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RESEARCH ARTICLE

Schizosaccharomyces osmophilus sp. nov., an osmophilic fission yeast occurring in bee bread of different solitary bee species

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One sentence summary: Schizosaccharomyces osmophilus sp. nov., a true osmophilic yeast that is associated with solitary bees is described.

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ABSTRACT

Eight yeast strains that asexually reproduce by cell fission were isolated from bee bread of different solitary bees in Germany. DNA sequence analysis revealed that the strains shared the same sequence in the D1/D2 domain of the nuclear large subunit (LSU) rRNA gene with a strain that was previously isolated from a fig snack from Spain. The closest related type strain was that of *Schizosaccharomyces octosporus*, which showed 98.2% sequence similarity (11 substitutions) with the new strains. By clone sequence analysis of the internal transcribed spacer (ITS) region (ITS1, 5.8S rDNA, and ITS2) a total of nine different copy types were identified. The new strains differed from *S. octosporus* by approximately 31% in the ITS region. Sequence analysis of the RNAse P gene further supported the description of a new species. The strains isolated during this study show some phenotypic characteristics that separate them from the closest related species, *S. octosporus* and *S. cryophilus*. Since all strains showed true osmophily the name of the new species is *S. osmophilus* (holotype: CBS 15793^{T}; isotype: CLIB 3267 ^{T} = NCAIM Y.02225 ^{T}, MycoBank no.: MB829586).

Keywords: Schizosaccharomyces osmophilus; beebread; solitary bees; osmophily

INTRODUCTION

In the genus *Schizosaccharomyces* cells reproduce asexually by fission and asci usually arise from conjugation of independent cells. Physiologically the genus is characterized by fermentation of different sugars, the inability to assimilate nitrate, a positive starch test in Wickerham's medium and negative Diazonium Blue B reaction. Either coenzyme Q9 or Q10 is formed (Vaughan-Martini and Martini 2011). In the latest edition of The Yeast, a Taxonomic Study, Vaughan-Martini and Martini (2011) accepted three species in the genus *Schizosaccharomyces*: *Schizosaccharomyces pombe* Lindner (1893), *S. octosporus* Beijerinck

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(1894) and *S. japonicus* Yukawa and Maki (1931). Shortly before the book was published but too late for inclusion in this edition a new species, *S. cryophilus* Helston *et al.* (2010) was described. The authors showed that the species is closely related to *S. octosporus*.

There is a long standing and ongoing discourse about species and variety delimitation and phylogeny in the genus *Schizosaccharomyces*. Since the description of *S. pombe* by Lindner (1893) a number of *Schizosaccharomyces* species have been described which are presently considered as synonyms of the four currently accepted species (Vaughan-Martini and Martini 2011) and proposals have been made to split the heterogeneous genus (Kudrjawzew 1960; Yamada and Banno 1987).

Synonyms described in the past include but are not limited to *S. liquefaciens* Osterwalder (1924) and *S. malidevorans* Rankin and Fornachon (1964) for *S. pombe* Lindner (1893), *S. slooffiae* Kumbhojkar (1972) for *S. octosporus* Beijerinck (1894) and *S. versatilis* Wickerham and Duprat (1945) for *S. japonicus* Yukawa and Maki (1931). After Kudrjawzew (1960) proposed to transfer the *Schizosaccharomyces* species with eight ascospores per ascus in the new genus *Octosporomyces* the phylogenetic relationship of the fission yeasts species as well as species delimitation have been investigated by means of morphological, biochemical and physiological characteristics in connection with numerical classification (Bridge and May 1984; Kocková-Kratochvílová *et al.* 1985), scanning electron microscopy of the ascospores (Yamada and Banno 1987), by hybridization studies and/or protoplast fusion (Sipiczki 1979; Johannsen 1981; Sipiczki *et al.* 1982; Naumov *et al.* 2015), by proton magnetic resonance spectroscopy (Spencer and Gorin 1969), analysis of coenzyme Q (Yamada, Arimoto and Kondo 1973; Yamada and Banno 1987), low temperature cytochrome profiles (Sipiczki *et al.* 1982), analysis of cellular fatty acids (Kock and Van der Walt 1986; Viljoen, Kock and Lategan 1986; Yamada and Banno 1987), nDNA/nDNA reassociation (Martini 1991), karyotyping (Robinow 1981; Brown *et al.* 2011; Naumov, Kondratieva and Naumova 2015), mitochondrial genome analysis (Bullerwell *et al.* 2003), sequence analysis of the RNA subunit of RNAse P (Tranguch and Engelke 1993; Helston *et al.* 2010) and rDNA sequence analysis (Kurtzman and Robnett 1991; Kurtzman and Robnett 1998). In spite of major differences in coenzyme Q content, in the linoleic acid content, in low temperature cytochrome profiles found in the genus splitting of the heterogeneous genus has never been broadly accepted (Kurtzman and Robnett 1991; Martini 1991; Sipiczki 1995; Kurtzman and Robnett 1998; Sipiczki 2000; Vaughan-Martini and Martini 2011).

The currently accepted *Schizosaccharomyces* species are well separated by the results of hybridization studies, rDNA/rDNA reassociation studies and analysis of D1/D2 large subunit (LSU) rDNA gene sequences but despite the application of various approaches to reveal the relationships within the genus there is still no common view on the validity of varieties (Vaughan-Martini and Martini 2011) such as *S. pombe* var. *pombe* (Sipiczki *et al.* 1982) and *S. pombe* var. *malidevorans* Sipiczki *et al.* (1982) or *S. japonicus* var. *japonicus* Sloof (1970), *S. japonicus* var. *versatilis* Sloof (1970) and *S. japonicus* var. *longobardus* Delfini (1989) although it was demonstrated that these varieties exhibit a certain degree of reproductive isolation (Sipiczki 1979; Sipiczki 2000; Sipiczki et al. 1982).

The example of *S. pombe* illustrates that the various approaches that have been used to delimitate the species have not led to a fully satisfactory species concept in *Schizosaccharomyces*, yet. The global genomic population structure of *S. pombe* is weak (Jeffares *et al.* 2017), which means that local populations do not differ dramatically from each other in their genomic sequences, although the chromosomal structural variation was shown to be high in several investigations (Brown *et al.* 2011; Naumov, Kondratieva and Naumova 2015; Jeffares *et al.* 2017). In strain CBS 2777 even a fourth chromosome was detected (Brown *et al.* 2011). A high level of chromosomal rearrangements is broadly accepted as one key mechanism in speciation (White 1968; Rieseberg 2001; Lee *et al.* 2008; Presgraves 2010; Hou *et al.* 2014) and its importance for fission yeast has been demonstrated (Fischer *et al.* 2000; Dujon 2010; Avelar *et al.* 2013; Zanders *et al.* 2014; Naumov, Kondratieva and Naumova 2015; Jeffares *et al.* 2017). In this context it is notable that a large part of the strains examined by Naumov, Kondratieva and Naumova (2015), which showed hybrid infertility were type strains of *Schizosaccharomyces* species described in the past that are currently regarded as to be conspecific with *S. pombe*. Following the biological species concept, it could be argued that the strains should be accepted as separate species. On the other hand, it was shown in experimental studies and population genomics that structural rearrangements may be gained or lost at rates in excess of point mutations (Jeffares *et al.* 2017). Therefore, the significance of hybrid infertility in species delimitation will require further careful investigation. As already highlighted by Naumov, Kondratieva and Naumova (2015), it will be crucial to include a larger number of strains of each karyotype in order to assess the taxonomic relevance of the hybrid infertility and to conclude if the strains already represent sexually isolated populations of new species.

