFZ1</td>
<td>Fructophilic</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>St. bacillaris PYCC6282</td>
<td>FFZ1</td>
<td>Fructophilic</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>St. bombicola PYCC5882</td>
<td>FFZ1</td>
<td>Glucophilic</td>
<td>Fructophilic</td>
<td>–</td>
</tr>
<tr>
<td>Torulaspora microellipsoides PYCC5189</td>
<td>Negative</td>
<td>Simultaneous</td>
<td>Fructophilic</td>
<td>–</td>
</tr>
<tr>
<td>Zygosaccharomyces bailii EVN101</td>
<td>FFZ2</td>
<td>Fructophilic</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Zs. bailii EVN1122</td>
<td>FFZ2</td>
<td>Simultaneous</td>
<td>Fructophilic</td>
<td>–</td>
</tr>
<tr>
<td>Zs. bailii ISA1024</td>
<td>FFZ2</td>
<td>Fructophilic</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Zs. bailii ISA1149ᵀ</td>
<td>FFZ2</td>
<td>Fructophilic</td>
<td>Fructophilic</td>
<td>–</td>
</tr>
<tr>
<td>Zs. bailii ISA1265</td>
<td>FFZ2</td>
<td>Fructophilic</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Zs. bailii ISA1307</td>
<td>FFZ2</td>
<td>Fructophilic</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Zs. bailii PYCC2470</td>
<td>FFZ2</td>
<td>Fructophilic</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Zs. bisporus ISA1868ᵀ</td>
<td>FFZ1</td>
<td>Fructophilic</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Zs. gambellarenensis CBS12191ᵀ</td>
<td>FFZ1</td>
<td>Fructophilic</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Zs. kambuchaensis CBS8849ᵀ</td>
<td>FFZ1</td>
<td>Fructophilic</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Zs. lentus PYCC5838ᵀ</td>
<td>FFZ2</td>
<td>Fructophilic</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Zs. machadoi CBS10264ᵀ</td>
<td>FFZ2</td>
<td>Simultaneous</td>
<td>Fructophilic</td>
<td>–</td>
</tr>
<tr>
<td>Zs. mells ISA1889ᵀ</td>
<td>FFZ2</td>
<td>Simultaneous</td>
<td>Fructophilic</td>
<td>–</td>
</tr>
<tr>
<td>Zs. rouxii CBS732ᵀ</td>
<td>FFZ1</td>
<td>Fructophilic</td>
<td>Fructophilic</td>
<td>–</td>
</tr>
<tr>
<td>Zs. roxii CBS9719</td>
<td>FFZ1</td>
<td>Fructophilic</td>
<td>Fructophilic</td>
<td>–</td>
</tr>
<tr>
<td>Zs. rouxii CBS12631</td>
<td>FFZ1</td>
<td>Simultaneous</td>
<td>Fructophilic</td>
<td>–</td>
</tr>
<tr>
<td>Zs. rouxii PYCC4372</td>
<td>FFZ1</td>
<td>Simultaneous</td>
<td>Fructophilic</td>
<td>–</td>
</tr>
<tr>
<td>Zs. rouxii PYCC5226</td>
<td>FFZ2</td>
<td>Simultaneous</td>
<td>Fructophilic</td>
<td>–</td>
</tr>
<tr>
<td>Zs. sapae CBS12607ᵀ</td>
<td>FFZ2</td>
<td>Simultaneous</td>
<td>Fructophilic</td>
<td>–</td>
</tr>
<tr>
<td>Zs. siamensis CBS12273ᵀ</td>
<td>FFZ2</td>
<td>Simultaneous</td>
<td>Fructophilic</td>
<td>–</td>
</tr>
<tr>
<td>Zygotorulaspora florentina PYCC4169ᵀ</td>
<td>Negative</td>
<td>Simultaneous</td>
<td>Fructophilic</td>
<td>–</td>
</tr>
<tr>
<td>Zt. mukii CBS4218ᵀ</td>
<td>Negative</td>
<td>Glucophilic</td>
<td>Glucophilic</td>
<td>–</td>
</tr>
</tbody>
</table>

Candida strains. The results obtained for C. magnoliae strains (Table 1) show that these yeasts are fructophilic only for high sugar concentrations; at low sugar concentrations, glucose and fructose were consumed at the same rate in C. magnoliae PYCC3191, whereas fructose was consumed at a lower rate than glucose in C. magnoliae PYCC2903ᵀ. C. magnoliae PYCC2903ᵀ (Fig. S1) had a more pronounced fructophilic behaviour than C. magnoliae PYCC3191 (data not shown).

