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Zygosaccharomyces seidelii sp. nov. a new yeast species from the Maldives, and a revisit of the single-strain species debate

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Abstract *Zygosaccharomyces seidelii,* a new species in the genus *Zygosaccharomyces* is described. The description of the species is based on a single strain that was isolated from flowers collected on the Maldives. On this occasion, the description of yeast species from single strains was revisited. Sequence analysis of the D1/D2 domain of the nuclear large subunit rRNA gene revealed that *Z. seidelii* is closely related to *Z. gambellarensis.* Both species differ by 2.6% (one indel of 7 bp and 9 substitutions) in the D1/D2 domain, 71 substitutions and 23 indels in the ITS1-5.8S-ITS2 (ITS) region and by several physiological tests. Two divergent copies of the ITS region were detected in *Z. seidelii*. Asexual and sexual reproduction as well as the physiological properties of *Z. seidelii* fit well in the genus *Zygosaccharomyces*. (Holotype strain: CBS 16021, Isotype strain: CLIB 3343; MycoBank no.: MB830900).

Keywords Zygosaccharomyces seidelii · Divergent copies ITS-region · Single-strain species

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Introduction

The genus *Zygosaccharomyces* Barker (1901) is characterised by conjugation prior to ascus formation (James and Stratford 2011). This indicates a haplontic life cycle, which means that the diploid phase is restricted to the zygote. Nevertheless, it was shown that some strains are diploid (Solieri et al. 2013a). Asexual reproduction is by multilateral budding. True hyphae do not occur. Physiologically, the genus is characterised by the fermentation of glucose and the inability to ferment galactose and to assimilate nitrate. (James and Stratford 2011).

In the latest edition of *The yeast, a Taxonomic Study* James and Stratford (2011) accepted 6 species: *Z. bailii* (Lindner) Guilliermond (1912), *Z. bisporus* Naganishi (1917), *Z. kombuchaensis* Kurtzman et al. (2001), *Z. lentus* Steels et al. (1999), *Z. mellis* Fabian and Quinet (1928) and *Z. rouxii* (Boutroux) Yarrow (Arx et al. 1977). *Z. machadoi* Rosa and Lachance (2005) was described before the book was published but too late for inclusion in the book. After the last edition was published *Z. gambellarensis* Torriani et al. (2011), *Z. siamensis* Saksinchai et al. (2012), *Z. sapae* Solieri et al. (2013b), *Z. parabailii,* and *Z. pseudobailii* Suh et al. (2013), as well as *Z. favi* (Čadež et al. 2015) were described. It is noteworthy that in *Z. rouxii* (Sujaya et al. 2003), *Z. sapae* (Solieri et al. 2007), *Z. favi* and *Z. gambellarensis* (Čadež et al. 2015) divergent copies of the ITS region were reported.

Strains of the genus *Zygosaccharomyces* have been isolated from many different natural habitats. Some species such as *Z. rouxii* are extremely osmotolerant and have been isolated from honey bee honey (Carvalho et al. 2010) and from the food provisions of other bee species (Brysch-Herzberg 2004; Spencer et al. 1970). The high osmotolerance makes these species important spoilage organisms of foods with low water activity (Deak 2008). The ecological relevance of osmotolerance in the genus was underlined by the description of the true osmophilic species *Z. favi* (Čadež et al. 2015), which was isolated from honey and other bee hive material. Other species like *Z. bailii* show high tolerance to weak organic acids e.g. acetic acid, benzoic acid or sorbic acid which may lead to issues in the preservation of some foods and beverages (Deak 2008).

The initial aim of the current study was to gain new strains of *Schizosaccharomyces octosporus* in order to evaluate their oenological properties. One strain that was isolated and characterised phenotypically and by rDNA sequence analysis belonged to the genus *Zygosaccharomyces*. The strain could not be assigned to any of the already described species within the genus because of its genetic distance and phenotypic differences compared to the currently accepted species. Therefore, a new species is described.

