Zygosaccharomyces gambellarensis sp. nov., an ascosporogenous yeast isolated from an Italian 'passito' style wine

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Yeast strains were isolated from Vin Santo of Gambellara, a sweet white wine with the specificity of Controlled Designation of Origin produced from off-vine overripened grapes in the Veneto region (Italy). Comparative sequence analysis of the 26S rRNA gene revealed that three representative strains (ZO03-5^T, CA06-8 and ME06-9) constitute a taxon related to, but distinct from, *Zygosaccharomyces machadoi*. Similarity between the 26S rRNA gene domain D1/D2 sequence of the three isolates and *Z. machadoi* was 97.9%; moreover, the morphological characteristics and the physiological behaviour also supported recognition of a novel taxon of osmophilic non-psychrophilic yeast showing a flower-like arrangement of budding cells that remain attached to each other. The name *Zygosaccharomyces gambellarensis* is proposed for the novel species, with ZO03-5^T (=CBS 12191^T=MUCL 53393^T) as the type strain.

INTRODUCTION

Vin Santo of Gambellara is a traditional sweet white wine obtained from off-vine overripened grapes of the Garganega variety that is produced in a small area (less than 40 km²) of the Veneto region (north-east of Italy). This wine obtained the specificity of Controlled Designation of Origin by the Italian government in 1970 (Gazzetta Ufficiale della Repubblica Italiana, 1970), which implies that its distinctive sensorial characteristics are derived from local environmental factors, as well as the particular winemaking procedure. In fact, the winemaking process includes many peculiar steps that have remarkable effects on the microbial population and, thus, on the wine properties. Briefly, grapes are partially dried in attics for 5/6 months; during this phase, saprophytic moulds, including *Botrytis cinerea* (noble rot), grow on the grapes and contribute positively to the chemical and sensory characteristics of the final product. Wine pressing is generally carried out during the Easter period and must fermentation proceeds very slowly as sugar levels are ≤ 350 g l⁻¹, reaching approximately 14 alcoholic degrees. The wine is aged for two or more years in oak barrels or barriques.

Few microbiological studies have been carried out on sweet white wines from partially dried grapes (e.g. Fleet *et al.*, 1984; Donèche, 1993; Fleet, 1998; Mills *et al.*, 2002). These

studies have shown that osmotolerant non-*Saccharomyces* yeasts are commonly found at the beginning of the fermentation, but they decrease as *Saccharomyces* species start dominating the process. However, some of the non-*Saccharomyces* species, such as *Candida* spp., *Dekkera* spp., *Hansenula* spp., *Kloeckera* spp., *Kluyveromyces* spp., *Pichia* spp. and *Zygosaccharomyces* spp. can survive and they can still be present during storage and ageing of wine, thus having an impact on the final wine quality (Ribéreau-Gayon *et al.*, 2006; Ciani *et al.*, 2010; Parapouli *et al.*, 2010). In particular, *Zygosaccharomyces* show peculiar features including specific nutritional requirements in terms of sugars (e.g. fructophily), osmotolerance, formation of heat-resistant ascospores and resistance to preservatives such as sulphite, sorbic acid and ethanol. Interestingly, these yeasts, even if often associated with wine spoilage (Fugelsang, 1996; Stratford, 2006), could also have a positive role in fermentation processes (Jolly *et al.*, 2006).

Considering natural selection and the progressive adaptation of micro-organisms to the climatic and physico-chemical conditions of the habitat, which create an ecological niche unique for every kind of wine-making, a study of the indigenous eukaryotic microbiota of Vin Santo of Gambellara was undertaken. During such analysis, 25 isolates were characterized from three wine samples produced in different vintages and diverse wineries. Isolates were shown to belong to the genus *Zygosaccharomyces* by cell morphology and asci formation (Kurtzman & Fell, 1998), but could not be allocated to any previously described species, i.e. *Zygosaccharomyces bailii*, *Z. bisporus*, *Z. kombuchaensis*, *Z. lentus*, *Z. mellis*, *Z. machadoi* and *Z. rouxii* (Kurtzman, 2003; Rosa & Lachance, 2005). Therefore, in the present study,

Abbreviation: RAPD, Randomly amplified polymorphic DNA.

