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# Biodiversity of *Zygosaccharomyces* species in food systems

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**ABSTRACTS**

*Zygosaccharomyces* species are among the most problematic food spoilage yeasts. The two most infamous species are *Zygosaccharomyces balii* and *Zygosaccharomyces rouxii*, although they may also take a positive role during the production of some fermented foods. DNA sequence based yeast identification aided by freely available reference databases of barcoding DNA sequences has boosted the description rate of novel yeast species in the last two decades. The genus *Zygosaccharomyces* has been considerably expanded as well. Especially the number of the extremely osmotolerant *Zygosaccharomyces* species, related to *Z. rouxii* and regularly found in high-sugar foods, has enlarged. A brief account of recent developments in the taxonomy and biodiversity of this important food associated genus is given in this review.

**KEYWORDS**

foodborne yeasts, food spoilage, osmophilic yeasts, *Zygosaccharomyces*

## 1. INTRODUCTION

Humans unknowingly and inadvertently daily ingest large populations of viable yeast cells by consuming different kind of foods and beverages. Significant amount of viable yeast cells are

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harboured e.g. in fresh fruits, fruit juices, salads, cheeses and other fermented dairy products, fermented meet products, alcoholic beverages, and traditional fermented foods (Fleet and Balia, 2006). Yeasts are introduced to foods and beverages deliberately as starter cultures, or unintentionally by the food ingredients or during the production, packaging, and transportation of foods. According to Fleet (2006), their significance in food and beverage production can be classified as follows: "production of fermented foods and beverages, production of ingredients and additives of food processing, spoilage of foods and beverages, biocontrol of spoilage microorganisms, probiotic and biotherapeutic agents, source of food allergens, and source of opportunistic, pathogenic yeasts". The biodiversity of yeasts in foods can be investigated by culture-based and culture-independent methods. The application of culture-independent methods revealed a number of hitherto unknown microorganisms in naturally fermented foods (Tamang et al., 2016). However, the application of culture-based methods is irreplaceable in cases when the behaviour of microorganisms in food matrices is to be investigated.

By the end of the previous millennium the sequences of the D1/D2 domain of nuclear rRNA gene for practically all yeast species known at that time were determined and guidelines for species delimitation were provided for ascomycetous (Kurtzman and Robnett, 1998) and basidiomycetous (Fell et al., 2000) yeast species as well. Although, unlike with the whole fungal kingdom (Schoch et al., 2012), in case of yeasts the D1/D2 domain of large subunit (LSU) nuclear rRNA gene has become the primary barcoding region, the DNA sequence of the ITS region, in itself or in combination with D1/D2 sequence has also increasingly been utilised for yeast identification. The application of DNA sequencing and the availability of freely accessible reference DNA sequence databases have provided unprecedented accuracy and rapidity of yeast identification. In addition to aiding identification, DNA barcode sequence comparisons also facilitate revealing novel yeast species. As a result the tempo of yeast species description has been accelerated in the last two decades, which was already reflected in the number of species (1,265) treated in the latest edition of the Yeasts, a Taxonomic Study (Kurtzman et al., 2011). Currently the number of known yeast species exceeds 2,000 (Boekhout et al., 2021). This boom of the number of yeast species has also affected foodborne yeasts. Due to their preservative resistance, osmotolerance, and strong fermentative ability, *Zygosaccharomyces* species are undoubtedly the most problematic spoilage yeasts encountered in food and drinks industries (James and Stratford, 2003). The aim of this review is to summarise the developments during the last ten years in the taxonomy and biodiversity of *Zygosaccharomyces*, a genus with prominent significance to food industry.

