Highlights

- We study yeast ‘cultivable’ biodiversity from grapes to wine in “Uva di Troia” (UdT).
- UdT is an Apulian autochthonous variety present in several regional wines.
- It is the first ‘grape to wine’ study on yeasts from Southern Italian production.
- We report and discuss the variation of yeast diversity in two geographical sites.
- We suggest the need of local based formulation for autochthonous starter cultures.

Running title: yeasts biodiversity and autochthonous grape variety

From grape berries to wine: population dynamics of cultivable yeasts associated to "Nero di Troia" autochthonous grape cultivar

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Keywords: Wine, *Saccharomyces cerevisiae*, non-*Saccharomyces*, starter cultures, autochthonous.

Abstract

The aim of this work was to study the biodiversity of yeasts isolated from the autochthonous grape variety called “Uva di Troia”, monitoring the natural diversity from the grape berries to wine during a vintage. Grapes were collected in vineyards from two different geographical areas and spontaneous alcoholic fermentations were performed. Different restriction profiles of ITS–5.8S rDNA region, corresponding to *Saccharomyces cerevisiae, Issatchenkia orientalis, Metschnikowia pulcherrima, Hanseniaspora uvarum, Candida zemplinina, Issatchenkia terricola, Kluyveromyces thermotolerans, Torulaspora delbrueckii, Metschnikowia chrysoperlae, Pichia fermentans, Hanseniaspora opuntiae* and *Hanseniaspora guilliermondii*, were observed. The yeast occurrences varied significantly from both grape berries and grape juices, depending on the sampling location. Furthermore, samples collected at the end of alcoholic fermentation (AF) revealed the great predominance of *Saccharomyces cerevisiae*, with a high intraspecific biodiversity. This is the first report on the population dynamics of ‘cultivable’ microbiota diversity of “Uva di Troia” cultivar from the grape to the corresponding wine (“Nero di Troia”), and more general for Southern Italian oenological productions, allowing us to provide the basis for an improved management of wine yeasts (with both non-*Saccharomyces* and *Saccharomyces*) for the production of typical wines with desired unique traits. A certain geographical-dependent variability has been reported, suggesting the need of local based formulation for autochthonous starter cultures, especially in the proportion of the different species/strains in the design of mixed microbial preparations.

Keywords: yeast; wine; biodiversity; non-*Saccharomyces*; autochthonous starter cultures

1. Introduction

The indigenous microbiota is very important in winemaking process, in reason of the possible positive or negative effects on wine quality. In particular, yeasts are essential for the carrying out of the alcoholic fermentation (AF), promoting the transformation of grape sugars into ethanol, carbon dioxide and hundreds of other metabolites (Romano *et al*. 2003a). Starter cultures based on selected strains of *Saccharomyces cerevisiae* are usually added by oenologists to control the fermentative process, in order to dominate yeasts belonging to the vineyard environment, winery facilities and cellar equipment. The International Organization of Vine and Wine (OIV) affirmed that *terroir* refers to 'an area in which collective knowledge of the interactions between the identifiable physical and biological environments and applied viticulture and oenological practices develops, giving distinctive characteristics for the products originating from this area' (International Organization of Vine and Wine 2010). The definition of "*terroir*" represents the foundation of the Appellation of Origin, with impact on the wine market and consumer choices. It has been demonstrated that the non-*Saccharomyces* yeasts contribute to wine qualities (Ciani et al. 2010; Jolly et al. 2014). Different studies have highlighted the important role of the microbiota associated with the "*terroir*" from which the grapes are grown, able to impart a unique quality to the wine (e.g. Csoma et al. 2010; Di Maio et al. 2012). In the grape/wine environment, Bokulich et al. (2014) have studied the "*microbial terroir"* and they showed the existence of a close relationship between microbial patterns, region of production and climate. On the above basis, an increasing number of scientific investigations have focused the attention on the cultivable micro-biodiversity connected with spontaneous fermentation, in order to select indigenous strains, displaying positive technological properties and quality traits, for their application in industrial fermentations (e.g. in Apulian region Cappello et al., 2008; Capozzi et al 2010; 2012; Grieco et al. 2011; Tristezza et al. 2012, 2013, 2014; Garofalo et al. 2015).