The GenBank/EMBL/DDBJ accession numbers for the partial 26S rRNA gene sequence of strains ZO03-5^T, CA06-8 and ME06-9 are FR725931, FR725932 and FR725933, respectively.

Two supplementary figures are available with the online version of this paper.

three representatives of the 25 isolates, namely CA06-8, ME06-9 and ZO03-5^T, were subjected to detailed molecular and physiological analysis. Based on results presented, a novel species is proposed.

METHODS

Yeast strains. Yeast strains were isolated from Vin Santo of Gambellara produced in three different wineries during diverse vintages. Wine samples were collected from oak barrels during ageing and either directly plated or first centrifuged at 8000 g for 10 min prior to transfer onto two selective cultivation media: Wallerstein laboratory nutrient agar (WL; Oxoid), supplemented with biphenyl (150 mg l⁻¹; Sigma) and chloramphenicol (100 mg l⁻¹; Sigma); and tryptone-glucose-yeast extract (TGY) agar (Beuchat, 1993). After incubation for 5 days at 27 °C, individual colonies were isolated and purified through repeated streaking. Isolates were maintained on yeast extract-peptone-glucose (YEPD; 10 g yeast extract l⁻¹, 20 g peptone l⁻¹ and 20 g glucose l⁻¹) agar slants prepared with 15 g agar l⁻¹ and kept at 4 °C before preservation by freeze-drying. Yeast isolates were routinely subcultured in YEPD at 27 °C for 72 h on a rotary shaker (200 r.p.m.).

Phenotypic characterization. The isolates were characterized following the usual criteria for spore formation and physiological tests according to Yarrow (1998). Sugar fermentation and assimilation tests were also performed using the API 20C AUX strip system (bioMérieux).

The effects of various conditions on growth were examined in test tubes containing 10 ml liquid medium, which were inoculated with about 10⁵ cells ml⁻¹. Tubes were incubated under both static and shaking conditions (200 r.p.m.) for 13 days.

The ability of the isolates to grow at different temperatures, i.e. 4, 8, 16, 20, 27, 34, 37 and 40 °C, was evaluated using YEPD broth. To test the effect of high sugar concentrations on growth, YEPD medium was supplemented with different amounts of glucose (200 and 400 g l⁻¹) and incubated at 27 °C. Growth in modified YEPD (mYEPD), which lacked glucose and contained fructose (20 g l⁻¹), was also examined at 27 °C. The increase in cell number was monitored by measuring OD₆₀₀.

DNA extraction, PCR and sequence analysis. Genomic DNA was extracted from overnight cultures applying the protocol of mechanical cell disruption as described by Cocolin *et al.* (2000). The obtained genomic DNA was suspended in 50 µl TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8) and maintained at -20 °C.

Randomly amplified polymorphic DNA (RAPD)-PCR fingerprinting of the isolates was carried out using primer M13 (5'-GAGGGT-GGCGGTCT-3') as described previously (Tofalo *et al.*, 2009). Conversion, normalization and numerical analysis of the patterns were performed using the software GelCompar 4.0 (Applied Maths, Kortrijk, Belgium).

The D1/D2 domain of the 26S rRNA gene was amplified according to Kurtzman & Robnett (1998) using primers NL1/NL4; the applied thermal cycling program comprised initial denaturation at 94 °C for 5 min, then 36 cycles at 94 °C for 1 min, 52 °C for 2 min and 72 °C for 2 min, followed by a final extension at 72 °C for 10 min.

Amplicons were purified using the NucleoSpin Extract II (Macherey-Nagel) following the manufacturer's instructions and sequenced at the BMR Genomics sequencing facility (Padua, Italy) using the same primers used for amplification.

Sequence similarity searches were performed using the BLAST network service on the NCBI website (<http://blast.ncbi.nlm.nih.gov/>).