## 2. *ZYGOSACCHAROMYCES* IN THE LATEST EDITION OF THE YEASTS, A TAXONOMIC STUDY – THE STATUS QUO

In the 5th edition of the Yeasts, a Taxonomic Study, six *Zygosaccharomyces* species were treated (James and Stratford, 2011), while the authors became aware of an additional one, *Zygosaccharomyces machadoi*, too late for inclusion in their contribution. On the basis of their physiological characters, the species were assigned to three sub-groups (numbering by me) as follows. *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* are characterised by extreme resistance to weak-acid food preservatives (1); *Zygosaccharomyces lentus* and *Zygosaccharomyces combuchaensis* show preference for slow growth at cooler temperatures (2), while

*Zygosaccharomyces rouxii* and *Zygosaccharomyces mellis* exhibit extreme osmotolerance (3). The two most infamous species among them are *Z. bailii* and *Z. rouxii*. *Z. bailii* is resistant to sorbic and benzoic acids and sulphur dioxide at levels permitted in food industry and is a notorious spoilage yeast of several foods and beverages including soft drinks, fruit juices and concentrates, wines, ciders, tomato sauce, salad dressing, and mayonnaise. *Zygosaccharomyces rouxii* is among the microorganisms capable of growing at low water activities down to 0.62 at least under a set of other environmental conditions. As a result of this remarkable property, it may cause spoilage of high-sugar foods and drinks, including honey and fruit juice concentrates (Pitt and Hocking, 2009). Some frequent isolation sources of *Zygosaccharomyces* species are shown in Table 1. Bold characters indicate the species not treated by James and Stratford (2011).

Although names of *Zygosaccharomyces* species, first of all *Z. rouxii* and *Z. bailii*, are mostly emerging in context of food spoilage, they also can exert beneficial effects during the production of some foods. For example *Z. rouxii* significantly contributes to the formation of aroma compounds during soy sauce fermentation (Devanthi and Gkatzionisand, 2019) and converts the sugars available in cooked grape must to ethanol, the substrate for acetic acid bacteria during the production of balsamic vinegar. In the latter case, *Z. rouxii* may be accompanied by other yeasts including further *Zygosaccharomyces* species (Solieri and Giudici, 2008). *Z. rouxii* has also been considered as starter culture for low-alcohol or alcohol-free beer (reviewed by Michel et al., 2016) and baking yeast (Boboye et al., 2008), while *Z. bailii* plays a positive role during kombucha fermentation by producing ethanol, which is subsequently converted to acetic acid by bacteria (Solieri, 2021).

## 3. THE CURRENT STAGE OF THE GENUS *ZYGOSACCHAROMYCES*

The genus *Zygosaccharomyces* has considerably been expanded in the last decade. The phylogenetic relationships among the currently recognised members of the genus deduced from the partial DNA sequences for the gene coding the large subunit (LSU) nuclear rRNA is depicted in Fig. 1. Species not treated by James and Stratford (2011) are indicated with bold characters. While no further species were added to group 2 comprising *Z. lentus* and *Z. combuchaensis*, two novel species closely related to *Z. bailii* (group 1) were described, and the group of extremely osmotolerant *Zygosaccharomyces* species (group 3) has significantly been enlarged. A brief compendium of the *Zygosaccharomyces* species not treated in the latest edition of the Yeast, a Taxonomic Study is provided below.

### 3.1. *Zygosaccharomyces parabailii* and *Z. Pseudobailii*

The comparison of barcoding DNA regions of several yeast strains maintained as *Z. bailii* revealed the existence of two groups characterised by DNA sequences divergent from the corresponding sequences of *Z. bailii* as well as from each other. The divergent regions included the nuclear rRNA gene cluster (partial LSU and ITS), as well as some protein-coding gens; $\beta$-tubulin, EF-1$\alpha$, RPB1, and RPB2. Although the investigated mitochondrial genes (mtSSU rRNA and COXII) were not variable enough to distinguish the two above-noted groups and *Z. bailii*, the two groups were interpreted as two distinct species and *Zygosaccharomyces parabailii* and *Zygosaccharomyces pseudobaillii* were proposed for them (Suh et al., 2013). The isolation sources of the two novel species are similar to the typical substrates for the isolation of

