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Yeast diversity on grapes in two German wine growing regions

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ABSTRACT

The yeast diversity on wine grapes in Germany, one of the most northern wine growing regions of the world, was investigated by means of a culture dependent approach. All yeast isolates were identified by sequence analysis of the D1/D2 domain of the 26S rDNA and the ITS region. Besides *Hanseniaspora uvarum* and *Metschnikowia pulcherrima*, which are well known to be abundant on grapes, *Metschnikowia viticola*, *Rhodosporidium babjevae*, and *Curvibasidium pallidicorallinum*, as well as two potentially new species related to *Sporidiobolus pararoseus* and *Filobasidium floriforme*, turned out to be typical members of the grape yeast community. We found *M. viticola* in about half of the grape samples in high abundance. Our data strongly suggest that *M. viticola* is one of the most important fermenting yeast species on grapes in the temperate climate of Germany. The frequent occurrence of *Cu. pallidicorallinum* and strains related to *F. floriforme* is a new finding. The current investigation provides information on the distribution of recently described yeast species, some of which are known from a very few strains up to now. Interestingly yeasts known for their role in the wine making process, such as *Saccharomyces cerevisiae*, *Saccharomyces bayanus* ssp. *uvarum*, *Torulaspora delbrueckii*, and *Zygosaccharomyces bailii*, were not found in the grape samples.

1. Introduction

Comprehensive overviews of the current knowledge on yeast diversity on grapes are given by Barata et al. (2012), Fleet et al. (2002) or Deak (2007). Unfortunately a great part of the older studies on yeast communities on grapes suffer from taxonomic uncertainty and the almost unmanageable number of synonyms. For example before *Candida zemplinina* was described in 2003 (Sipiczki, 2003), a lot of the strains belonging to this species were misidentified as *Candida stellata* (Csoma and Sipiczki, 2008). For this reason it is very difficult to draw conclusions about the frequency of *C. stellata* on grapes from literature published before the description of *C. zemplinina*. This problem will not be alleviated with the recent renaming of this species as *Starmerella bacillaris* (Duarte et al., 2012).

A new species belonging to the genus *Metschnikowia*, *Metschnikowia viticola*, was isolated from grapes in Hungary. *M. viticola* is a genetically well separated species within the genus *Metschnikowia*. Until now very little is known about the distribution of *M. viticola* since the species description (Peter et al., 2005) was based on two strains from one grape sample only and the species has been detected only a very few times since then.

Many new species have recently been described in the *Metschnikowia pulcherrima* clade. These include *Metschnikowia chrysoperlae* (Suh et al., 2004), *Metschnikowia fructicola* (Kurtzman and Droby, 2001) and *Metschnikowia andauensis* (Molnar and Prillinger, 2005), among others. The difficulties associated with the species delimitation in the *M. pulcherrima* clade were discussed by Lachance (2011). The finding of Sipiczki et al. (2013) that the type strains of *M. andauensis* and *M. fructicola* possess divergent copies of the rDNA gene will lead to further investigations of the species concept in the clade.

The examples given above emphasize the importance of unambiguous yeast identification in any study of the yeast diversity of grapes. Because of the numerous changes in yeast taxonomy in the last decades and due to the fact that no studies of grape yeast diversity have been conducted in Germany based on state-of-the-art methods for yeast identification, it seems appropriate to reinvestigate this area. The current investigation focuses on the identification of frequent and abundant yeast species found on grapes in Germany.

2. Material and methods

2.1. Sampling sites and sampling

Six samples were taken in the vineyards of the winery "Weingut Maximin Grünhaus Schlosskellerei C. von Schubert" in the Ruwer valley in the Mosel vine growing region of Germany. All other samples were taken in vineyards of the village of Ochsenbach, located in the Kirbach valley, which is part of the wine growing region Württemberg, Germany. Samples were taken from 10 different grape varieties. Healthy and sour rotten grapes were collected separately. From each of the grape varieties healthy grapes were collected only at the stage of full ripeness just before harvest. In total 18

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grape samples were taken. Healthy and rotten grapes were collected separately. A bunch was regarded as healthy if not a single berry was infected or damaged. Bunches regarded as rotten contained more than 3/4 of heavily infected berries. In total 12 samples consisted of healthy grapes and 6 samples of sour rotten grapes (Table 1). Samples were taken aseptically into sterile plastic bags, stored in the cold, and processed in the laboratory within 12 h. At each sampling site grapes were collected from areas measuring approximately 50 × 50 m. The vines from which the grapes were taken were equally distributed in the sampling areas. 20 grapes were collected per sampling site.