Sequence alignments were performed with CLUSTAL W 2.0 (Larkin *et al.*, 2007) and adjusted manually. Phylogenetic analysis was performed using the software package MEGA4 (Tamura *et al.*, 2007) using the Jukes-Cantor distance model, neighbour-joining (Saitou & Nei, 1987) and minimum evolution (Nei & Kumar, 2000) methods for tree reconstruction, as suggested by Kämpfer *et al.* (2003). Sequences from *Saccharomyces cerevisiae* and *Wickerhamomyces anomalus* (formerly '*Pichia anomala*'; Kurtzman, 2011) were included as the outgroup.

The statistical reliability of the phylogenetic tree topology was evaluated using bootstrapping with 1000 replicates (Felsenstein, 1985).

RESULTS AND DISCUSSION

A low number of colonies with very similar morphology was detected from each sample of Vin Santo of Gambellara on the two cultivation media (WL and TGY), indicating the presence of osmophilic or osmotolerant yeast populations. Independent clones from samples from the three wineries were isolated: eight from cellar A, nine from cellar B and eight from cellar C.

The collection of 25 isolates was subjected to RAPD-PCR using primer M13 and numerical analysis of fingerprinting profiles was performed. The resulting dendrogram (Supplementary Fig. S1, available in IJSEM Online) revealed the presence of three different genomic clusters; three strains, i.e. ZO03-5^T, ME06-9 and CA06-8, were chosen as representatives of each cluster and further analysed.

Firstly, the 26S rRNA gene D1/D2 domain was sequenced and analysed for each of the three strains. The amplified fragments had identical nucleotide sequences indicating their conspecificity. The phylogenetic relationship with all *Zygosaccharomyces* species, also including *Zygosaccharomyces pseudorouxii* as in Rosa & Lachance (2005), revealed that strains ZO03-5^T, ME06-9 and CA06-8 formed a distinct, highly supported clade with *Z. machadoi*, *Z. mellis*, *Z. pseudorouxii* and *Z. rouxii* (Fig. 1), with *Z. machadoi* as the closest relative. As for sequence similarity values, the selected strains and *Z. machadoi* showed differences in nine positions out of 423 nt compared (2.1%). Usually, 26S rRNA gene D1/D2 domain sequences differing by more than 1% are considered indicative of different species (Kurtzman & Robnett, 1998). Therefore, phenotypic and physiological analyses were performed to verify and support this hypothesis.

Growth in different environmental conditions was tested. When cultured in YEPD medium under static and shaking conditions, the novel strains showed a lag phase of about 46 h. Also, a preference for aerobic conditions was observed, which is similar to other *Zygosaccharomyces* species, i.e. *Z. rouxii*, *Z. bailii* and *Z. bisporus* (Steels *et al.*, 1999). This behaviour distinguishes the novel strains from *Z. lentus*, a species that is also characterized by a slow growth rate, but which does not grow in shake cultures with high levels of oxygen (Steels *et al.*, 1999).

Fig. 1. Phylogenetic tree based on 26S rRNA gene sequences depicting the relationship between strains ZO03-5^T, CA06-8 and ME06-9, and *Zygosaccharomyces* species; sequences from *Saccharomyces cerevisiae* and *Wickerhamomyces anomalus* were used as the outgroup. The tree was calculated using the Jukes–Cantor distance formula and minimum evolution algorithm. Bootstrap values (%; 1000 replicates) are given at nodes (values below 60 % are not shown). Bar, number of substitutions per site.

With regard to growth in the presence of different glucose concentrations, the three strains showed slow growth in the presence of 20 and 200 g glucose l⁻¹, whereas a higher sugar concentration (400 g l⁻¹) allowed faster growth. Growth kinetics of the novel isolates were similar in YEPD and mYEPD, which contained fructose (20 g l⁻¹) instead of glucose, revealing they have no clear preference for either of the two sugars (Supplementary Fig. S2, available in IJSEM Online).

Finally, effects of temperature on growth were examined at 4, 8, 16, 20, 27, 34, 37 and 40 °C. Strains were unable to grow at 4, 8 and 40 °C during the time period studied (13 days). This behaviour distinguishes these strains from *Z. machadoi*, a species with similar physiological characteristics: the novel isolates grew at 37 °C, whereas *Z. machadoi* was not able to develop at this temperature (Rosa & Lachance, 2005).