During the spontaneous fermentation process, a dynamics of different yeast species occur: the non-*Saccharomyces* yeasts (mainly belonging *Hanseniaspora*/*Kloeckera*, *Candida*, *Pichia*, *Zygosaccharomyces*, *Schizosaccharomyces*, *Torulaspora*, *Kluyveromyces* and *Metschnikowia genera*) dominate the beginning of AF and then they are replaced by *Saccharomyces cerevisiae* that complete sugars conversion in ethylic alcohol (Fleet 2008; Ciani et al. 2010; Jolly et al. 2014).

The selection of non-*Saccharomyces* is very important for the preparation of new starter cultures, since they are able to produce several secondary compounds that can have a positive influence on the quality of the wine (Fleet 2008; Ciani et al. 2010; Bely et al. 2008; De Benedictis et al., 2011). In fact, non-*Saccharomyces* species may also have an application to improve the wine technological proprieties and to enhance the unique sensorial qualities of typical productions (Fleet 2008; Ciani et al. 2010; Bely et al. 2008; De Benedictis et al. 2011;). Non-*Saccharomyces* can also be used as agents for the biological control of moulds or spoilage microorganism, such as lactic acid bacteria or *Brettanomyces bruxellensis* (Capozzi et al. 2015; Oro et al. 2014). However, non-*Saccharomyces* utilization is also associate with such as production of biogenic amines, off-flavors (acetic acid, esters, acetaldehydes, H_2S) and with competition for the nutrients availability with *S. cerevisiae* strains able to complete AF (Capozzi et al. 2015).

Even though, several studies had been already performed in order to characterized autochthonous microbes from Apulian wines (Capozzi et al 2010, 2011; Grieco et al., 2011; Tristezza et al., 2012, 2013, 2014; Garofalo et al 2015), the aim of this work was to study, for the first time in a Southern Italian wine, the biodiversity of 'cultivable' yeasts isolated from the grapes ("Uva di Troia", an autochthonous regional variety common denominator of several wines produced in North-Apulian region) up to corresponding wines (so called "Nero di Troia").

2. Material and methods

2.1 Yeast isolation from grape berries, musts and wines

Grape berries were directly collected in the vineyard with the aim to avoid contamination yeast the commercial culture strains used in the cellar. For the yeast isolation, 1.00 Kg of grape berries were collected aseptically in North Apulia area from two vineyards (Lucera and Ascoli Satriano areas, see Figure 1) (18° Babo, 0.25 g/L total acidity, 4 g/L malic acid, pH 3.8, free ammonium 165 mg/L and 17° Babo, 0.3 g/L total acidity, 3.6 g/L malic acid, pH 3.8 free ammonium 155 mg/L, respectively for Lucera and Ascoli Satriano area). The grape were pressed for 20 minutes using a Bag Mixer® (Interscience, France), then spontaneous fermentation of grape juices were carried out in laboratory at 28 °C temperature and monitored over 1 month. Yeast sampling were accomplished at different stages, first from grape berries surface, then during alcoholic fermentation, at the beginning and at the end of fermentation, which were determined on the basis of alcohol content, about 1%, at the beginning of AF, and 9%, in the final phases of AF. Yeast from grape surface were isolated according to method of Prakitchaiwattana et al. (2004), Fifty grams of berries were rinsed in 450 ml of 0.1% peptone water with 0.01% Tween 80 by orbital shaking in a flask at 150 rpm for 30 min. Aliquots of 0.1ml from serially diluted samples in physiological solution were plated either on Wallerstein Laboratory (WL) and on nutrient agar (Oxoid, USA) and Lysine medium (Oxoid, USA), both added with 10 mg/L chloramphenicol, that respectively allowed the isolation and identification of non-*Saccharomyces* and *Saccharomyces* species. Selection of non-*Saccharomyces* isolates were chosen on the basis of their different colony morphology, whereas the *Saccharomyces* strains were isolated randomly.