Starmerella strains. St. bacillaris PYCC6282 and St. bacillaris PYCC3044 (previously named C. zemplinina) displayed fructophilic behaviour even at low sugar concentrations (Table 1; Fig. 1e, f), although fructose was consumed faster at high concentrations. St. bombicola PYCC5882 presented a different profile (Table 1, Fig. 1a, b), being fructophilic only at high concentrations, as with C. magnoliae.

Zygosaccharomyces strains. Although Zs. rouxii CBS732ᵀ showed an accentuated fructophilic behaviour at both sugar concentrations (Leandro et al., 2014), the other strains tested, Zs. rouxii PYCC4372, Zs. rouxii PYCC5226, Zs. rouxii CBS9719 and Zs. rouxii CBS12631, were fructophilic only in medium with high sugar concentrations (Table 1). The representative behaviour of Zs. rouxii PYCC5226 is shown in Fig. 1(c, d).

Zs. machadoi CBS10264ᵀ, Zs. mells ISA1889ᵀ (Fig. S1), Zs. sapae CBS12697ᵀ and Zs. siamensis CBS12273ᵀ behaved as for the majority of the Zs. rouxii strains tested (Table 1). By contrast, Zs. bisporus ISA1868ᵀ (Table 1), Zs. gambellarenensis CBS12191ᵀ (Table 1), Zs. kambuchaensis CBS8849ᵀ (Fig. S2) and Zs. lentus PYCC5838ᵀ (Fig. S2) presented a preference for fructose even at low concentrations, as with Zs. rouxii CBS732ᵀ (Leandro et al., 2014).

All the Zs. bailii strains tested presented fructophilic behaviour even at low sugar concentrations (Table 1, Fig. 2c, d), except Zs. bailii EVN1122 (Table 1), which presented fructophilic behaviour only at high sugar concentrations.

Lachancea, Torulaspora and Zygotorulaspora strains. Although L. fermentatiISA1888ᵀ, T. microellipsoides PYCC5189,
Zt. florentina PYCC4169\textsuperscript{T} and Zt. mrakii CBS4218\textsuperscript{T} were previously assigned to the genus Zygosaccharomyces they did not show a preference for fructose (Table 1). This reinforces our initial hypothesis for the existence of a direct correlation between the presence of FFZ genes and fructophilic behaviour, as FFZ genes were not amplified in any of these strains. To discount the possibility that these strains might present fructophilic behaviour at even higher concentrations of hexoses, assays were performed in YP medium with 150 g fructose l\textsuperscript{-1} + 150 g glucose l\textsuperscript{-1}, the concentrations that Yu et al. (2006) used for C. magnoliae KFCC11023. Under these conditions, the behaviour of L. fermentati ISA1888\textsuperscript{T} (Table 1), T. microellipsoides PYCC5189 (Table 1, Fig. S3) and Zt.

Fig. 1. Growth curves and sugar (fructose and glucose) consumption profiles of St. bombicola PYCC5882 (a, b), Zs. rouxii PYCC5226 (c, d) and St. bacillaris PYCC3044 (e, f) in YNB with 10 g fructose l\textsuperscript{-1} + 10 g glucose l\textsuperscript{-1} (a, c, e) and YP with 100 g fructose l\textsuperscript{-1} + 100 g glucose l\textsuperscript{-1} (b, d, f). Fructose is represented by filled triangles (▲), glucose by open circles (⊙) and OD\textsubscript{640} by a dashed line. Data are representative of at least two independent experiments. Error bars (SD) are shown.
florentina PYCC4169T (Table 1) changed from simultaneous consumption of the two sugars to a preference for glucose.