In conclusion, phylogenetic analysis, phenotypic data and overall differences between the novel isolates and other *Zygosaccharomyces* species support the proposal for a novel osmophilic non-psychrophilic species, for which the name *Z. gambellarensis* is proposed, with ZO03-5^T (=CBS 12191^T=MUCL 53393^T) as the type strain.

Latin diagnosis of *Zygosaccharomyces gambellarensis* Torriani, Lorenzini, Salvetti et Felis sp. nov.

Cultura in extracto malti post 3 dies ad 27 °C, cellulae ellipsoideae aut globosae (5 µm), singulae, binae et fasciculatae, per gemmationem multipolarem reproducentes. Pseudohyphae et hyphae verae non fiunt post 15 dies ad 27 °C in medio agar acetato formantur. Asci per conjugationem cellularum vegetativarum oriuntur, conjugati, 1–4 ascoporas ovales contententes, non liberi. In WL post 7 dies ad 27 °C, incrementum fuscum pallidum, nitens, butyrosum; margo uniformis. Glucosum et fructosum fermentantur at non sucrosum, maltosum

nec trehalosum. Glucosum, glycerinum, D-sorbitolum, D-galactosum, sucrosum, trehalosum, maltosum, adonitolum et D-xylosum assimilantur at non cellobiosum, lactosum, raffinolum, melezitolum, L-arabinolum, N-acetyl-D-glucosaminum, methylum α-D-glucopyranosidum, inositolum, xylitolum, D-mannitolum, D-gluconatum sodicum, DL-lactatum, citratum, melibiosum, L-arabinolum, D-ribosum, L-rhamnosum, methanolum, ethanolum, meso-erythritolum nec 2-keto-gluconatum calcicum. Crescit in medio cum 40 % glucoso.

Typus depositus in collectionis Centraalbureau voor Schimmelcultures (CBS), *cum numero* CBS 12191^T *et in collectionis* Mycothèque de l'Université catholique de Louvain BCCM/MUCL, *cum numero* MUCL 53393^T.

Description of *Zygosaccharomyces gambellarensis* Torriani, Lorenzini, Salvetti & Felis sp. nov.

Zygosaccharomyces gambellarensis (gam.bel.la.ren'sis. L. adj. *gambellarensis* refers to the Gambellara area).

On 5 % malt extract agar medium, after 3 days of incubation at 27 °C, cells are spherical (5 µm diameter) and occur singly, in pairs or in small groups. Budding is multilateral; budding cells remain attached to each other and often form a flower-like arrangement (Fig. 2a). Neither hyphae nor pseudohyphae are produced. Ascospores are observed on agar acetate medium after 15 days at 27 °C. Asci are persistent and contain one to four spheroidal to ovoid ascospores that may appear smooth (Fig. 2b). Ascosporeulation may be preceded by conjugation between two cells and many cells show the 'dumbbell' configuration typical of *Zygosaccharomyces* species. On WL agar, after 7 days of incubation at 27 °C, colonies are small, opaque and raised; colony margins are smooth and regular.

Ferments only glucose and fructose, but not sucrose, maltose or trehalose. Assimilates glucose, glycerol, D-sorbitol,

Fig. 2. Photomicrographs of budding cells (indicated by arrows) of *Z. gambellarensis* ZO03-5^T grown at 27 °C for 3 days on 5% malt extract agar medium (a) and ascospores (indicated by arrows) of *Z. gambellarensis* ZO03-5^T grown at 27 °C for 15 days on agar acetate medium (b). Bars, 10 µm.

D-galactose, sucrose, trehalose, maltose, adonitol and D-xylose. Cellobiose, lactose, raffinose, melezitose, L-arabinose, N-acetyl-D-glucosamine, methyl α -D-glucopyranoside, inositol, xylitol, D-mannitol, sodium D-gluconate, DL-lactate, citrate, melibiose, L-arabinose, D-ribose, L-rhamnose, methanol, ethanol, meso-erythritol and calcium-2-ketogluconate are not assimilated. Growth occurs in the presence of 400 g glucose l⁻¹.

The type strain is ZO03-5^T (=CBS 12191^T=MUCL 53393^T).

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