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Diversity of *Candida zemplinina* isolates inferred from RAPD, micro/minisatellite and physiological analysis

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ABSTRACT

Among non-*Saccharomyces* wine yeasts, *Candida zemlpinina* is one of the frequently isolated and oenologically important species. It is mostly known from European winemaking areas and it has become one of the key species of non-*Saccharomyces* wine yeasts to study. Investigating the diversity of *C. zemplinina* isolates is important for a deeper understanding of the non-*Saccharomyces* wine yeasts and for the yeast starter industry, as numerous researches have pointed to the potential use of this species in winemaking. For assessing the biodiversity of a larger number of strains, RAPD and micro/minisatellite PCR is often the method of choice, however, this technique is often unstandardized. Whereas some laboratories use these methods for species identifications, others apply RAPD primers for determining intraspecies diversity. In this study, we have tested 5 different RAPD and micro/minisatellite primers on strains of *C. zemplinina* isolated from different locations. We show that after a rigorous PCR-optimization aimed at reproducibility and comparability of band patterns with these PCR-reactions, diversity of different strains from a wide range of geographic locations is relatively low. The analysis of several oenologically important physiological traits of the strains showed a relatively low level of diversity as well. We also demonstrate that the intraspecific diversity of *C. zemplinina* observable with different techniques (RAPD, micro/minisatellite or physiological analysis) may be fairly different and not necessarily comparable.

1. Introduction

Candida zemplinina was described by Sipiczki (2003) as a novel, osmo- and psychrotolerant, fructophilic and acidogenic anamorphous yeast species very similar to *Candida stellata* Kroemer and Krumbholz. Both species can be found in grape must prior to fermentation and also during the fermentation process (Nisiotou et al., 2007; Urso et al., 2008; Zott et al., 2008). As it was demonstrated by Csoma and Sipiczki (2008), the similar characteristics and co-occurrence in wine environment caused many strains of *C. zemplinina* to be regarded as members of the *C. stellata* species earlier. The unequivocal discrimination of these species is only possible by molecular techniques (Sipiczki, 2004).

It is more and more widely acknowledged that the yeast ecology of wine must fermentation is a complex process (not exclusively characterized by *Saccharomyces* species) and this view has led to an increasing number of publications on non-*Saccharomyces* wine yeasts and their sequential changes in the grape juice biota. Thus *C. zemplinina* gradually became one of the key species to study in the field of wine microbiology, mostly in that of sweet wines (e.g. Urso et al., 2008; Tofalo et al., 2009; Magyar and Tóth, 2011). *C. zemplinina* seems to be more abundant in the wine musts studied so far, sometimes occurring together with *C. stellata* (Magyar and Bene, 2006), but many times without a detectable *C. stellata* population (Nisiotou et al., 2007; Lopandic et al., 2008; Urso et al., 2008; Tofalo et al., 2009; Andorrà et al., 2010; Esteve-Zarzoso et al., 2010).

C. zemplinina is apparently favoured by low temperatures during fermentation (Zott et al., 2010). The strains of *C. stellata* that were later reclassified as *C. zemplinina* (Csoma and Sipiczki, 2008) were known as fructophilic, enologically important yeasts (Ribéreau-Gayon and Peynaud, 1960; Minárik et al., 1978; Ciani and Ferraro, 1998; Soden et al., 2000; Clemente-Jimenez et al., 2004; Solieri et al., 2006). This fructophilic character of *C. zemplinina* was further emphasized by Mills et al. (2002) and Magyar and Tóth (2011) who also stated that the strains tested by them possessed an extremely poor ethanol yield from the sugar consumed. Recently, the potential usefulness of *C. zemplinina* strains as co-starters (through sequential inoculation) in wine industry was suggested and physiological characterization of dozens of strains was carried out to facilitate the utilization of this species (Tofalo et al., 2009, 2012).