*Table 1.* Common isolation sources of *Zygosaccharomyces* species

| Species* | Isolation source | Reference |
|---|---|---|
| *Z. bailii* | Grape must, Italy; wine, France; Swiss wine yeast; cloudy wine, South Africa; apple juice, mayonnaise and vinegar containing products, The Netherlands; vinegar, Spain; sour red wine, USA; Brazilian orange juice concentrate; sorghum-brandy mash; Worcester sauce; lees of pear must; honey; Institute of Brewing, Tokyo; sweet pickle, UK; salad cream; grape and blackcurrant juice | Barnett et al. (2000); James and Stratford (2011) |
| *Z. bisporus* | Tea-beer fungus, Java; fermenting cucumbers, USA | Barnett et al. (2000); James and Stratford (2011) |
| ***Z. favi*** | Bee bread, polyfloral honey, Hungary | Čadež et al. (2015) |
| ***Z. gambellarensis*** | Sweet white wine, Veneto region, Italy | Torriani et al. (2011) |
| *Z. kombuchaensis* | Kombucha tea, Russia, USA | James and Stratford (2011) |
| *Z. lentus* | Spoiled orange juice, UK; spoiled orange squash; spoiled tomato ketchup, UK; spoiled pear and blackcurrant squash drink, Ireland | James and Stratford (2011) |
| ***Z. machadoi*** | Garbage pellets of a stingless bee *Tetragonisca angustula* in Brazil | Rosa and Lachance (2005) |
| *Z. mellis* | Alpechin, Spain; honey, Italy, USA, Canada; wine grapes, Germany; syrup containing root ginger, strawberry juice | Barnett et al. (2000); James and Stratford (2011) |
| ***Z. osmophilus*** | Sugar, Mauritius; honey, and larval food of bees (*Scaptotrigona* cfr. *bipunctata* and *T. angustula*) in Brazil | Kreger-van Rij (1966); Matos et al. (2020) |
| ***Z. parabailii*** | Imported citrus concentrate, The Netherlands; citrus paste, The Netherlands; salad dressing, USA | Suh et al. (2013) |
| ***Z. pseudobailii*** | Worcestershire sauce, Japan; pickle relish | Suh et al. (2013) |
| *Z. rouxii* | Concentrated black grape must, Italy; wine, fermenting jam and bonbon of bitter orange syrup, France; wine grapes, Germany; Portuguese white wine; salted beans, The Netherlands; raw cane sugar; pineapple jam; wort; dates, Tunisia; ginger cake, marzipan, candied fruit, salted beans and molasses, The Netherlands; malt extract, UK; pickles, miso, sweat cream cake, Japan; marmalade, Belgium; Cuban molasses; fermenting cucumbers and maple syrup, USA; honey, Canada; cane sugar, maize, soya product, apple jelly, soy sauce, soft drinks | Barnett et al. (2000); James and Stratford (2011) |
| ***Z. sapae*** | Traditional balsamic vinegar, Italy | Solieri et al. (2013) |
| ***Z. seidelii*** | Flowers, Maldives | Brysch-Herzberg et al. (2020) |
| ***Z. siamensis*** | Honey, Thailand | Sakhincai et al., (2012) |

*Species not treated by James and Stratford (2011) are marked with bold characters.