**Hanseniaspora guilliermondii NCYC2380.** *H. guilliermondii* NCYC2380 presented a fructophilic character at high sugar concentrations (Table 1, Fig. 2b), although amplification of *FFZ* genes was not found. However, *H. guilliermondii* fructophilic behaviour (Fig. 2b) was not as pronounced as that in *Zs. bailii* ISA1307 (Fig. 2c, d) or in *Zs. rouxii* CBS732T (Leandro et al., 2014), which may be explained by differences in relative uptake capacities for fructose and glucose among these yeasts. We measured the kinetic parameters of uptake of hexoses of *H. guilliermondii* grown under fructophilic conditions (medium with high sugar concentrations) and Fig. 3 depicts the differences in $V_{\text{max}}$ for fructose and glucose among *H. guilliermondii* NCYC2380, *Zs. bailii* ISA1307 (Sousa-Dias et al., 1996) and *Zs. rouxii* CBS732T (Sousa-Dias, 2000). The ratio of $V_{\text{max}}$ values for fructose and glucose for *H. guilliermondii* was 1.45, much lower than the ratios obtained for *Zs. bailii* (3.71) and *Zs. rouxii* (10.17).

**Phylogenetic analysis**

A phylogenetic tree reconstructed using the **MEGA** software version 6 (Tamura et al., 2013) shows that the protein sequences from the *FFZ* fragments amplified using degenerate primers grouped with the putative Ffz proteins from filamentous fungi, and there was also clear separation between Ffz1-like proteins and Ffz2-like proteins (Fig. 4). Moreover, as reported by Leandro et al. (2011), these proteins form a new family of hexose transporters distinct from the SP family [which include Hxt-like hexose facilitators and Fsy1-like fructose/H$^+$ symporters (Coelho et al., 2013; Leandro et al., 2013)] and DHA1 family (which includes drug/H$^+$ antiporters). Accession numbers of proteins considered in the phylogenetic tree are provided in Table S2.
For all the strains in which FFZ-like genes were detected, fructophilic behaviour was observed under at least one of the tested conditions, as per our initial hypothesis. The yeast strains in which FFZ-like genes were not detected (Sa. cerevisiae, De. hansenii, L. fermentati ISA1888, T. microellipsoïdes PYCC5189, Zt. florentina PYCC4169 and Zt. mrakii CBS4218) did not exhibit a preference for fructose under any conditions tested, preferentially consuming glucose over fructose or in some cases consuming both sugars simultaneously. H. guilliermondii NCYC2380 was an exception, having a preference for fructose in medium with high sugar concentrations, despite no FFZ-like genes being amplified under the conditions tested. This result does not exclude the hypothesis that an FFZ-like gene is present in the genome. To explore this hypothesis, studies of transport kinetics were performed. Comparing the $V_{\text{max}}$ ratio for fructose and glucose transport, the ratio for H. guilliermondii was much lower than that for Zs. bailii and Zs. rouxii (Fig. 3), which might explain the less pronounced fructophilic behaviour of H. guilliermondii (Fig. 2).

The phylogenetic tree shown in Fig. 4 clearly demonstrates the emergence of a new and important family of Ffz transporters phylogenetically distinct from the SP and DHA1 families. Given the behaviour of H. guilliermondii NCYC2380, we propose that the presence of FFZ genes is a marker for fructophilic behaviour although their absence is not always synonymous with glucophilic behaviour.

In the glucophilic yeast Sa. cerevisiae, both the transporter proteins Hxts and the hexokinases (Hxk) display a higher affinity for glucose than for fructose, the hexokinases involved in hexose sensing and signalling, and therefore playing an important role in this yeast’s preference for glucose (Berthels et al., 2008). In addition, other factors such as response to ethanol and nitrogen availability and utilization seem to be important for sugar preference in yeasts, indicating that hexose utilization depends on several environmental and biological parameters (Liccioli et al., 2011).

Measurement of the activity of a hexokinase from Zs. bailii strain 210, expressed in a Sa. cerevisiae hexokinase deletion mutant strain, showed that ZbHxk had a preference for fructose phosphorylation, with a higher affinity for fructose ($K_m$ 8.6 mM) than for glucose ($K_m$ 19.1 mM), but with lower affinity for both fructose and glucose, when compared with Sa. cerevisiae hexokinases (Sütterlin, 2010).

In the glucophilic yeast Yarrowia lipolytica it was reported that hexokinase activity is a limiting factor in production of lipids from fructose, as overexpression of hexokinase improves sugar utilization and lipid accumulation from fructose (Lazar et al., 2014).

Thus, at least for some yeasts, the basis of the preference for fructose may result not only from the presence of particular fructose carriers but also from the second step of sugar metabolism, the phosphorylation step, and therefore it cannot be discounted that (some) fructophilic yeasts may...
also harbour hexokinases with a higher affinity for fructose than for glucose. If this was the case, their expression in wine strains, together with ZrFFZ1, could further improve fructose fermentation capacity.