As the latest edition of the "International Code of Nomenclature for algae, fungi and plants" (McNeill et al., 2012) abolished the rule effective for a long time that anamorphic yeasts with ascomycetous affiliation have to be classified to *Candida*, the species was recently

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moved to the genus *Starmerella* leaving the name *C. zemplinina* as obligate synonym (Duarte et al., 2012).

The genetic diversity of *C. zemplinina* in different wine environments is a question that could be interesting in the wine industry, especially in the exploration of sweet wine yeast biota and also in the wine starter industry. Tofalo et al. (2012) noted that the physiological characterization and the assessing of biodiversity in different wine regions and wine types could represent the first step for the selection of *C. zemplinina* strains to be used as starters (in co-culture or in sequential inoculation) to improve the complexity and to enhance the particular characteristics of wines. Indeed, there is growing evidence that non-*Saccharomyces* yeasts can produce significant amounts of aroma compounds (e.g. Jolly et al., 2006; Ciani et al., 2010). Magyar and Tóth (2011) evaluated some enologial properties of several *C. zemplinina* and *C. stellata* wine yeasts and Rantsiou et al. (2012) observed that *C. zemplinina* can reduce the acetic acid production of *Saccharomyces*. However, studies in real winemaking environments or microvinification experiments with *C. zemplinina* are still uncommon.

So far, studies on the diversity of *C. zemplinina* and other non-*Saccharomyces* wine yeasts have been conducted using RAPD-PCR (Random Amplification of Polymorphic DNA) or the related micro/minisatellite-based PCR method (Lopandic et al., 2008; Rantsiou et al., 2012; Tofalo et al., 2012), SAU-PCR (a PCR-based method involving restriction digestion of genomic DNA with the enzyme Sau3AI and selective amplification with primers whose core sequence is based on the Sau3AI recognition site) (Rantsiou et al., 2012), AFLP-fingerprinting (Amplified Fragment Length Polymorphism) (Esteve-Zarzoso et al., 2010) and TRtRNA-PCR (Tandem Repeat-tRNA PCR) (Barquet et al., 2012). So far, of the many widely used micro/minisatellite and RAPD-primers, only a few have been tested for the usefulness for characterization of the diversity of strains within the species *C. zemplinina*. One of these primers, the M13 core sequence (a primer for minisatellite-based PCR), was used to evaluate the autochthonous yeast population during spontaneous fermentations of grape musts in Austrian wine-producing areas, but in this study, only two *Candida zemplinina* isolates and the type strain of the species was tested along with many other *Saccharomyces* isolates and non-*Saccharomyces* wine yeasts. All three *C. zemplinina* strains produced very similar band patterns using this primer. PCR with this primer was found to result in characteristic band patterns when different wine yeast species were compared (Lopandic et al., 2008). The primer M13 was also used to characterize *C. zemplinina* isolates by Tofalo et al. (2009), where among other yeasts, 15 *C. zemplinina* isolates from Italian wine musts were compared and based on the band patterns, 2 clusters and 6 subclusters were differentiated. The two main clusters had a ~80% similarity. The primer M13 was used by Rantsiou et al. (2012) as well, combined with SAU-PCR. In the study conducted by Tofalo et al. (2012), the RAPD-primers R5 and RF2 were used to compare altogether 36 *C. zemplinina* isolates (33 of them Italian isolates). The combined data from the two RAPD-PCRs allowed the differentiation of 6 clusters and interestingly resolved all the 36 strains. The usefulness of other commonly used RAPD-primers in the analysis of genetic diversity among *C. zemplinina* isolates, although many have been tested on other wine-yeast (e.g. Baleiras Couto et al., 1994, 1996; El-Fiky et al., 2012), or in discriminating them from common species found in must, has not been analyzed yet.

Our aim in this study was to characterize *C. zemplinina* isolates of diverse geographical origin with the use of micro/minisatellite and RAPD-PCR and physiological tests to assess biodiversity in the species. By using different RAPD and microsatellite primers, we also aimed to investigate, which of these may be used to characterize the biodiversity of different isolates of the same species and which may be used to easily discriminate this species from the closely related *C. stellata*.