In honor of Martin Seidel, scientist and lecturer at the Heilbronn University of Applied Sciences (Germany) the species is named *Zygosaccharomyces seidelii*. Martin Seidel has contributed to the understanding of the distribution of *Saccharomyces* and *Schizosaccharomyces* species in the natural landscape in Germany.

Materials and methods

Isolation procedure

The flowers examined in the current study were collected from oleander (*Nerium oleander*) and other undetermined shrub species growing directly at the beach (coordinates: 5° 31′ 12.9″ N 73° 26′ 30.6″ E) in a touristic holiday housing area on the Maldives.

The description is based on a single strain that was isolated from flowers originating from the Maldives. Unfortunately, it was neither possible for the author to visit the natural habitat from which the type strain originates, in order to gain additional strains of the same type nor was it possible to receive additional material from the Maldives. Various attempts to isolate more strains of the new species from similar or related substrates such as flowers, honey, bee-bread and different bees by the same isolation technique failed for several years in Germany (data not shown).

Flowers were collected aseptically in polyethylene bags, stored cold and brought to the laboratory within 48 h. Five samples consisting of about 10 flowers each were processed. An enrichment step in the 60% sugar medium described here was performed because *S. octosporus* is a highly osmotolerant yeast species (Deak 2008; Phaff et al. 1978; Skinner et al. 1980) and because the authors have used the same medium successfully for the isolation of *S. octosporus* from raisins (data not shown).

The enrichment medium consisted of 0.5% (w/w) peptone, 0.5% (w/w) yeast extract, 5% (w/w) glucose and 55% (w/w) fructose. The authors have experienced in earlier enrichment trials for which a medium consisting of 60% glucose as the sole sugar was used that glucose easily crystallizes when it gets in contact with the sample material. In the current study fructose was used as the main sugar because its solubility is much higher and in aqueous solution it causes about the same reduction of water activity as glucose (Tokuoka and Ishitani 1991).

Glucose and fructose were dissolved in water and autoclaved separately from the peptone/yeast extract solution. After autoclaving at 115 °C for 15 min both solutions were mixed under sterile conditions. Whole flowers with corolla and calyx leaves were placed in 150 ml of the enrichment medium in 200 ml Erlenmeyer flasks and overlaid with 1 cm of rapeseed oil to prevent growth of osmotolerant moulds. The flasks were incubated at 25 °C up to four weeks. If gas was formed in the medium a drop of medium was streaked on 45% (w/w) fructose agar (1.5%) containing glucose, peptone and yeast extract as given above. Each colony type was repeatedly streaked on a medium with gradually lower osmotic pressure as recommended before (Phaff and Starmer 1980) because sensitive strains could get lost otherwise (Brysch-Herzberg et al. 2019; Phaff and Starmer 1980) The concentration was lowered in steps of 10% (w/w) until a total sugar concentration of 2% (w/v) was reached.

Phenotypic and molecular genetic characterisation

The strains isolated in this study were phenotypically characterised by the standard methods (Kurtzman et al. 2011). In order to prevent cross reactions between strains in one petri dish, twelve well plates were prepared with agar medium. One strain was inoculated per well by means of a multiple point inoculator for the assimilation tests. The fermentation tests were performed in liquid media with the aid of Durham tubes.

Amplification, sequencing and sequence analysis of the D1/D2 domain of the nuclear large subunit rRNA gene and of the ITS1-5,8S-ITS2 region (ITS region) was done as described before (Brysch-Herzberg and Seidel 2015) with primers NL1 and NL4 (Kurtzman and Robnett 1998). Direct sequencing of the ITS region with the primer pair ITS1-ITS4 (White et al. 1990) and with the alternative primer pair ITSF and NL1rev (Nguyen and Boekhout 2017) resulted in superimposed signals after about 415 bp or 106 bp depending on the sequencing primer used. In both sequencing directions the superimposed signals started after a homopolymer A/T repeat indicating that copies of the ITS region exist that differ in the length of the A/T repeat. In order to reconfirm these results, the ITS region was amplified with Q5 proof-reading polymerase (New England Biolabs GmbH) and cloned into the pJET1.2/blunt vector (CloneJET™ PCR-Cloning Kit, Thermo Scientific™) following the manufacturer's instructions. Five clones were sequenced. Sequences were determined by StarSEQ GmbH, Mainz, Germany. Phylogenetic trees were calculated with the maximum likelihood method and the Jukes Cantor nucleotide substitution model. Bootstrap values were calculated from 1000 iterations.