Taking all this together it gets apparent that a deep understanding of the speciation processes will be required in order to come to a true natural species concept for *Schizosaccharomyces* and that not even mating experiments alone may be suited to reveal if strains belong to a certain species or not. For this reason, for the description of new species all available information from molecular genetics, physiology, morphology and ecology need to be taken into account and a broad set of strains of closely related species from various geographical origins should be included.

The basis of the current study were attempts to isolate *S. octosporus* from bee bread of solitary bees in Germany in order to investigate the oenological properties of autochthonous *S. octosporus* strains. Bee bread was used as a substrate because the presence of *S. octosporus* in this material was reported earlier (Helston *et al.* 2010). Since *S. octosporus* is known as a highly osmotolerant yeast species (Phaff, Miller and Mrak 1978; Skinner, Passmore and Davenport 1980; Deak 2008) and because the same medium that is described here had been successfully used in order to isolate *S. octosporus* from raisins by the authors earlier (data not shown) an enrichment step with a 60% sugar medium was performed. From bee bread of different solitary bee species strains were isolated that resemble *S. octosporus* microscopically. DNA sequence analysis of the partial LSU rRNA (D1/D2 domain) gene revealed that the strains belong to the genus *Schizosaccharomyces* but because of a sequence divergence of more than 1% compared to any of the already described species it was suspected that the strains may represent a new yet undescribed species. A blast search against the GenBank database showed that a strain with an identical D1/D2 sequence was present in the Hungarian National Collection of Agricultural and Industrial Microorganisms. The strain had been isolated from a fig snack from Spain. It was included in the current study as well. All strains were characterized morphologically, physiologically and by DNA sequence analysis of the D1/D2 domain, the internal transcribed spacer (ITS) region and the RNA subunit of RNAse P. In addition to the strains of the undescribed species additional strains of the currently recognized four

Schizosaccharomyces species from different geographic origins were included in the present study in order to compare intraspecies and interspecies variation. An exception was *S. cryophilus* that could not be examined in this respect because according to the authors' knowledge only one strain is currently available. Based on phenotypic, molecular genetic and ecological data a new species is described. As all strains of the proposed species that were investigated clearly grew better on high osmotic growth medium than on standard medium (GPY) containing 2% glucose the new species was named as *Schizosaccharomyces osmophilus*.

MATERIALS AND METHODS

Strains and isolation procedure

Strains included in this study are listed in Table 1. Beside the type strains of the already described *Schizosaccharomyces* species additional strains from the National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary, from the Westerdijk Fungal Biodiversity Institute (CBS), Utrecht, The Netherlands, from the VTT Technical Research Centre of Finland Ltd, Finland and the strain collection of the Laboratory for Wine Microbiology, University of Applied Science Heilbronn, Germany, were included in the study.

Most strains of *S. osmophilus* were isolated from fresh bee bread of different solitary bee species using an enrichment medium containing 0.5% (w/w) peptone, 0.5% (w/w) yeast extract, 5% (w/w) glucose and 55% (w/w) fructose. For preparation of the medium, 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. In April to June 2017 nesting tubes of solitary bees were taken to the lab, opened under aseptic conditions and a volume of 200–400 µl bee bread was added into 50 ml screw cap tubes filled with 30 ml of the enrichment medium. A total of 20 samples were examined. In order to prevent growth of osmotolerant molds the medium was overlaid with about 10 mm rapeseed oil. The tubes were incubated at 25°C up to four weeks. If gas formation was observed in the medium it was microscopically checked for fission yeast cells. If those were present a drop of enrichment medium was streaked on 45% (w/w) fructose agar containing glucose, peptone and yeast extract as given above. The medium was solidified by the addition of 1.5% (w/w) agar. Colonies that developed were examined microscopically. In case if cell fission was observed the colonies were further cultivated and purified by streaking. With each inoculation step the fructose concentration was lowered in steps of 10% (w/w) until a total sugar concentration of 20–30% was reached.

Phenotypic characterization

All strains were characterized by replica plating following the standard methods of Kurtzman *et al.* (2011). In order to prevent cross reactions between strains in one petri dish 12 well plates were used for replica plating. One strain was inoculated per well. As the strains of the new species grew poorly or not at all on standard medium the osmotic pressure had to be increased in the basal test medium. To this end, different substances such as glycerol, mannitol, xylitol and sorbitol were added at concentrations in a wide range from 10% (w/w) to 60% (w/w). Because the strains were not able to assimilate sorbitol alone but did grow well when 2% glucose was added the authors chose sorbitol as the agent to elevate the osmotic pressure. For fermentation tests the liquid media were supplemented with 30% (w/w) sorbitol. As the tests on solid media gave inconsistent results when 30% (w/w) sorbitol was added to the medium, the test was repeated on media containing 50% (w/w) sorbitol, which significantly improved the results. Exceptions were the tests for tolerance of 10 and 16% NaCl as well as the tests for tolerance of 50 and 60% glucose. No additional solute was added in those cases. All strains not belonging to *S. osmophilus* were tested under standard conditions (Kurtzman *et al.* 2011). Thus, for all strains the physiological properties were tested under an osmotic pressure that was found to be favorable for the growth of the respective species. A similar approach was successfully used before by Čadež *et al.* (2015: 645–54) who described the true osmophilic species *Zygosaccharomyces favi*. Using the same solute concentration for each species would have meant that some of the strains would have been tested far from their physiological optimum. For example, *S. japonicus* is not able to grow at 50% glucose (Vaughan-Martini and Martini 2011; this study), which means that it cannot be judged as osmotolerant.

In order to determine the optimal solute concentration a series of GPY (2% glucose, 0.5% peptone, 0.5% yeast extract) media with various glycerol concentrations were prepared. The glycerol concentrations varied from 0 to 70% in steps of 5%. The pre-test culture medium contained 35% glycerol. All test media were inoculated simultaneously. After two weeks the intensity of growth was estimated in four groups reaching from weak to very strong.

In a second trial the cultures were repeatedly inoculated on a new medium containing less glycerol a few days after substantial growth was observed. With each inoculation step the glycerol concentration in the medium was lowered by 5%. Again, the pre-test culture medium contained 35%. The aim of this trial was to determine the lowest glycerol concentration at which the different strains showed visible growth in case the cultures had time to adapt stepwise to low glycerol concentrations.