2. Materials and methods

2.1. Yeast strains

Strains used in this study are listed in Table 1. The majority of the strains were described and identified in Sipiczki (2003, 2004) and in Csoma and Sipiczki (2008). Cultures were maintained on YPGA (2% glucose, 2% agar, 1% yeast extract and 1% peptone; all w/v).

2.2. Molecular identification of the strains

Yeast cells were grown aerobically at 24 °C for 1 day. DNA was isolated and purified according to the method described in Hanna and Xiao (2006). The 5.8S internal transcribed spacer (ITS) region was amplified in a thermocycler with primers ITS1 and ITS4 and the D1/D2 domains of the large-subunit (LSU) rRNA genes were amplified with primers NL-1 and NL-4 and sequenced as described by Sipiczki (2003). The BLAST network service of the NCBI database (http://ncbi.nlm.nih.gov/blast) was used for DNA sequence similarity searches with previously undetermined strains, and the sequences of the amplified fragments were also compared to the sequences of the type strains (obtained from the CBS database). The ITS1-5.8S-ITS2 regions were tested with RFLP method. The amplified DNA was digested with the restriction endonucleases *Dra*I and *Mbo*I to discriminate between *C. stellata* and *C. zemplinina*, as described by Sipiczki (2004). D1/D2 domain sequences of newly identified strains are deposited with the following accession numbers: 11-658 (KC846100), 11-148 (KC846099), 11-31 (KC846098), 11-479 (KC846097).

2.3. PCR primers used for the analysis of molecular diversity of the strains

RAPD primers used were primer 24 (5′-GCG TGA CTT G) (Baleiras Couto et al., 1996), primer 1283 (5′-GCG ATC CCC A-3′) (Akopyanz et al., 1992) and RF2 (5′-CGG CCC CTG T-3′) (Paffetti et al., 1995). Mini/microsatellite primers used were M13 (5′-GAG GGT GGC GGT TCT-3′) and $(GTG)_5$ (Lieckfeldt et al., 1993).

2.4. Conditions for micro/minisatellite and RAPD-PCR

Concentration of the genomic DNA for RAPD-PCR was measured with an UVS-99 Micro-Volume UV/Vis Spectrophotometer (ATC-Gene) and DNA-concentrations were set to 100 ng/μl subsequently. For each reaction, 50 ng was used. PCR reactions were performed with the following programmes: 94 °C for 5 min, 30× (94 °C 50 s, T_m 50 s, 72 °C 50 s), 72 °C 5 min. T_m was set to 38 °C for primers RAPD24 and RAPD1283, to 45 °C for primer M13 and to 50 °C for $(GTG)_5$. For RF2, the programme and T_m (36 °C) of Paffetti et al. (1995) used also by Tofalo et al. (2012) was applied. For amplification, 1.5 units of GoTaq® DNA Polymerase was used with GoTaq Green Buffer (Promega) for reactions with primers RAPD24 and RAPD1283 and 1.5 units of DreamTaq® DNA Polymerase with DreamTaq Green buffer (Thermo Scientific) for reactions with primers RF2, M13 and $(GTG)_5$. Both buffers were supplemented with additional $MgCl_2$ (Thermo Scientific) to a 2.5 mM end concentration and with 1 μg of BSA (Biolabs). 25 pmol of primers were used per reaction. PCR reactions were conducted using an Applied Biosystems 2720 thermal cycler in a final volume of 25 μl.

2.5. Gel electrophoresis and analysis

PCR-products were loaded onto 2% (w/v) (reactions with RAPD24, RAPD1283, $(GTG)_5$) or 1.2% (w/v) (RAPD-RF2, M13) agarose gels stained with ethidium-bromide and electrophoresis was carried out with 90 V for 60 min in 1× TBE buffer, visualization