In order to detect more strains of *Z. seidelii* sp. nov. that might have been isolated by other researchers, a Blast search against GenBank (NCBI, USA) with the sequences of the D1/D2 domain and the ITS region was performed as well as a search at the CBS database (Westerdijk Fungal Biodiversity Institute, The Netherlands).

Results and discussion

To obtain new *S. octosporus* strains to investigate the oenological properties of strains from different geographical origins, yeasts were isolated by means of a highly osmotic medium from flower samples from the Maldives. One of the isolated strains belongs to the genus *Zygosaccharomyces* judged from molecular genetic data, its way of sexual reproduction, and its physiological characteristics. The molecular genetic data and the physiological characteristics of the strain suggested that it represents a separate yet undescribed species. Thus, the new species *Zygosaccharomyces seidelii* sp. nov. is proposed here.

Isolation of yeasts

Beside the strain described here as *Z. seidelii* sp. nov., species of the genus *Starmerella* (Santos et al. 2018)-like *St. bombi*, *St. etchellsii*, *St. orientalis*, and *St. caucasica* were isolated. Because an enrichment step with a high osmotic medium was applied, it seems almost sure that other yeast species with lower tolerance to osmotic stress have been excluded. Additionally, the number of samples examined was very low. Thus, it was not possible to describe the whole yeast community in flowers on the Maldives.

Phenotypic characterisation

It needs to be stressed that the characterization is based on a single strain. For this reason, no estimate about the phenotypic and molecular genetic variability within the species can be provided.

Under the microscope *Z. seidelii* sp. nov. resembles other species of the genus. Asexual cells are globose or ovoid measuring 4–8 µm × 4–8 µm. Asexual growth is by multilateral budding (Fig. 1a). Pseudohyphae or true hyphae were not observed. Formation of asci was observed after 2 weeks at 25 °C on GPY (Glucose-pepton-yeast extract) agar (Kurtzman et al. 2011) in pure culture. Asci formation is preceded by conjugation of a cell and its bud (Fig. 1b, c). The asci contain two globose ascospores (3–4 µm) (Fig. 1d). The fact that conjugation always preceded ascus formation and asci formation took place in pure culture suggests that the strain is haploid and homothallic. The way of sexual reproduction can be interpreted in a way that the diploid phase is reduced to the zygote, which is typical for the genus *Zygosaccharomyces*.

The inability to utilize nitrate as the sole nitrogen source and the inability to ferment galactose is typical for the genus *Zygosaccharomyces* (James and

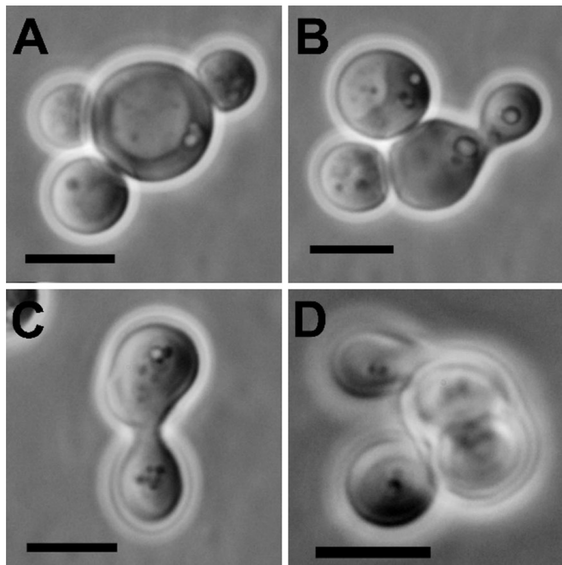


Fig. 1 Micromorphology of *Zygosaccharomyces seidelii* sp. nov.; **a–b** multilateral budding after 24 h at 25 °C on GPY Agar; **b–c** conjugation between a cell and its bud; D: ascus with two ascospores; (**b–d** 2 weeks at 25 °C on GPY Agar). The bar indicates 5 μm