Molecular genetic characterization

Amplification, sequencing and sequence analysis of D1/D2 domain of the nuclear LSU rRNA gene was done as described before (Brysch-Herzberg and Seidel 2017). All sequences were determined by StarSEQ GmbH, Mainz, Germany.

As several attempts to directly sequence the PCR product of the ITS1-5.8S-ITS2 region (ITS-region) failed because of multiple signals that occurred from certain points onward, the ITS region of the type strain of the novel species was cloned in a common vector and 15 different clones were sequenced. Amplification of the ITS region for cloning was done with Q5 proofreading polymerase (New England Biolabs GmbH) with the primer pair ITSF and NL1rev (Nguyen and Gaillardin 2005) following the manufacturer's instructions. Sequencing was done by Eurofins Genomics, Ebersberg, Germany.

In addition to the rDNA loci the RNA subunit of RNAse P that was already used by Helston *et al.* (2010: 779–86) for the description of *S. cryophilus* was analyzed. RNAse P gene fragment from the type strain of *S. osmophilus* was amplified by using 5′- TACGGACAAACGCCGCAC (forward) and 5′-TAGTCGGAATCGAGTTGCCTG (reverse) primers. The primers were designed using NCBI's Primer-BLAST tool (Ye *et al.* 2012) on the basis of sequences of *S. cryophilus* (GU470883) and *S. octosporus* (X52531). The PCR reactions were carried out as follows: an initial denaturing step of 3 min at 95°C was followed by

Table 1. Strains included in the study.

Species	Strain numbers in this study	CBS strain number	CIRM strain number	Substrate	Geographical origin	D1/D2 accession number	ITS sequences accession number	RNAseP accession number
S. osmophilus	SZ132-FG-D	CBS 15792	CLIB 3266	bee bread of *Osmia rufa*	Germany	MK253004*		MK813874
S. osmophilus	SZ134-FG-A[T]	CBS 15793[T]	CLIB 3267[T]	bee bread of *Osmia florisomnis*	Germany	MK253005*	MK589403-MK589411*	MK405475*
S. osmophilus	SZ137-FG-A	CBS 15794	CLIB 3268	bee bread of *Osmia florisomnis*	Germany	MK253006*		MK813875
S. osmophilus	SZ138-FG-A	CBS 15795	CLIB 3269	bee bread of *Osmia florisomnis*	Germany	MK253007*		MK813869
S. osmophilus	SZ144-FG-C	CBS 15796	CLIB 3270	bee bread of *Osmia florisomnis*	Germany	MK253008*		MK813870
S. osmophilus	SZ150-FG-A	CBS 15797	CLIB 3271	bee bread of *Osmia florisomnis*	Germany	MK253009*		MK813871
S. osmophilus	SZ154-FG-A	CBS 15798	CLIB 3272	bee bread of *Megachile* sp.	Germany	MK253010*		MK813872
S. osmophilus	SZ158-FG-A	CBS 15799	CLIB 3273	bee bread of *Osmia florisomnis*	Germany	MK253011*		MK813873
S. osmophilus	NCAIM Y.02191	CBS 15810	CLIB 3291	fig snack	Spain	MG250353*		MK405476*
S. octosporus	CBS 371[T]	CBS 371[T]	CLIB 832[T]	unknown	unknown	U76525	CBS[†]	X52531
S. octosporus	SZ40-FG-A	–	CLIB 3274	raisins	Australia	MK690460*		
S. octosporus	SZ41-FG-A	CBS 15800	CLIB 3275	raisins	Greece	MK690461*		
S. octosporus	SZ42-FG-A	–	CLIB 3276	raisins	South Africa	MK690462*		
S. octosporus	SZ46-FG-A	CBS 15801	CLIB 3277	raisins	California, USA,	MK690463*		
S. octosporus	SZ48-FG-A	–	CLIB 3278	raisins	Turkey	MK690464*		
S. octosporus	SZ51-FG-A	–	CLIB 3279	raisins	South Africa	MK690465*		
S. octosporus	SZ60-FG-A	–	CLIB 3280	raisins	Chile	MK690466*		
S. octosporus	SZ75-FG-B	–	CLIB 3281	raisins	Argentina	MK690467*		
S. octosporus	SZ82-FG-A	–	CLIB 3282	raisins	South Africa	MK690468*		
S. cryophilus	CBS 11777[T]	CBS 11777[T]	–	unknown	unknown	GU470882	NR_121468.1	GU470883
S. japonicus	CBS 354[T]	CBS 354[T]	CLIB 831[T]	wine	Japan	U94943.1	NR_121199.1	GU470884
S. japonicus	S437-OB	CBS 15802	CLIB 3283	forest soil under *Picea abies*	Ruwer Valley, Germany	MK690475*		
S. japonicus	S457-OA	CBS 15803	CLIB 3284	forest soil under *Larix* sp.	Ruwer Valley, Germany	MK690476*		
S. japonicus	S465-OAII	CBS 15804	CLIB 3285	bark of *Malus domesticus*	Ruwer Valley, Germany	MK690477*		
S. japonicus	S544-OB	CBS 15805	CLIB 3286	Sap flux of *Prunus avium*	Ruwer Valley, Germany	MK690478*		
S. japonicus	S628-OA	CBS 15806	CLIB 3287	Sap flux of *Prunus avium*	Ruwer Valley, Germany	MK690479*		
S. japonicus	S666-OA	CBS 15807	CLIB 3288	Soil under *Pinus* spec	Roussillion, France	MK690480*		
S. japonicus	S672-OA	CBS 15808	CLIB 3289	Soil under *Quercus* spec.	Abbaye de Saint-Roman, France	MK690481*		
S. japonicus	S685-OB	CBS 15809	CLIB 3290	bark of *Quercus* spec.	Dentelles de Montmirail, France	MK690482*		
S. pombe	CBS 356[T]	CBS 356[T]	CLIB 833[T]	unknown	unknown	U40085	MK749863*	X04013
S. pombe	CBS 352	CBS 352	–	arak factory	Indonesia	MK749856*		
S. pombe	CBS 357	CBS 357	CLIB 834	molasse	Jamaica	CBS[†]		
S. pombe	CBS 1057	CBS 1057	–	brewer's yeast	Skäne, Sweden	MK749857*		
S. pombe	CBS 1059	CBS 1059	–	food	Mauritius	MK749858*		
S. pombe	CBS 2628	CBS 2628	–	wine	Pakistan	MK749859*		
S. pombe	CBS 2776	CBS 2776	–	molasses	Japan	MK749860*		
S. pombe	CBS 5557	CBS 5557	–	Listan grapes	Spain	MK749861*		
S. pombe	CBS 5682	CBS 5682	–	beer	South Afica	MK749862*		
S. pombe	VTT-C-80103	CBS 10391	–	millet beer	Africa	CBS[†]		
S. pombe	VTT-C-05656	CBS 10393	–	fermenting apple juice	unknown	CBS[†]		
S. pombe	VTT-C-05657	CBS 10394	–	fermenting toddy	Sri Lanka	CBS[†]		
S. pombe	VTT-C-82133T	CBS 1042	–	sulphited grape juice	Germany	CBS[†]		

[T] = type strain; * = sequence determined in the current study; [†] = DNA sequence from CBS database; VTT = Technical Research Centre of Finland, Espoo, Finland; CIRM = Center for Microbial Resources, Cedex, France; CBS, Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; NCAIM, National Collection of Agricultural and Industrial Micro-organisms, Budapest, Hungary.