2.2 RFLP analysis and sequencing of 5.8S rRNA gene and the two ribosomal internal transcribed region

The RFLP analysis of 5.8S rRNA gene and the two ribosomal internal transcribed spacer was performed according to method of Esteve-Zarzoso et al. (1999), with some modifications. The

Amplification reaction were performed using PCR reaction mix containing 0.5 µM of each primer (ITS1 and ITS4), 200 µM dNTP, buffer 10X, solution Q and 1.25 unit Taq DNA Polymerase (Taq PCR Core; Qiagen, USA). PCR was performed in a thermocycler (I-Cycler, Bio-Rad), using the following program: initial denaturation at 95 °C for 10 min, followed by 35 cycles of denaturing at 94 °C for 1 min, annealing at 55.5 °C for 2 min and extension at 72 °C for 2 min; and a final extension at 72 °C for 10 min, then samples were conserved at 4 °C. Amplification products were previously analysed on 2% agarose gels, with 1x TBE buffer and stained with ethidium bromide. After electrophoresis, gels were visualized under UV light and photographed (Versa Doc, BIO-RAD). Sizes were estimated by comparison against a DNA length standard (50 bp ladder; Promega, USA) with Quantity One Software (Bio-Rad, USA). Then PCR products were digested without further purification with the fast restriction endonucleases *HaeIII*, *HhaI*, *HinfI* and *DdeI* (Thermo Scientific, USA), following the manufacture's instruction. The restriction fragments were separated on 3% agarose gel with 1X TBE buffer and stained with ethidium bromide. For each sampling point, two PCR products obtained with primers ITS1-ITS4 for each obtained pattern were randomly selected and sequenced (PRIMM, Italy) to confirm the specie assignment.

2.3 Genetic characterization of *Saccharomyces cerevisiae* strains

The genetic variability of *S. cerevisiae* isolates was evaluated by amplification of δ region, using the primers δ12 (5'TCAACAATGGAATCCCAAC3') and δ21 (5'-CATCTTAACACCGTATATGA-3') (Legras and Karst 2003). The protocol described by Capece et al. (2012) was adopted with some modifications. The amplification of δ region was performed directly from the colony, using a reaction mix containing 1 µM primers (δ12 and δ21) and 1.5 unit of Taq DNA Polymerase (Qiagen, USA). The PCR conditions were the following: initial denaturation at 97° C for 10 min, then reaction mixture was cycled 35 times with 30 s denaturation at 94° C, 1 min primer annealing at 42° C and 2 min primer extension at 72° C, followed by a 10-min final extension step at 72° C. After

electrophoresis gel were visualized under UV light, scanned with (Versadoc System; Bio-Rad, USA) and analysed by using the FP Quest TM software (BioRad, USA). The electrophoresis patterns were grouped, and analysed for the similarity and cophenetic correlations through the Dice coefficient. Cluster analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA). Cophenetic correlation was the measure of how faithfully the tree represents the dissimilarities among observations.

2.4 Statistical analyses

Molecular data has been analyzed by One-way ANOVA, Turkey test ($P < 0.005$). Ecological indices, such as the Shannon-Wiener index of general diversity (H), the richness (S) of the microbial community, Simpson's diversity indices (D and 1-D) and Evenness (e^H/S) were calculated according to Tristezza et al., 2013. All statistical analyses were performed using Past, version 3.05 (Hammer et al. 2001).

3. Results

3.1 Yeast species identification from grape berries

Samples were collected from two different vineyards located in north Apulia region (**Figure 1**) during vintage 2012. A total of 136 colonies were isolated from grape berries of Uva di Troia variety and subjected to a PCR-RFLP analysis of the 5.8SITS rDNA region. The yeast species identified and the isolation frequencies obtained are shown in **Table 1**. The PCR products, showing variations in length ranging from 400 to 880 bp, were digested with *Hha*I (*Cfo*I), *Hae*III, *Hinf*I and *Dde*I enzymes. The produced fragments were compared with those described previously in literature (Esteve-Zarzoso et al. 1999). In general, we observed 12 different restriction profiles of ITS–5.8S rDNA region, corresponding to *Saccharomyces cerevisiae, Issatchenkia orientalis,*