Stratford 2011). Compared to *Z. gambellarensis* (Torriani et al. 2011) and *Z. favi* (Čadež et al. 2015) which are the species closest related to *Z. seidelii* sp. nov. based on the results of the molecular genetic analysis, *Z. seidelii* sp. nov. differs from the two species in its ability to assimilate methyl-α-D-glucoside, cellobiose, melibiose, lactose, raffinose, melezitose, *N*-acetyl-D-glucosamin and its inability to assimilate sorbitol and ribitol. Beside those characteristics that differentiate *Z. seidelii* sp. nov. from both species other characteristics differentiate *Z. seidelii* sp. nov. from one or the other species. A summary of all differentiating physiological test results is given in Table 1.

Molecular genetic characterisation

In *Z. seidelii* sp. nov. the D1/D2 domain of the nuclear large subunit rRNA gene is 580 bp long (MK393753). Because GenBank included two substantially different D1/D2 sequences for the type strain of *Z. gambellarensis,* the ex-type MUCL 53393T was sequenced for this region at BCCM/MUCL and by the authors (JN874489; unpublished). The obtained sequence was used in the current study for comparison and the phylogenetic analysis. The D1/D2 domain of *Z. seidelii* sp. nov. differs by 2.8% from that of *Z. gambellarensis,* the most similar species judged from pairwise D1/D2 domain sequence comparison. In the calculation 9 SNPs and a 7 bp long indel are taken into account. If the difference in length is not considered, the D1/D2 domains differ by 1.6%. From their sequence analysis of ascomycetous yeast species Kurtzman and Robnett (1998) concluded that strains exhibiting more than 1% substitutions supposedly represent different species and that strains showing less than 0.5% substitutions belong to the same species or to a sister species. This assessment was later reconfirmed by Vu et al. (2016) who did not analyse the D1/D2 domain sequences of type strains only, but of several strains per species if they were available. The distance of 2.8% between *Z. gambellarensis* and the new strain strongly indicates that it represents a new yet undescribed species. In the phylogenetic analysis based on the D1/D2 domain *Z. seidelii* sp. nov. is placed in a clade together with *Z. gambellarensis*, *Z. favi* and *Z. machadoi*. The bootstrap support of this clade is 100%. The results of the analysis are illustrated in Fig. 2.

Direct sequencing of the ITS region was not possible as it always resulted in superimposed signals after approximately 415 bp or 106 bp depending on the sequencing direction. Cloning the ITS region in a common vector revealed two divergent copy types in the genome (MK791270, MK791271). The copies differ by one indel located in a 14 or 15 bp long homopolymer A/T repeat located in the ITS1 region. The position of the variable site corresponds well with the results of the attempts to directly sequence the PCR product. The superimposed signals retrieved from direct sequencing started at the same position at which the divergent length of the homopolymer A/T repeat was detected by cloning analysis.

Because of this and because a proof reading DNA-polymerase was used for amplification of the ITS region prior to cloning it can be excluded that the difference between the copies of the ITS region is an artefact.

Divergent copies of the ITS region were described before in *Z. sapae* (Solieri et al. 2013b), *Z. gambellarensis* and *Z. favi* (Čadež et al. 2015). Compared to the ITS region of *Z. gambellarensis* the ITS region of *Z. seidelii* differs by 71 substitutions and 23 indels. The results of the phylogenetic analysis are displayed

Table 1 Growth characteristics differentiating *Zygosaccharomyces seidelii* sp. nov. from the most closely related species *Z. favi* and *Z. gambellarensis*. Data for *Z. favi* and *Z. gambellarensis* are taken from the original species descriptions (Čadež et al. 2015; Torriani et al. 2011)