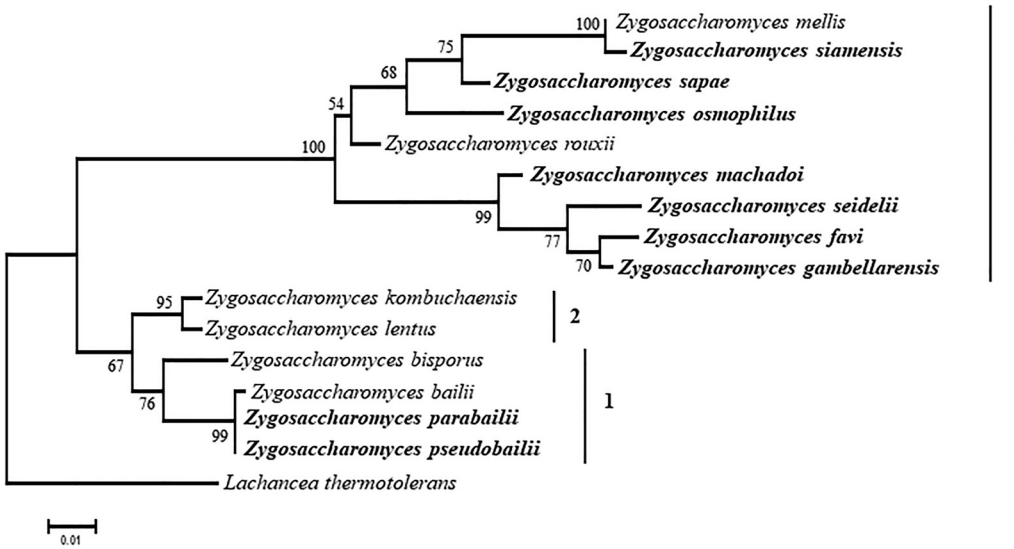


*Fig. 1.* Phylogenetic relationships among the currently recognised *Zygosaccharomyces* species determined from analysis of LSU rRNA gene D1/D2 domain. Bar, 1% nucleotide sequence divergence. Evolutionary analysis was conducted in MEGA6 (Tamura et al., 2013)

*Z. bailii* (Table 1), and it may be assumed that they possess similar food spoilage potential as *Z. bailii*. Indeed, one of the investigated *Z. parabailii* strains was a causative agent of spoilage outbreak of salad dressing in the USA (Suh et al., 2013). *Z. bailii, Z. parabailii*, and *Z. pseudobaillii* are not clearly distinguished from one another based on conventional physiological tests (Suh et al., 2013) and may be referred to as the *Z. bailii* species complex (Braun-Galleani et al., 2018). Analyses of genome sequences revealed that *Z. parabailii* is a hybrid of *Z. bailii* and a yet unknown *Zygosaccharomyces* species with approximately 93% overall genome sequence identity to *Z. bailii* (Ortiz-Merino et al., 2017). Similarly, genome sequence comparisons unveiled that *Z. pseudobaillii* is a hybrid of *Z. bailii* and an other unidentified *Zygosaccharomyces* species (Braun-Galleani et al., 2018).

## 3.2. The expanding group of extremely osmotolerant *Zygosaccharomyces* species

The group of extremely osmotolerant *Zygosaccharomyces* species delimited by *Z. mellis* and *Zygosaccharomyces gambellarensis* in Fig. 1 forms a subclade within the genus with 100% statistical support. Recently added species to this group are introduced in chronological order of their description.

*Zygosaccharomyces machadoi* has been proposed for a strain isolated from garbage pellets of the stingless bee *Tetragonisca angustula* in Brasil (Rosa and Lachance, 2005). The cell count of the species was in the order of $10^5$ CFU $g^{-1}$ pellet suggesting that the species is metabolically active in this substrate and that it may be an agent of spoilage in nests of stingless bees. The species, like *Z. rouxii* and *Z. mellis* grows in medium containing 50% glucose, but differs from them by its incapability of growing at the presence of 10% NaCl (Rosa and Lachance, 2005).

Given the similar physiological characters to *Z. rouxii* and *Z. mellis*, the occurrence of *Z. machadoi* in honey made by European honey bee (*Apis mellifera*) sympatric to the stingless bee *T. angustula* would not be unexpected.

During the course of a study in Thailand, 186 yeast strains were isolated from 37 honey samples of 12 different bee species. Among the isolated strains, 6 proved to represent an undescribed *Zygosaccharomyces* species closely related to *Z. mellis*. For the placement of the 6 strains, 4 of which originated from honey of European honey bee, a novel species *Zygosaccharomyces siamensis* was introduced (Sakhincai et al., 2012). It has been recognised for a long time that *Z. mellis* consists of two genetically divergent subpopulations (Kurtzman 1990; Suezawa et al., 2008). Sakhincai et al. (2012) also noted that the so called $\beta$-subpopulation of *Z. mellis* (Suezawa et al., 2008) belongs to *Z. siamensis*. As the yeast count in honey ranged from $10^2$ to $10^4$ CFU ml$^{-1}$, the authors came to the conclusion that *Z. siamensis* was metabolically active and able to grow in honey, which may be its normal environment, where, together with *Z. mellis*, it may be an agent of spoilage.