175 *Metschnikowia pulcherrima, Hanseniaspora uvarum, Candida zemplinina, Issatchenkia terricola,*
176 *Kluyveromyces thermotolerans, Torulaspora delbrueckii, Metschnikowia chrysoperlae, Pichia*
177 *fermentans, Hanseniaspora opuntiae* and *Hanseniaspora guilliermondii* (Table 1). Two ITS
178 fragments for each obtained pattern were randomly selected and sequenced and the obtained data
179 were compared with sequences available at the NCBI database (GenBank) using the standard
180 nucleotide_nucleotide homology search Basic Local Alignment Search Tool (BLAST,
181 http://www.ncbi.nlm.nih.gov/BLAST) (corresponding gene accession numbers are reported in
182 Table 1). Several yeast species such as *M. pulcherrima, C. zemplinina, H. guilliermondii, H.*
183 *uvarum* and *I. terricola* represented a common denominator of the two vineyards studied (Table 1).
184 The **Figure S1** reportes the frequencies of strains identified from grape berries from the two
185 different vineyards, during vintage 2012.
186 Among the non-*Saccharomyces* characterized in this study, the most abundant genera on berries
187 surface were *Hanseniaspora* (about *H. uvarum* 22%, *H. guilliermondii* 13%, and *H. opuntiae* 1%)
188 and *Metschnikowia* (35%, *M. pulcherrima* 34% and *M. chrysoperlae* 1%) (Figure S1). The analysis
189 of non-*Saccharomyces* diversity in the two different areas revealed a great variability, showing, in
190 several cases, statistically significant differences among locations (Figure S1). *S. cerevisiae, K.*
191 *thermotolerans, T. delbrueckii, M. chrysoperlae, P. fermentans, I. orientalis and H. opuntiae* were
192 isolated only from grape berries collected from Lucera (respectively 5, 7, 1, 1, 3, 1, and 1 %). *H.*
193 *guilliermondii* was isolated only from Ascoli Satriano (about 26%) samples. The frequency of *M.*
194 *pulcherrima* showed differences between Lucera (42%) and Ascoli Satriano (28%) vineyards. *H.*
195 *uvarum* ecotypes have been isolated with higher frequency from Ascoli Satriano (about 36%),
196 rather than in Lucera vineyards (only 7%). *C. zemplinina* and *I. terricola* frequency did not show
197 significant changes (respectively about 10 and 2%).
198
199 **3.2 Yeast species identification from fermenting grape juice**

A total of 133 colonies were isolated from grape juice at the beginning of alcoholic fermentation (about 1% EtOH) and subjected to a PCR-RFLP analysis of the 5.8SITS rDNA region as above described. In general, we observed 11 different restriction profiles of ITS–5.8 S rDNA region, corresponding to *S. cerevisiae, I. orientalis, M. pulcherrima, H. uvarum, C. zemplinina, I. terricola, K. thermotolerans, T. delbrueckii, P. fermentans, H. opuntiae* and *H. guilliermondii* (**Table 2**). The differences in yeast frequency and diversity highlighted in the two locations studied might also be addressable to dissimilarities in composition (data reported in material and method section, 2.1), in fact grape juice obtained from grape collected in Lucera area showed higher sugars and free ammonium contents. As described above, two ITS fragments for each obtained pattern were randomly chosen, sequenced and subjected to comparative analysis to confirm species assignation (Table 2). Several yeast species such as *S. cerevisiae, M. pulcherrima, C. zemplinina, H. uvarum* and *I. terricola* represented a common denominator between the studied vineyards. Otherwise, some species were isolated only from one vineyard, respectively *I. orientalis, K. thermotolerans, T. delbrueckii, P. fermentans, H. guilliermondii* and *H. opuntiae* from Lucera (Table 2). The predominance of non-*Saccharomyces* yeasts was observed for all the samples analyzed, nevertheless *S. cerevisiae* strains has been isolated from both vineyards studied, their frequencies were higher in Lucera (about 33%) than Ascoli Satriano (about 10%). The **Figure S2** describes the frequencies of strains identified from grape juice from the two different vineyards, during vintage 2012.

Among the non-*Saccharomyces* characterized in this study, the most abundant genera at the beginning of AF were *Hanseniaspora* (about 38%, *H. uvarum* 35%, *H. guilliermondii* 1.5%, and *H. opuntiae* 1.5%) and *Metschnikowia* (*M. pulcherrima* 25%) (Figure S2). The analysis of non-*Saccharomyces* diversity in the two different areas revealed a great variability, showing, in several cases, statistically significant differences among locations (Figure S2). *I. orientalis, K. thermotolerans, T. delbrueckii, P. fermentans, H. opuntiae and H. guilliermondii* were isolated only