Assimilation of	*Z. seidelii*	*Z. favi*	*Z. gambellarensis*
Maltose	dw	−	+
Sucrose	dw	−	+
α, α-trehalose	w	−	+
Methyl α-D-glucoside	w	−	−
Cellobiose	w	−	−
Salicin	dw	−	n
Arbutin	dw	−	n
Melibiose	dw	−	−
Lactose	w	−	−
Raffinose	dw	−	−
Melezitose	dw	−	−
Inulin	w	−	n
N-acetyl-D-glucosamin	w	−	−
Xylose	−	−	+
Ethanol	−	s, sw	−
Xylitol	−	s, w, sv	−
Sorbitol	−−	v	+
ribitol/Adonitol	−	w, sv	+
D-mannitol	+	+, s	−
D-gluconate	−	+, s	−
Ethylamin	+	+	n
Cadaverin	+	+	n
Creatinine	dw	−	n
50% glucose	+	+	n
60% glucose	+	+	n
16% NaCl	−	+	n

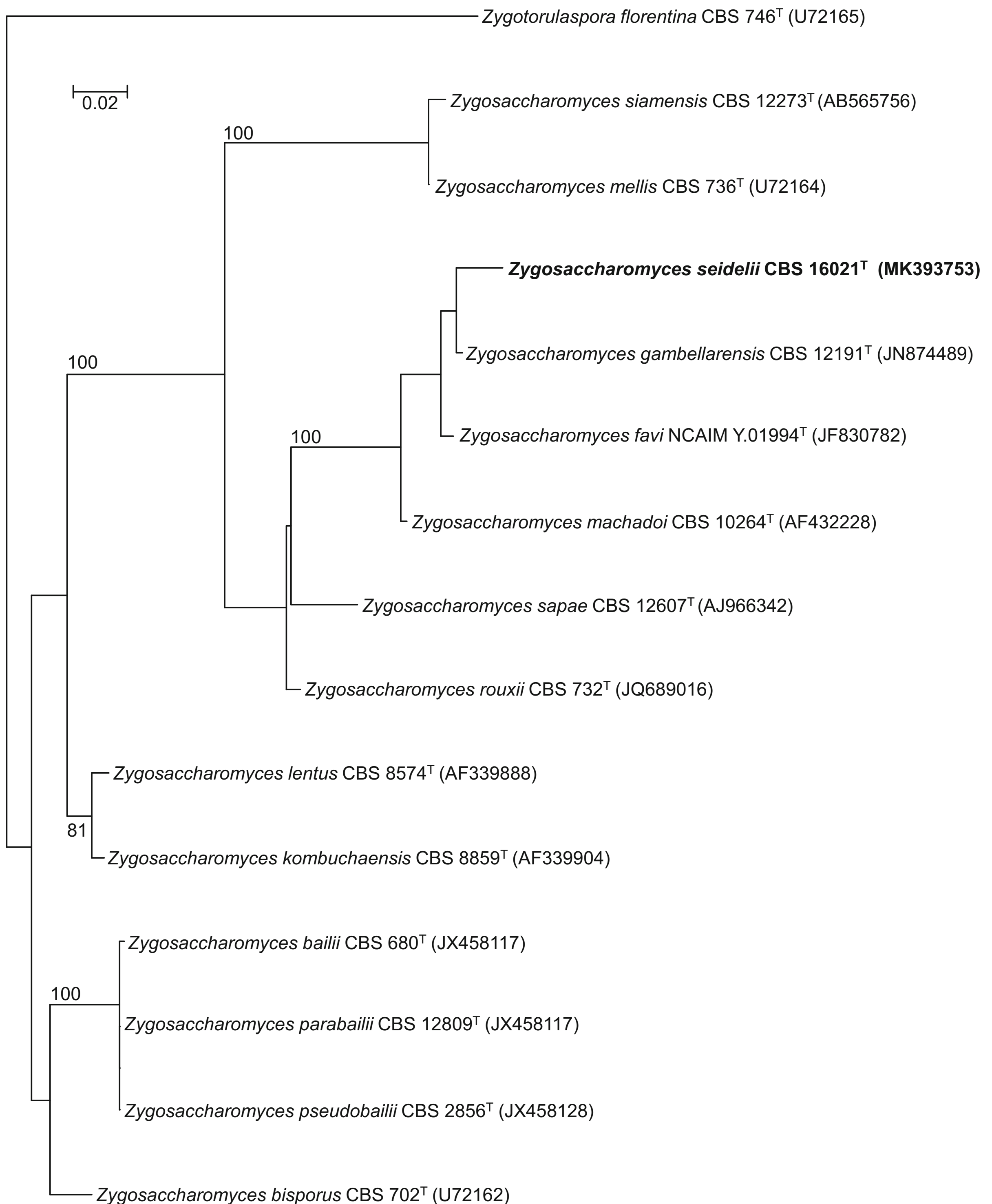
+ = positiv; − = negative; d = delayed; w = weak; v = variable; n = no data