32 cycles of 30 s at 95°C, 30 s at 58°C and 40 s at 72°C and terminated with a final extension step of 5 min at 72°C. The sequences were determined directly by a commercial sequencing facility (BIOMI Ltd., Hungary and StarSEQ GmbH, Mainz, Germany).

For all three loci the phylogenetic relationships were calculated with the maximum likelihood method and the Jukes Cantor nucleotide substitution model. Bootstrap values were calculated from 1000 iterations.

RESULTS AND DISCUSSION

Phenotypic characterization

The results of the physiological tests are given in Table 2. For comparison and since not all tests were performed with all species in the current study results reported by Barnett *et al.* (2000) and in case of *S. cryophilus* results obtained by Helston *et al.* (2010) are also included in the Table. The outcome of the physiological tests performed for the current investigation corresponds well with the data of Barnett *et al.* (2000) and exhibited only some minor differences compared to those of Helston *et al.* (2010).

As the sequence analysis of the D1/D2 domain of the LSU rDNA (see below) revealed that *S. osmophilus* is more closely related to *S. cryophilus* and *S. octosporus* than to *S. pombe* or *S. japonicus* the phenotypic characteristics of these three species are discussed in the following:

From *S. octosporus, S. osmophilus* is separated by its ability to grow on gluconate, DL-lactate and creatinine. Barnett *et al.* (2000) reported *S. octosporus* not to grow on glycerol while Vaughan-Martini and Martini (2011) found variable growth responses of *S. octosporus* to glycerol. This inconsistency suggests that only a very few strains of *S. octosporus* are able to grow on glycerol. In contrast all strains of *S. osmophilus* showed strong growth with this carbon source.

The main differences between *S. cryophilus* and *S. osmophilus* are the inability of *S. osmophilus* to ferment Me-α-D-glucoside, raffinose and inulin, which are fermented by *S. cryophilus* at least to some extent. Additionally, unlike *S. cryophillus S. osmophilus* is not able to grow on sucrose, trehalose, cellobiose, raffinose, palatinose and in the presence of 0.001% cycloheximide. Growth of *S. cryophilus* on sucrose, trehalose, cellobiose, raffinose and palatinose was not observed by Helston *et al.* (2010). *Schizosaccharomyces osmophilus* grows on gluconate, DL-lactate, glycerol and 60% glucose, while *S. cryophilus* gives negative results with these tests. All strains of *S. osmophilus* are able to grow at 33°C and no strain grow at 36°C. At 34 and 35°C the growth response is weak and variable among the strains. In contrast *S. cryophilus* is not able to grow at 32°C or at higher temperatures. In general, it is difficult to compare *S. cryophilus* to any strains or species as its phenotypic characterization is based on one strain only and to our knowledge no additional strain of the species has been isolated since the species description was published in 2010 (Helston *et al.* 2010). It is unclear if the single strain represents the species well or not and naturally the phenotypic variability of *S. cryophilus* cannot be estimated.

The ability of *S. osmophilus* to grow on DL-lactate and to assimilate creatinine is a unique property in the genus *Schizosaccharomyces*. The ability to hydrolyze urea, to ferment different sugars and the inability to assimilate nitrate fit well into the phenotypic characteristics of the genus.

The capability of the strains to grow on GPY-Medium containing various concentrations of glycerol was tested. The results are given in Table 3. All strains of the novel species clearly showed the strongest growth at glycerol concentrations between 20 and 30% in case they were inoculated simultaneously on the media with different glycerol concentrations.

In order to figure out if the strains could adapt to lower osmotic pressure glycerol concentration was lowered by steps of 5% with each inoculation step starting at 35%. When this procedure was applied all strains grew even on standard medium containing 2% glucose and no glycerol. However, growth was always very week and stopped soon. This was true even if the cultures were repeatedly inoculated from one agar medium with a low solute concentration to another agar medium with the same low concentration indicating that the cultures have not been able to fully adapt to the low solute concentration. The results indicate that all strains are true osmophilic.

On GPY-agar containing 50% glucose colonies are cream to slightly tan in colour. The shape of the colony is irregular with an undulate to lobate margin. The surface is dull and rough to papillate. After one week the texture of colonies is friable. For several weeks to several months from old tan colored colonies single cream colored fresh growing papillae arise. At 25°C, on GPY-agar containing 40–50% glucose the cultures may survive for more than 9 months.

Under the microscope *S. osmophilus* resembles to *S. octosporus* and *S. cryophilus*. Asexual cells are globose, ovoid or irregular in shape (2.5–10 μm x 2.5–10 μm) (Fig. 1J–N). Elongated cells are rare (3–4 μm x 6–8 μm) (Fig. 1O and P). Very rarely cells of the *S. pombe* type which are elongated and do exhibit pronounced corners at the ends of the cell can be observed. Asexual reproduction proceeds by fission (Fig. 1I–O). Fission takes place in cells of different shape and size. True hyphae were not observed. Sometimes the cells adhere to each other after fission. The formation of asci takes place after the conjugation of two independent cells (Fig. 1A and B), which could be interpreted in a way that cells before conjugation are haploid. Most asci contain eight ascospores. The number of spores can vary from 2 to 8 (Fig. 1B–H). Ascospores are globose to ovoid, sometimes kidney or drop shaped (Fig. 1B–H). Ascospores can vary in size from 2.5–5 × 2.5–5 μm. Frequently asci can be observed that contain one or more underdeveloped spores (Fig. 1F–G). Thus, most likely the varying number of spores is caused by incomplete development of some spores after meiosis.

Molecular genetic characterization

The D1/D2 domain of *S. osmophilus* is 607 bp long; it differs by 11 substitutions (1.8%) from that of the type strain of *S. octosporus* which has the closest related D1/D2 sequence. From the next closest species, *S. cryophilus*, *S. osmophilus* differs by 18 substitutions and 2 indels (3.5%). According to Kurtzman and Robnett (1998) strains belonging to the same biological species generally exhibit less than 1% variance in their D1/D2 domain. This assessment was later reconfirmed by Vu *et al.* (2016) who did not analyze the D1/D2 domain sequences of the type strains only but of several strains per species if they were available. On the other hand, it should be emphasized that species are not solely defined by D1/D2 divergence values and that substantial deviations from the '1% rule' have been reported (Lachance 2018). Therefore, the ITS-region and the RNA subunit of RNAse P was analyzed beside the D1/D2 domain and physiological characteristics as well as information about the ecology of the new strains are taken into account (see below).