2.2. Yeast isolation

The grapes were crushed thoroughly by hand in the closed plastic bags and shaken for 5 min. One hundred microliters of the juice was diluted 1:1000 and 1:10,000 with sterile tap water. One hundred microliters of the dilutions were plated on commercial rose bengal agar with chloramphenicol. The plates were incubated for one week at 22 °C. After incubation all plates were examined under a binocular loupe. The number of colonies with similar morphologies was noted. For each colony type one representative colony was transferred to GYP agar. In case of doubt, similar colonies were subcultured and identified separately. The cultures were purified by streaking and stored at −60 °C in 15% glycerol.

2.3. Identification of yeast cultures

All strains were identified by sequencing of the D1/D2 domain of the small subunit rRNA gene. When necessary the ITS region (ITS1-5,8s-ITS2) was analyzed also. The D1/D2 domain and the ITS region were amplified as described before by Kurtzman and Robnett (1998) and Fell et al. (2000). Sequencing of the PCR products was done by Seqlab GmbH, Göttingen, Germany. For strain identification, the local pairwise alignment search offered on the The Centraalbureau voor Schimmelcultures (CBS) Fungal Biodiversity Centre's (The Netherlands) homepage (http://www.cbs.knaw.nl/Collections/) was performed. The sequence of the closest related type strains was compared with the respective sequence of our own strains. If deemed necessary additional sequences of other strains described in the literature were included in the comparison. Phylogenetic relationships were calculated with the maximum likelihood method and the Jukes Cantor nucleotide substitution model. Bootstrap values were calculated from 1000 iterations.

The relative abundance of the species for each sample was calculated by the number of colony forming units (cfus) of each species divided by the total number of cfu. The frequency of each species was calculated by the number of samples containing a certain species divided by the total number of grape samples.

3. Results

3.1. Overview of results

A total of 146 yeast strains belonging to 23 species were isolated from 18 samples derived from 10 grape varieties. Table 2 provides an overview of the species found, their frequency, and their relative abundance. All strains which were isolated from different grape samples and which belong to separate species or represented a different genotype within the same species were deposited at the CBS culture collection. For each species the number of differences in the sequence of the D1/D2 domain and the ITS region compared to those of the closest related type strain is given. The sequences of the D1/D2 domain and the ITS region were deposited at GenBank. CBS strain numbers and GenBank accession numbers are included in Table 2.

Table 1 gives an overview of the samples, their origin, the sanitary state, the grape variety, the number of isolates retrieved from each sample, and the number of species detected per sample. The number of species per sample ranged from 3 to 9.

3.2. Basidiomycetes

About four fifth of the samples harbored strains with identical D1/D2 sequence belonging to the *Rhodotorula glutinis* sensu stricto group. In their D1/D2-sequences these strains were identical with the type strain (CBS 2826) of *Rhodotorula graminis* and showed one substitution compared to the type strains of *Rhodosporidium babjevae* (CBS 7808) and of *Rh. glutinis* (CBS 20). In their D1/D2 sequence our strains were in full agreement with compatible mating partners (e.g. CBS 7809, CBS 9072 or CBS 322) of the type strain (CBS 7808) of *R. babjevae* (Sampaio, 2011). Thus the strains could not be unequivocally assigned to a one of the species in the *Rh. glutinis* sensu stricto group based on D1/D2 sequence analysis only. Therefore we analyzed the ITS region as well.

The ITS region of our strains resembled the type strain of *R. babjevae* (CBS 7808) and *Rh. glutinis* (CBS 20) closely showing only 3–4 differences. From the type strain of *Rh. graminis* (CBS 2826) our strains differed at 5–6 sites. From strain CBS 7809 the mating partner of strain CBS 7808 our strains differed by 1–2 substitutions only.

Table 1
Grape varieties from which the samples were retrieved, grape sanitary state, number of isolates per sample, number of species per sample, location where the sample was taken and sampling date.

Grape variety	Grape sanitary state		Number of isolates per sample	Number of species per sample	Location		Sample date
	Healthy	Sour rotten			Kirbach Valley	Ruwer Valley	
Müller-Thurgau	x		7	4	x		25.09.2014
Pinot meunier	x		6	4	x		25.09.2014
Portugieser	x		6	6	x		25.09.2014
Müller-Thurgau	x		3	3	x		06.10.2014
Müller-Thurgau		x	13	5	x		06.10.2014
Sauvignon blanc	x		9	5	x		06.10.2014
Samtrot	x		12	6	x		06.10.2014
Portugieser	x		13	8	x		06.10.2014
Auxerrois	x		7	6		x	11.10.2014
Auxerrois		x	7	5		x	11.10.2014
Pinot blanc		x	6	6		x	11.10.2014
Pinot noir	x		7	6		x	11.10.2014
Pinot noir		x	10	9		x	11.10.2014
Pinot noir	x		7	5		x	11.10.2014
Pinot meunier		x	8	7	x		14.10.2014
Pinot meunier	x		9	7	x		14.10.2014
Gewürztraminer		x	9	8	x		14.10.2014
Trollinger	x		4	4	x		14.10.2014