in Fig. 3. *Z. seidelii* is placed at a very basal position in a clade with a rather low bootstrap support, which indicates that its ITS region is not closely related to the ITS regions of the other species. The substantial differences in the ITS region compared to other species in the genus support the description of *Z. seidelii* sp. nov.

The species is well separated by molecular genetic data from the already described species in the genus.

Pros and cons of species described based on a single strain

The characterization of *Z. seidelii* sp. nov. is based on one strain only, because it was neither possible for the authors to isolate additional material nor did a Blast search against GenBank and the CBS database reveal any strains that matched *Z. seidelii* sp. nov.. Oppositional views have been expressed concerning species descriptions based on single isolates (Fell et al. 2000). The main arguments that have been put forward against single strain species descriptions are that neither the genetic nor the phenotypic variability can be estimated and thus, proper delineation of a new species based on a single strain from its sister species is not possible. Additionally, based on a single strain a species description can not include any meaningful data on the ecology of a yeast species (Lachance 2011a). If the coverage of the genetic and phenotypic variability as well as serious data on the ecology of a species would be a prerequisite for the description of a new species hardly any new species would be described and the vast majority of species descriptions that have been published so far would not have been possible. In this context the question arises if the often



requested minimum of five strains (Christensen et al. 2001; Janda and Abbott 2002) would improve the information provided with the original species description substantially. It is beyond question that

◀**Fig. 2** Phylogenetic tree showing the position of *Zygosaccharomyces seidelii* within the genus *Zygosaccharomyces*. The tree is based on the D1/D2 domain of the nuclear large subunit (LSU) rRNA gene and obtained by maximum likelihood analysis. Percentage bootstrap values of 1000 replicates are given at the nodes. Only values above 80 percent are displayed. GenBank accession numbers are indicated after strain designation. *Zygotorulaspora florentina* was used as an outgroup species. The bar indicates 20% nucleotide sequence divergence

each additional strain is valuable since it may contribute to a better understanding of a new species but in order to quantify the intraspecies variability and to provide meaningful information on the ecology of a species rather hundreds of isolations are needed from different substrates and different geographical origins.

Because species consist of one or in most cases of numerous populations, a single strain is always less suited to represent a species than a large set of strains of different geographic origins and from different habitats (Fell et al. 2000; Kurtzman 2010; Lachance 2011a). While nobody would like to argue against this view there is no consensus about what to do with single strains that undoubtedly represent a new species. It has been argued that single strains that cannot be assigned to an already described species should be deposited in a culture collection and its barcoding sequences be deposited in GenBank. In case additional strains of that type are isolated later by others a joined species description could be published (Fell et al. 2000; Lachance 2011a).

It was estimated that the original species descriptions of about one-third of the currently described species were based on single strains (Kurtzman 2010), which means that these species descriptions have greatly contributed to our understanding of yeast diversity and will also do so in the future.