In the current investigation the D1/D2 domains of nine strains of *S. octosporus* that were isolated from raisins that came from five continents were analyzed. Despite the great distance

Table 2. Phenotypic characters of *Schizosaccharomces* species.

		S. pombe		*S. japonicus*		*S. octosporus*		*S. cryophilus*		*S. osmophilus*
		This study	Barnett *et al.* (2000)	This study	Barnett *et al.* (2000)	This study	Barnett *et al.* (2000)	this study	Helston *et al.* (2010)	this study
Fermentation	D-Glucose	+	+	+	+	+	+	+	+	+
	D-Galactose	v	v	–	–	–	–	–	–	–
	Rhamnose	–	–	–	n	–	–	–	n	–
	Sucrose	+	+	+	+	–	v	+	w	d
	Maltose	v	v	v	v	+	v	+	+	d
	Trehalose	–	–	–	–	v	d	–	n	–
	Me-α-D-Glucoside	v	v	–	d	v	–	+	n	–
	Cellobiose	–	–	–	–	v	–	–	n	–
	Melibiose	–	+	+	v	–	–	–	n	–
	Lactose	–	–	–	–	–	–	–	n	–
	Raffinose	+	v	+	+	–	–	w	n	–
	Melezitose	–	–	–	–	–	–	–	n	–
	Inulin	v	v	v	–	–	–	w	n	–
	Starch	–	–	–	–	–	–	–	n	–
Assimilation	Glucose	+	+	w	+	+	v	+	+	+
	Galactose	–	v	–	–	–	–	–	–	–
	Sorbose	–	–	–	–	–	–	–	–	–
	D-Glucosamine	–	–	–	–	–	–	–	–	–
	D-Ribose	–	–	–	–	–	–	–	–	–
	Xylose	v	–	–	–	v	–	–	–	–
	L-Arabinose	–	–	–	–	–	–	–	–	–
	D-Arabinose	–	–	–	–	–	–	–	n	–
	L-Rhamnose	–	–	–	–	–	–	–	–	–
	Saccharose	+	+	+	v	–	v	+	–	–
	Maltose	v	v	–	v	v	v	d	+	v
	Trehalose	v	v	–	–	v	v	w	–	–
	Methyl α-D-glucoside	+	v	–	–	v	v	+	d	v
	Cellobiose	v	–	–	–	–	v	w	–	–
	Salicin	–	–	–	–	–	–	–	n	–
	Arbutin	–	–	–	–	–	–	–	n	–
	Melibiose	–	v	–	v	–	–	–	–	–
	Lactose	–	–	–	–	–	–	–	–	–
	Raffinose	+	+	d	v	–	–	+	–	–
	Melezitose	v	–	–	–	–	–	–	–	–
	Inulin	v	v	–	v	–	v	d	n	v
	Starch	–	v	–	–	–	v	–	n	–
	Glycerol	v	v	–	–	–	–	–	–	+
	Erythritol	–	–	–	–	–	–	–	–	–
	Ribitol	–	–	–	–	–	–	–	n	–
	Xylitol	–	–	–	–	–	–	–	n	–
	Arabitol	–	–	–	–	–	–	–	n	–
	D-Glucitol	v	–	–	–	–	–	–	–	–
	D-mannitol	–	–	–	–	–	–	–	–	–
	Galactitol	–	–	–	–	–	–	–	n	–
	myo-Inositol	–	–	–	–	–	–	–	–	–
	D-Glucono-1,5-lactone	–	v	–	–	–	v	–	–	–
	2-Keto-D-Gluconate	–	v	–	–	–	–	–	–	–
	5-Keto-D-Gluconate	–	–	–	–	–	–	–	n	–
	D-gluconate	–	v	–	–	–	–	–	n	+
	D-Glucuronate	–	–	–	–	–	–	–	–	–
	D-Galacturonate	–	–	–	–	–	–	–	n	–
	DL-Lactate	–	–	–	–	–	–	–	n	+
	Succinate	–	–	–	–	–	–	–	n	–
	Citrate	–	–	–	–	–	–	–	n	–
	Methanol	–	–	–	–	–	–	–	n	–
	Ethanol	–	v	–	–	–	–	–	n	–
	Propane-1,2-diol	–	–	–	–	–	–	–	n	–
	Butane-2,3-diol	–	–	–	–	–	–	–	n	–
	Quinate	–	–	–	–	–	–	–	n	–
	Saccharate	–	–	–	–	–	–	–	–	–
	Palatinose	v	n	–	n	v	n	d	–	–

Table 2. Continued

		S. pombe		*S. japonicus*		*S. octosporus*		*S. cryophilus*		*S. osmophilus*
		This study	Barnett *et al.* (2000)	This study	Barnett *et al.* (2000)	This study	Barnett *et al.* (2000)	this study	Helston *et al.* (2010)	this study
	N-Acetylglucosamine	–	n	–	n	–	n	–	–	–
	Nitrate	n	–	n	–	n	–	n	n	–
	Nitrite	n	–	n	–	n	–	n	n	–
	Ethylamine	n	v	n	–	n	v	n	n	–
	L-Lysine	n	v	n	v	n	–	n	n	–
	Cadaverin	n	v	n	–	n	v	n	n	+
	Creatine	n	–	n	–	n	–	n	n	–
	Creatinine	n	–	n	–	n	–	n	n	+
	Glucosamine	n	–	n	–	n	–	n	n	–
	Imidazole	n	–	n	–	n	–	n	n	–
Tolerance	Cyloheximid 0.001%	+	n	–	n	–	–	+	n	–
	Cyloheximid 0.01%	v	v	–	–	–	v	–	–	–
	Cyloheximid 0.1%	–	–	–	–	–	–	–	–	–
	Acetic acid 1%	v	v	–	–	–	v	+	–	–
	NaCl 10%	–	v	–	–	+	+	–	–	+
	NaCl 16%	–	–	–	–	+	v	–	–	v
	Glucose 50%	+	+	–	–	+	v	+	–	+
	Glucose 60%	v	v	–	–	+	v	–	–	+
	Urea hydrolysis	+	v	+	+	w	+	+	d	d
Growth at	4°C	n	n	n	n	n	n	n	n	+
	12°C	n	n	n	n	n	n	n	n	+
	15°C	n	n	n	n	n	n	n	n	+
	18°C	n	n	n	n	n	n	n	+	+
	21°C	n	n	n	n	n	n	n	n	+
	25°C	+	+	+	+	+	+	+	+	+
	30°C	n	+	n	+	n	+	n	–	+
	32°C	n	n	n	n	n	n	n	–	+
	33°C	n	n	n	n	n	n	n	n	+
	34°C	n	n	n	n	n	n	n	n	v
	35°C	n	+	n	+	n	+	n	n	v
	36°C	n	n	n	n	n	n	n	–	–
	37°C	n	+	n	+	n	v	n	n	–
	40°C	n	v	n	v	n	–	n	n	–
	42°C	n	v	n	v	n	–	n	n	n
	45°C	n	–	n	–	n	–	n	n	n

+ = positive result; – = negative result; v = variable response; n = not tested

Table 3. Growth response of *Schizosaccharomyces osmophilus* strains to various glycerol concentrations on GPY agar.