*Zygosaccharomyces gambellarensis*, the next member of the osmotolerant *Zygosaccharomyces* species, was recovered from a traditional sweet white wine produced in a small area of the Veneto region, Italy. The wine called Vin Santo of Gambellara is made from grapes, partially dried in attics for 5–6 months. During this drying process, saprophytic moulds, including *Botrytis cinerea,* grow on the grapes and contribute positively to the characteristics of the wine. At the beginning, osmotolerant non-*Saccharomyces* yeasts are commonly found in the fermenting must, which are gradually replaced by *Saccharomyces* species (Torriani et al., 2011). In a study of indigenous eukaryotic microbiota of Vin Santo of Gambellara, 25 isolates originating from different wineries proved to belong to an undescribed *Zygosaccharomyces* species. Following detailed physiological and molecular characterisation of 3 selected strains, Z. *gambellarensis* was proposed to accommodate the novel species, but no possible contribution to the chemical and sensory characteristics of the wine was mentioned (Torriani et al., 2011).

*Zygosaccharomyces sapae* related to *Z. rouxii* and *Z. mellis* was described for some halo- and osmotolerant yeast strains associated with the alcoholic fermentation of traditional balsamic vinegar, a condiment produced in some northern provinces of Italy (Solieri et al., 2013). *Z. sapae* shows unusually high, more than 20%, intragenomic ITS variability (Solieri et al., 2007) and might be a hybrid (Solieri et al., 2013). Several sugar-tolerant *Zygosaccharomyces* species contribute to the alcoholic fermentation step of traditional balsamic vinegar production (Solieri and Giudici, 2008), and *Z. sapae* may be involved as well.

Although the group of *Zygosaccharomyces* species delimited by *Z. mellis* and *Z. gambellarensis* in Fig. 1 are osmotolerant, all but one species can also grow in high water activity environment. To the contrary, *Zygosaccharomyces favi* recovered from bee bread and honey in Hungary has an absolute requirement for non-ionic solutes and is unable to grow in/on high water activity culture media (Čadež et al., 2015), therefore qualifies itself as an osmophilic yeast species according to the definition of Dakal et al. (2014). *Zygosaccharomyces favi* has been assumed to have spoilage potential in high-sugar food products (Čadež et al., 2015).

Very recently, *Zygosaccharomyces seidelii* was described based on a single strain isolated from flowers collected on the Maldives (Brysch-Herzberg et al., 2020). Although flowers are associated with honey, the relevance of *Z. seidelii* to foods remains to be determined.

Finally, *Kluyveromyces osmophilus* was transferred to the genus *Zygosaccharomyces* as *Zygosaccharomyces osmophilus* (Matos et al., 2020). *K. osmophilus* was described by Kreger-van Rij (1966)

based on a single strain from sugar of Mauritius. James et al. (2005) raised the possibility that actually it represents a novel *Zygosaccharomyces* species. Several additional conspecific strains were isolated from honey and larval food of bees in Brazil. The phylogenetic position of *K. osmophilus* was determined by DNA barcode sequence and phylogenomic analyses. As a result, *K. osmophilus* was reassigned as *Z. osmophilus* (Matos et al., 2020). Although, according to the above cited definition of Dakal et al. (2014), *Z. osmophilus* is not osmophilic, its association with high-sugar foods is obvious.

## 4. CONCLUSIONS

DNA sequence based yeast identification has aided the discovery and description of numerous novel yeast species in the last two decades. The significant food associated genus *Zygosaccharomyces* has considerably been expanded as well, and the trend may be anticipated to continue in the future. Accumulating data will shed light to the particular impact of the newly described species on the quality of the foods of their origin.

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