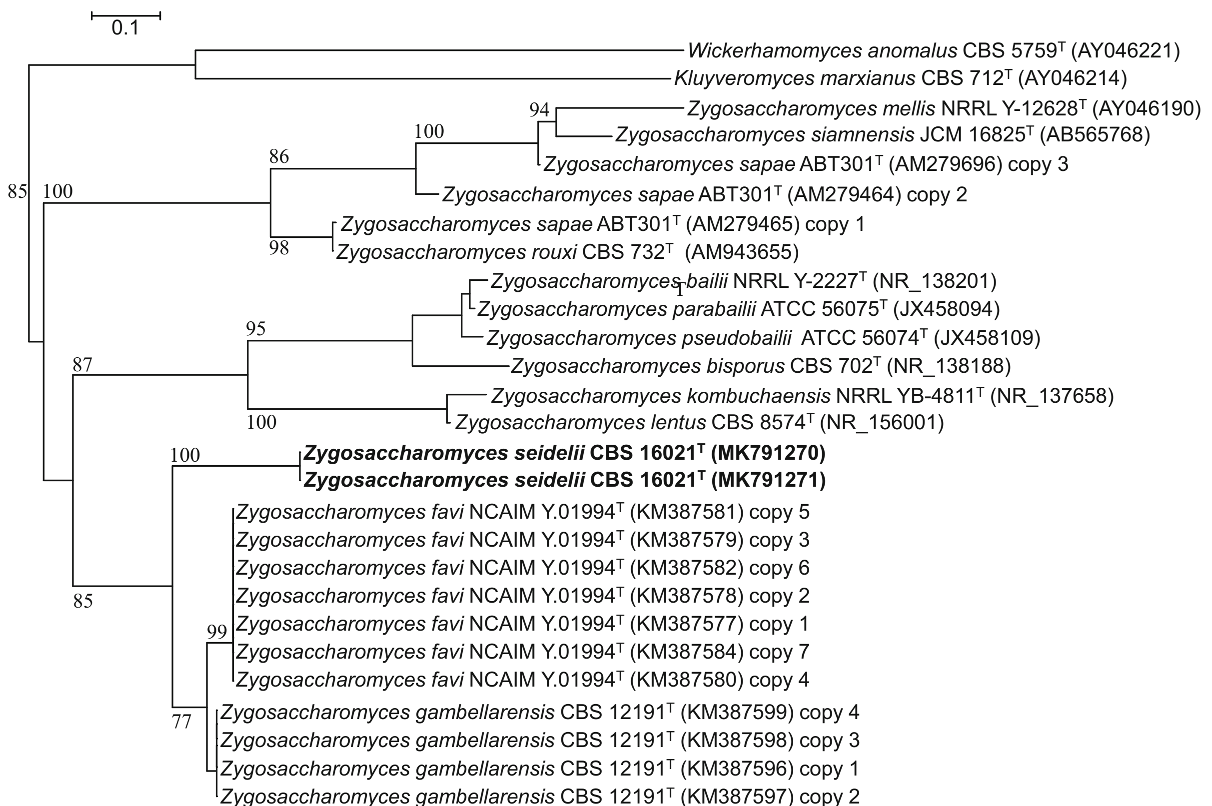


Fig. 3 Phylogenetic tree showing the position of *Zygosaccharomyces seidelii* sp. nov. within the genus *Zygosaccharomyces*. The tree is based on partial ITS regions and obtained by maximum likelihood analysis. Percentage bootstrap values of 1000 replicates are given at the nodes. Only values above 80 percent are displayed. GenBank accession numbers are indicated after strain designation. *Wickerhamomyces anomalus* and *Kluyveromyces marxianus* were used as an outgroup species. The bar indicates 10% nucleotide sequence divergence