	Glycerol concentration												
Strain	0%	5%	10%	15%	20%	25%	30%	35%	40%	45%	50%	55%	60%
CBS 15 792	–	–	–	+++	+++	+++	+++	+++	+	w	–	–	–
CBS 15793^T	–	+	+	+++	+++	+++	+++	+++	++	+	–	–	–
CBS 15 794	–	–	–	+++	+++	+++	+++	+	+	w	–	–	–
CBS 15 795	–	–	++	+++	+++	+++	+++	+++	+++	++	+	–	–
CBS 15 796	–	–	–	+++	+++	+++	+++	++	+	–	–	–	–
CBS 15 797	–	++	++	+++	+++	+++	+++	++	+	–	–	–	–
CBS 15 798	–	++	++	++	+++	+++	+++	+++	+++	+++	+	w	–
CBS 15 799	–	–	–	+++	+++	+++	+++	++	+	–	–	–	–
CBS 15 810	–	–	–	+++	+++	+++	+++	+++	+++	+	–	–	–

– = no growth; w = hardly any visible growth at all; + = growth easily visible; ++ = strong growth; +++ very strong growth

between their geographic origin all strains exhibited identical D1/D2 sequences, which were also identical with that of the type strain (CBS 371^T). The results indicate that *S. octosporus* has a high level of D1/D2 homogeneity. Similarly, the nine strains of *S. osmophilus* did not exhibit any differences in their D1/D2 sequences although the Spanish strain (NCAIM Y.02191) originated from a location that was more than 1000 km away from the region in which the eight German strains were isolated. The absence of any variations between the strains can be interpreted in a way that within *S. osmophilus* the D1/D2 domains are highly

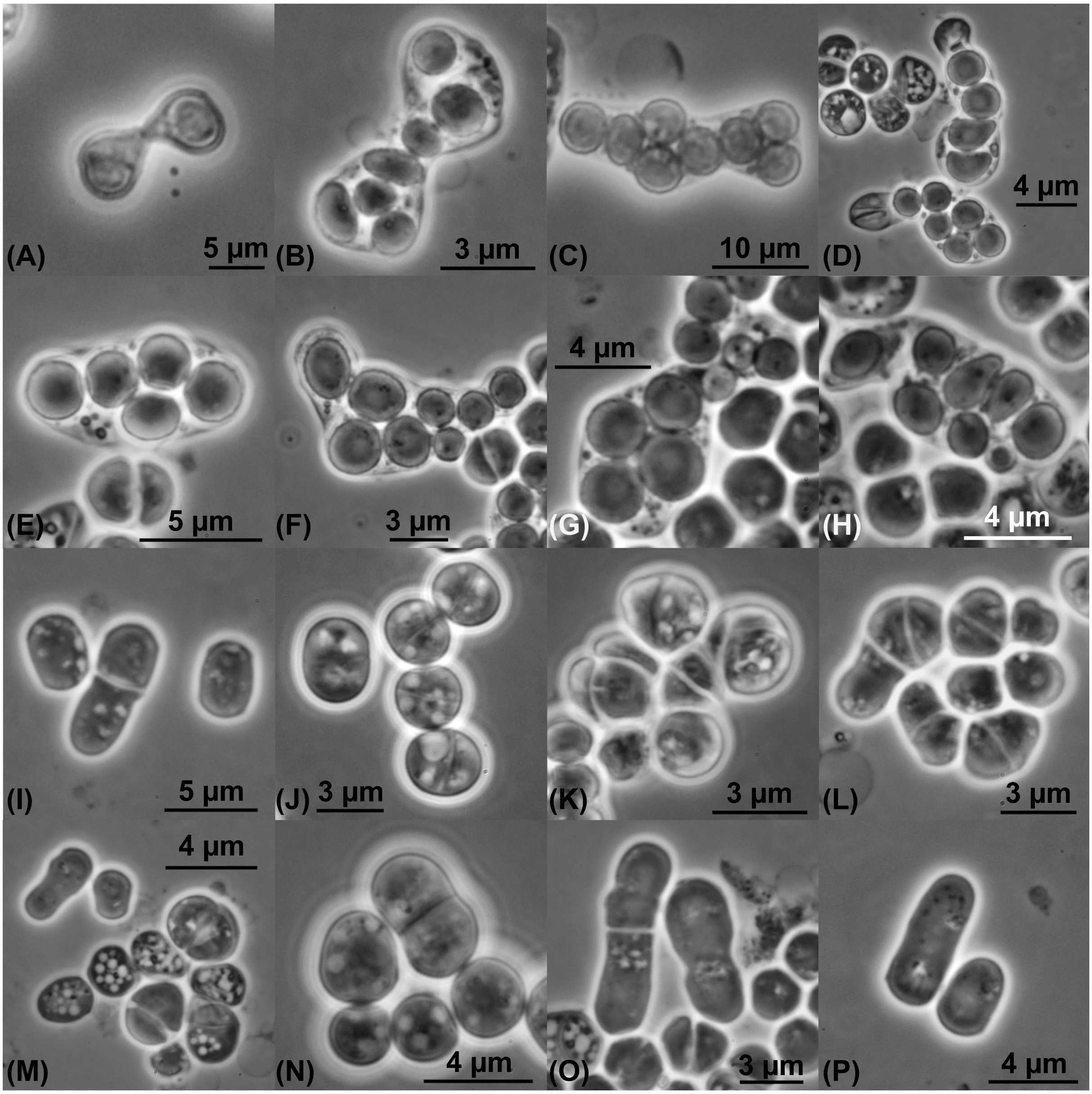

Figure 1. Micromorphology of Schizosaccharomyces osmophilus CBS 15793T; A: conjugation of two indipendent cells; B–E: asci with 4 to 8 Ascospores; F–G: asci with underdeveloped or dead ascospores; B, C, D, H: Asci with round, ovoid, kidney shaped and drop shaped ascospores; I–P different sizes and shapes of asexual cells.

uniform, too. A similar high degree of D1/D2 sequence conformity was found in *S. pombe* and *S. japonicus*. Due to the absence of several strains *S. cryophilus* cannot be assessed in this respect. The very low intraspecific variability combined with an interspecific divergence of the different *Schizosaccharomyces* species that is clearly above the level, which is usually found within yeast species provides a strong argument to assign the nine osmophilic strains to the new species *S. osmophilus*. The phylogenetic placement of the new species within the genus on the basis of D1/D2 sequences is illustrated in Fig. 2.