ZrFFZ1 (specific for fructose) was shown to play a key role in the fructophilic behaviour of Zs. rouxii CBS732\(^2\), whereas ZrFFZ2 (a fructose/glucose facilitator) had no impact on its fructophilic behaviour (Leandro \textit{et al.}, 2014). As we observed fructophilic behaviour in yeasts in which only FFZ2-like genes were detected by PCR, it is possible that at least one FFZ1 gene may also be present in these strains, although it was not detected in the PCR screening. This might be due to the fact that the degenerate primers amplified preferentially only one of the FFZs when both were present in the yeast genomes, as was observed for the control strains Zs. rouxii CBS732\(^2\) and Zs. bailii ISA1307. In these cases, although we know that both FFZ1 and FFZ2 are present, only one of them was amplified (Table 1).

The presence of Ffz transporters in several \textit{Zygosaccharomyces} yeasts may confer an advantage to these microorganisms isolated from food products with high sugar content, namely fructose. The sugar gradient produced by the high sugar concentrations in the extracellular space is used to transport the sugar without loss of energy via facilitated diffusion by low-affinity, high-capacity Ffz-like transporters (Sousa-Dias \textit{et al.}, 1996).

Besides taxonomic problems in the \textit{Zygosaccharomyces} clade, \textit{St. bacillaris} (previously \textit{C. zemplinina}) and \textit{St. bombicola} have been misclassified as \textit{Candida stellata} (synonym: \textit{Torulopsis stellata}). \textit{C. stellata} is found in the fermentation of sweet botrytized wines (in which grapes are affected by \textit{Botrytis cinerea}) and overripe grapes (Rosini \textit{et al.}, 1982; Sipiczki, 2003) and is considered the main agent responsible for the wine aroma-compounds produced (Soden \textit{et al.}, 2000). Nevertheless, it has been demonstrated that in most cases \textit{St. bacillaris} (\textit{C. zemplinina}) and \textit{St. bombicola} (\textit{Candida bombicola}) have been incorrectly classified as \textit{C. stellata} (Sipiczki, 2003, 2004; Sipiczki \textit{et al.}, 2005; Csoma & Sipiczki, 2008). The aroma-compounds may thus be produced by \textit{St. bacillaris} and/or \textit{St. bombicola}. \textit{St. bombicola} is also found in honey of \textit{Bombus} species (bumble-bee) and its osmotolerance seems to be related to the association of this yeast with nectar-feeding insects (Rosa & Lachance, 1998). Similarly, \textit{St. bacillaris} PYCC6282 is found in \textit{Drosophila melanogaster} that may play a key role as a vector, transporting the yeast cells from the winery to the ripening grapes in the vineyard (Csoma & Sipiczki, 2008).

\textit{C. magnoliae}, belonging to the \textit{Starmerella} clade, shares the ecological niches in which \textit{Starmerella} species are found, namely in association with bees and their habitats (Kurtzman, 2011). The fact that \textit{St. bacillaris}, \textit{St. bombicola} and \textit{C. magnoliae} are also present in environments with high sugar content is in line with what happens with \textit{Zygosaccharomyces} species. Ffz transporters may represent an advantage in these environments. Our results demonstrate a general relationship in yeasts between FFZ genes and fructophily. We also suggest a relationship with the presence of these yeasts in niches with high sugar content.

These fructophilic yeasts have a strong biotechnological potential that should be further explored, as their ability to consume fructose preferentially is an important trait that could accelerate the consumption of all available sugars, consequently reducing the chances of stuck fermentations occurring. Besides \textit{Zs. rouxii} and \textit{Zs. kombuchaensis}, which are already used for the manufacture of some food products, such as miso paste and kombucha, respectively, \textit{Zs. bailii} has also been reported as a good candidate for biotechnological processes due to its high biomass yield, environmental resilience and specific growth rate (Sá-Correia \textit{et al.}, 2014).

The use of \textit{Zs. bailii} strains, isolated from winery environments, to cure stuck fermentations has been tested as an alternative approach to remove residual sugar from red wines, and the results obtained are equivalent to those achieved using \textit{Sa. cerevisiae} EC1118, a strain commonly used to restart fermentations (Zuehlke \textit{et al.}, 2015).

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