Although species descriptions based on single strains are not ideal in many ways it should not be overlooked that they often served as a starting point from which the knowledge about the newly described species evolved and facilitated the work of later authors. A typical example for such a gradual increase in knowledge is the case of *Metschnikowia viticola*. The original species description was based on two strains which were derived from the same grape sample and therefore had a high chance to be clonal (Peter et al. 2005). Because of the low number of strains the authors were not able to draw any conclusion concerning the ecology of the new species. Later, others isolated *M. viticola* from grapes and grape related samples in Austria (Lopandic et al. 2008), the Slovak Republic (Brezna et al. 2010), Italy (Bovo et al. 2009) and Dagestan (Kachalkin et al. 2015), from flowers in Korea (Hyun et al. 2014), and Egypt (Moubasher et al. 2018), from plant material in Israel (Abu-Ghosh et al. 2014), spring water in Portugal (Pereira et al. 2013), and the intestines of wild salmonids in Chile (Raggi et al. 2014). The original species description made it easy for the later authors to assign the newly isolated strains to the species and to compare their results with those obtained by the others. After the description of a new species, additional strains are often identified by others also in different habitats within short time, although the case of *Metschnikowia lunata* demonstrates this is not always the case. After the original species description, which was based on a single strain (Golubev 1977) only one additional strain was assigned to *M. lunata* (Lachance 2011b). However, one could argue that single strain species descriptions often facilitate and accelerate research in yeast biodiversity and yeast ecology no matter on how many strains they were based.

Most likely the deposition of barcode sequences in public databases like GenBank and the deposition of the strains in culture collections instead of a formal species description would not have a similar effect. It is much more effective to assign strains to formally described species than dealing with a diffuse group of strains of an uncertain taxonomic status, which were isolated by many different researchers. In this context it should be considered that a common name is much more than just a label that can be put on a yeast isolate. The great advantage is that all information on phenotypic characteristics, molecular genetic data, ecological data, economical application etc. can be connected with a single epithet, no matter which strain was used in a certain investigation.

Even if no further strains of a new species are isolated in the future, the formal species description will make the species visible to the scientific community and researchers of other fields than taxonomy are stimulated to use the strain for their purposes. *Schizosaccharomyces cryophilus* can serve as an example here. The description of *S. cryophilus* was based on just one strain (Helston et al. 2010). After the description the type strain of *S. cryophilus* was an important part of other investigations (e.g. Rhind et al. 2011; Zhu and Niu 2013).

For the reasons given above the authors are of the opinion that the description of *Z. seidelii* based on a single strain is justified.

Description of *Zygosaccharomyces seidelii* Brysch-Herzberg sp. nov

Zygosaccharomyces seidelii (sei.de´li.i. N.L. gen. masc. n. *seidelii* of Seidel, in honour of biologist Martin Seidel, in recognition of his contributions to the ecology of yeasts).

After 7 days on GPY agar cells are globose to ovoid. Budding is multilateral. No pseudomycelium or mycelium is formed. Ascospores are observed after 14 days on GPY agar at 25 °C. Ascus formation usually is preceded by conjugation between a cell and its bud. Ascospores are globose. On GPY agar after 7 days colonies are cream coloured, convex with an entire margin.

Glucose fermentation is delayed. Galactose, sucrose, maltose, trehalose, me-α-D-glucoside, cellobiose, mellibiose, lactose, raffinose, melezitose, inulin, and soluble starch are not fermented.

Glucose, galactose (weak), sucrose, maltose (delayed, weak), trehalose (weak), methyl-α-D-glucoside (weak), cellobiose (weak), salicin (delayed, weak), arbutin (delayed, weak), melibiose (delayed, weak), lactose (weak), raffinose (delayed, weak), melezitose (delayed, weak), inulin (weak), glycerol (weak), mannitol, palatinose (delayed, weak), *N*-acetyl-D-glucosamin (weak), ethylamine, cadaverin, and creatinine (delayed, weak) are assimilated.

Sorbose, glucosamin, ribose, xylose, L-arabinose, D-arabinose, ramnose, soluble starch, erythritol,

ribitol, xylitol, arabitol, sorbitol, gluconic acid sodium salt, glucuronate, DL-lactate, succinate, citrate, methanol, ethanol, propane-1,2-diol, 2,3-butanediol, quinic acid, saccharic acid, nitrate and nitrite are not assimilated. No growth was observed on 0.001% cycloheximide, 1% acetic acid and 16% NaCl. Growth occurred on 10% NaCl and 60% glucose. Growth was observed at 30 °C but not at 37 °C.

Holotype: CBS 16021^T; Isotype: CLIB 3343; both metabolically inactive. Mycobank number: MB830900. The type culture was isolated from flowers originating from the Maldives.

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Complaince with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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