Due to the intraspecific homogeneity of the D1/D2 sequences the D1/D2 domain can be used as a reliable tool for the detection of new *Schizosaccharomyces* species. This is noteworthy since for some other species like the members of the *Metschnikowia pulcherrima* species complex it was shown that neither the D1/D2 domain nor the ITS region is suitable for genetic barcoding due to divergent copies of both loci (Sipiczki, Horvath and Pfliegler

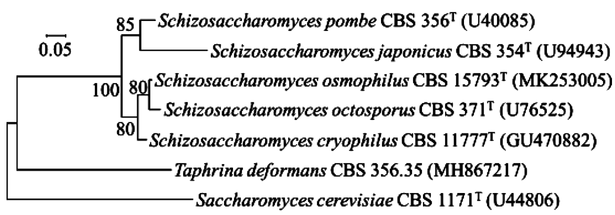

Figure 2. Phylogenetic placement of *Schizosaccharomyces osmophilus* within the genus Schizosaccharomyces. The tree is based on DNA sequence analysis of the D1/D2 domain of the nuclear large subunit (LSU) rRNA gene and obtained by maximum likelihood analysis. Percentage bootstrap values of 1000 replicates are given at each node. GenBank accession numbers are indicated after strain designation. Bar, 5% nucleotide sequence divergence. *Saccharomyces cerevisiae* was used as an outgroup species.

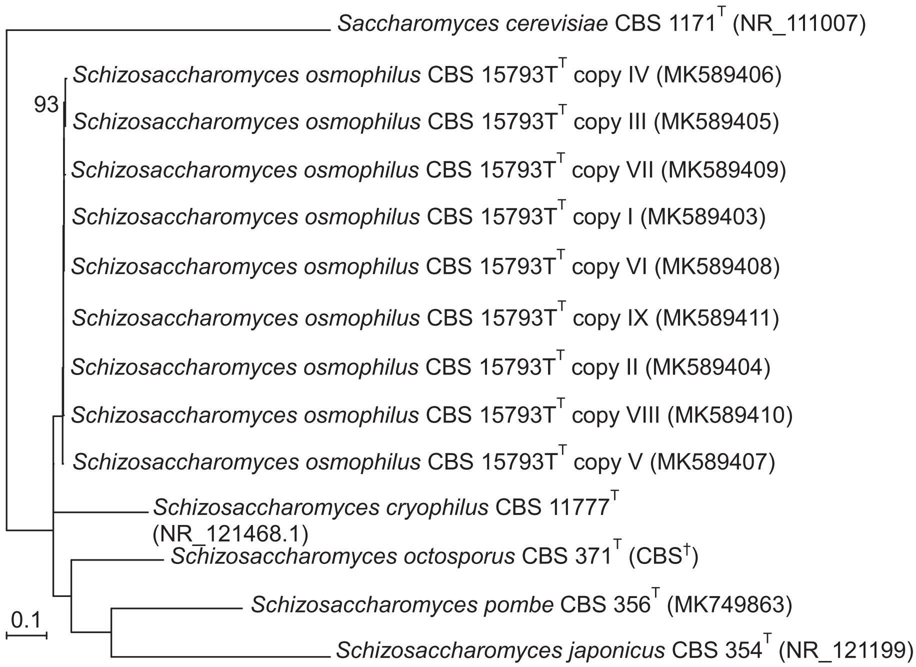

Figure 3. Phylogenetic position of *Schizosaccharomyces osmophilus* within the genus Schizosaccharomyces. The tree is based on the DNA sequences analysis of the ITS1-5.8S-ITS2 rDNA region and obtained by maximum likelihood analysis. Percentage bootstrap values of 1000 replicates which exceed 80 are given at each node. GenBank accession numbers are indicated after strain designation. Bar, 10% nucleotide sequence divergence. *Saccharomyces cerevisiae* was used as an outgroup species.

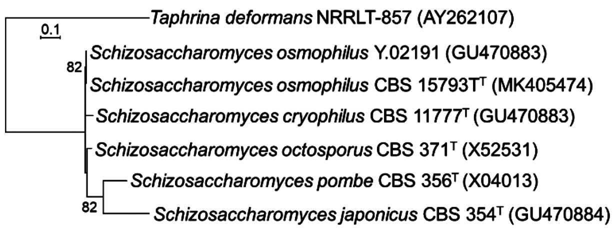

Figure 4. Phylogenetic placement of *Schizosaccharomyces osmophilus* within the genus Schizosaccharomyces. The tree is based on the DNA sequence analysis of RNA subunit of RNAse P and obtained by maximum likelihood analysis. Percentage bootstrap values of 1000 replicates that exceed 80 are given at each node. GenBank accession numbers are indicated after strain designation. Bar, 10% nucleotide sequence divergence. *Taphrina deformans* was used as an outgroup speciesT

2018). Additionally, other species like *Kazachstania aerobia* and *Kazachstania solicola* share identical D1/D2 domains (Wu and Bai 2005). In some other yeast groups like the *Rhodosporidium babjevae* clade or the *Filobasidium floriforme* clade it was shown that a reliable identification of strains based on both the D1/D2 domain and the ITS region is not always possible (Brysch-Herzberg and Seidel 2017).

Direct sequencing of the ITS region of the type strain CBS 15793^{T} was not possible because divergent copies of the ITS region were present in its genome. A total of nine different ITS copies were detected. The maximum sequence difference among the divergent copies was 1.8% (seven substitutions; six indels). Most indels were located in long homopolymer regions and short microsatellite like sections. Four sites which showed length variations consisted of single nucleotide repeats up to 12 bp (A/T repeats). A short TGA/ACT-repeat consisted of three or four repeats while an ATT/TAA-repeat consisted of six or seven repeats in different copies. The intragenomic ITS region copy diversity in *S. osmophilus* is somewhat higher than the predicted general threshold for species delimitation based on ITS-region sequences of 1.59% proposed by Vu *et al.* (2016: 91–105). However, the minimum distance to *S. octosporus* which has the closest related ITS region was about 17 times higher (∼30%). The minimum distance to *S. cryophilus* was 36%. Thus, based on the ITS region *S. osmophilus* is well separated from the other *Schizosaccharomyces* species. The phylogenetic placement of the new species within the genus on the basis of ITS region sequences is illustrated in Fig. 3. Divergent copies of the ITS region have been reported from species of other yeast genera as for example *Metschnikowia* (Sipiczki, Horvath and Pfliegler 2018) or *Zygosaccharomyces* (Solieri *et al.* 2013; Solieri, Dakal and Giudici 2013; Čadež *et al.* 2015) but to our knowledge this is the first report on intragenomic variance of ITS regions in genus *Schizosaccharomyces*.

The RNA subunit of RNAse P has repeatedly been analyzed for phylogenetic studies in yeasts and ascomycetous fungi (Tranguch and Engelke 1993; Seif *et al.* 2003). Helston *et al.* (2010) sequenced this locus in the four currently accepted *Schizosacchaormyces* species for the description of *S. cryophilus*. The type strain of *S. osmophilus* (CBS 15793^{T}) differs from the type strain of *S. cryophilus* (CBS 11777^{T}) by nine substitutions and one indel (4.4%). From the type strain of *S. octosporus* (CBS 371^{T}) it differs by eight substitutions and two indels (4.4%), whereas the type strains of *S. octosporus* and *S. cryophilus* differ by 15 substitutions and 3 indels (6.1%). The *S. osmophilus* type strain (CBS 15793^{T}) that was isolated in Germany and the strain originating from Spain (NCAIM Y.02191) merely differ by one gap of 2 bp length (0.9%) which may represent a single mutation event. All eight German strains share identical RNAse P sequences. From the RNAse P sequences of *S. pombe* and *S. japonicus* *S. osmophilus* differs by more than 22%. Thus, at least in the currently available nine strains of *S. osmophilus* the intraspecific variability in this locus is substantially lower than the divergence from the type strains of the other *Schizosaccharomyces* species. The phylogenetic placement of the new species within the genus on the basis of the RNA subunit of RNAse P sequences is illustrated in Fig. 4.

The two strains of S. *osmophilus* with different sequences were placed in one clade with a bootstrap support of 82%. A broad set of sequence results for the RNA subunit of RNAse P is currently missing for *Schizosaccharomyces* species, which makes it difficult to draw taxonomic conclusions based on these sequences alone. Nevertheless, the results hint into the same direction as the results for the ITS region and the D1/D2 domain and support the description of the new species.

Ecology

The single strain originating from Spain was isolated from a fig snack. Thus, only the substrate from which the German strains were isolated may provide hints about the niche in which S. *osmophilus* propagates in nature. The strains were isolated from bee bread of different solitary bee species. Bee bread consists of a mixture of pollen with a small amount of floral nectar. Just as the sugar concentration in floral nectar the sugar concentration in the bee bread is in equilibrium with the relative air humidity. Thus, for any yeast that propagates in this substrate a strong osmotolerance is a prerequisite. The pronounced osmophily of S. *osmophilus* is a strong indicator that the occurrence of this species in nature depends on a high osmotic environment. Beside some sugar rich fruit floral nectar, honey and bee bread provide such conditions in Germany. Therefore, it seems reasonable to assume that the bee bread of solitary bees is at least one important habitat of S. *osmophilus*.

Schizosaccharomyces pombe and S. *octosporus* have been reported to be present in honey (Lochhead and Farrell 1931; Relan and Vyas 1971; Kumbhojkar 1972; Gilliam *et al*. 1974; Benito *et al*. 2013) but both species have been isolated from other substrates such as raisins, grapes and vineyard soil (Kudrjawzew 1960; Relan and Vyas 1971; Davenport 1974; Florenzano, Balloni and Materassi 1977; Barata, Malfeito-Ferreira and Loureiro 2012), different kind of fruits other than wine grapes such as plums (Lund 1958; Skinner, Passmore and Davenport 1980), blueberries (*Vaccinium myrtillus*) (Kudrjawzew 1960) apple, oil palm sap, palm wine (Faparusi 1974; Sanni and Lönner 1993), maple sap (Lund 1958) or cocoa fermentation (Ravelomanana *et al*. 1984; Mazigh 1994). In connection with other yet unpublished investigation the authors have examined the yeast communities of different substrates such as raisins, grapes, rotten grapes, apples, black currents, red currents, cherries, forest soil, orchard soil, slime fluxes of different tree species by the enrichment technique described above without detecting S. *osmophilus* (data not shown). This does not necessarily mean that S. *osmophilus* is never present in/on such substrates but it is a hint that the species is not frequent or abundant in/on these substrates and from the literature cited above it can be concluded that S. *pombe* and S. *octosporus* have a somewhat broader distribution.

As the selection of divergent usually extreme phenotypes has been described as the driving force in ecological speciation (Rundle and Nosil 2005) the obvious specialization of S. *osmophilus* to a high osmotic environment can be considered as a further argument to describe the investigated strain as a separate species.

CONCLUSION

Analyses of the DNA sequences for the D1/D2 domain, the ITS-region and the RNA subunit of RNAse P provide strong arguments that the new strains characterized in this study represent a new species of *Schizosaccharomyces*. Some of the physiological properties of S. *osmophilus* are unique in *Schizosaccharomyces* e.g. the ability to assimilate DL-lactate or creatinine. The true pronounced osmophily in combination with the highly specific high osmotic microhabitat bee bread suggests the idea of a highly specialist yeast species that is most likely separated from other *Schizosaccharomyces* species by its supposed narrow distribution in nature.

Description of *Schizosaccharomyces osmophilus* Brysch-Herzberg, Tobiás, Seidel, Wittmann, Fischer, Dlauchy & Péter sp. nov.

MycoBank no.: MB829586

N.L. masc. adj. *osmophilus* (from Gr. n. osmos, pressure and Gr. masc. adj. philos, loving), osmophile, pressure loving

On 50% glucose GPY Agar at 25°C the form of the colonies is irregular with an undulate to lobate margin. The surface is dull and rough to papillate. After one week the texture of colonies is friable. True mycelium is not formed. Cells are globose, ovoid, irregular (2.5–10 μm x 2.5–10 μm) or elongated (3–4 μm x 6–8 μm). Asexual reproduction proceeds by fission. Asci usually arise from conjugation of independent cells. Two to eight ascospores are formed per ascus (Fig. 1). Ascus formation takes place on different growth media including GPY (30% glucose) after 3–4 days.

Glucose, sucrose (delayed) and maltose (delayed) are fermented. Galactose, trehalose, me-α-D-glucoside, cellobiose, melibiose, lactose, raffinose, melezitose, inulin and starch are not fermented. Glucose, maltose (variably), methyl α-D-glucoside (variably), inulin (variably), glycerol, gluconate, DL-lactate, cadaverine, creatinine are assimilated.

Galactose, sorbose, D-glucosamine, ribose, xylose, L-arabinose, D-arabinose, rhamnose, sucrose, trehalose, cellobiose, salicin, arbutin, melibiose, lactose, raffinose, melezitose, soluble starch, erythritol, ribitol, xylitol, arabitol, sorbitol, mannitol, galactitol, myo-inositol, glucono-1,5-lactone, 2-keto-D-gluconate, 5-keto-D-gluconate, glucuronate, galacturonate, succinate, citrate, methanol, ethanol, propane 1,2 diol, 2,3 butanediol, quinate, saccharate, palatinose, N-acetyl-D-glucosamine, nitrate, nitrite, ethylamine, lysine, creatine, glucosamine (as nitrogen source), imidazole are not assimilated. No growth occurs in the presence of 0.001% cycloheximide and 1% acetic acid. Growth occurs with 16% NaCl, 60% glucose and in vitamin-free medium. Urea is hydrolyzed.

Holotype: CBS 15793^{T}; isotype: CLIB 3267 ^{T} = NCAIM Y.02225 ^{T}, all are permanently preserved in a metabolically inactive state. The type culture was isolated from bee bread of *Osmia florisomnis* in July 2017 in the region Württemberg, Germany.

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Conflict of interest. None declared.

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