from grape juice collected from Lucera (respectively 1.5, 5, 3, 1.5, 3 and 3 %). The presence of *M. pulcherrima* is different in Lucera and Ascoli Satriano vineyards, respectively 14 and 4%, furthermore significant differences were reported also with total frequency (about 9%). *H. uvarum* ecotypes have been isolated with higher frequency from Ascoli Satriano (about 42%), contrariwise it frequency was about 28% in Lucera vineyards. Its frequency was not comparable with those reported for the totality of yeast isolated. *C. zemplinina* frequency showed significant differences from Lucera and Ascoli Satriano vineyard, respectively 6 and 42%. As reported in Table 3, the species richness was highest in the yeast population from Lucera (S=12) than in the population from Ascoli Satriano (S=6). However, biodiversity not rely merely on the numbers of species but likewise on its relative abundance and dominance. The Shannon diversity index (H), that takes into account the number of individuals as well as number of taxa, was higher for the yeast population from Lucera (H=1.793) being representative of a more diverse community than that from Ascoli Satriano (H=1.512) (Tab. 3). Moreover, the Evenness index measures the uniformity with which individuals are divided among the taxa present in the population. This index was higher in Ascoli Satriano yeast community than in Lucera population, with values of 0.7559 and 0.5005 respectively (Tab.3).

3. 3 Yeast species identification from wines and genetic characterization of *Saccharomyces cerevisiae* strains

In the final phases of AF (9% ethanol content), we selected only *S. cerevisiae* strains (Table S1). A total number of 146 yeast isolates identified as *S. cerevisiae* were subjected to genotypic characterization by analysis of δ sequences. These ecotypes had been isolated from the first stage of alcoholic fermentation and in the last phases (Tab. S1). PCR analysis of inter-delta region produced 119 different profiles (Table S**1**). The relationship among strains according to patterns obtained

249 with amplification of inter-delta region was evaluated using cluster analysis. According to the
250 resulting dendrogram (Figure 2), the strains were distributed in 10 main similarity groups. Only the
251 groups C, D and E include strains of the same isolation area, respectively Lucera and Ascoli
252 Satriano all collected from wine. Contrariwise, other groups contain strains collected from grape
253 berries, grape juice and wine of the two vineyards studied. Cluster B contain only one strain,
254 isolated from wine collected from Ascoli Satriano vineyard. In addition, 5 groups including strains
255 with identical profiles were found. Identical profiles generally has been isolated in the same area,
256 with the exception of profile 16, obtained from two strains collected from both the vineyards
257 studied.
258 The two *S. cerevisiae* populations from Lucera and Ascoli Satriano showed low indices of
259 dominance (D= 0.2596) and relative high diversity (H= 1.528 and 1.640, respectively; Table 4).

260

261 **4. Discussion**

262 Non-*Saccharomyces* yeasts isolated from grapes, musts and wines show potential effects on the
263 organoleptic qualities of the final products (Romano et al. 2003a; Ciani et al. 2010). A major
264 understanding of non-*Saccharomyces* biodiversity in fermenting wines is an essential criterion for
265 quality improvement programs in the oenological productions, and more specifically in the sector of
266 typical wine and oenological geographical indications (Fleet et al. 2008). In the present study, for
267 the first time in a Southern Italian wine, we study the biodiversity of 'cultivable' yeasts isolated
268 from the the grapes ("Uva di Troia", an autochthonous regional grape variety common denominator
269 of several wines produced in North-Apulian region) up to corresponding wines (so called "Nero di
270 Troia").
271 The majority of the strains isolated belong to *M. pulcherrima*, a species common on wine grapes at
272 the time of harvest and in grape must during the early stages of wine fermentation. This species

occurs more frequently on damaged berries, on berries used to produce ice wine, and in botrytized (noble-rotted) wines (Oro et al. 2014). Several authors have investigated the potentiality of *M. pulcherrima* for wine fermentation. In particular, the absence of relevant changes in fermentation rate and chemical composition has been often observed (Jolly et al. 2014; Comitini et al. 2010). Furthermore, Comitini et al. (2010) noted in the final wines a significant decrease in volatile acidity and in total acidity. Other yeast of oenological interest isolated from grape surfaces of "Uva di Troia" belonged to *Hanseniaspora* spp., mainly *H. guilliermondii* and *H. uvarum*. Our results confirmed findings previously reported on literature, showing that the apiculate *H. uvarum/K. apiculata* may be the predominant species on either the berries and at the beginning of spontaneous must fermentations (e.g. Fleet 2008; Tristezza et al. 2013). All samples collected at the beginning of AF show the predominance of non-*Saccharomyces* yeast, nevertheless *S. cerevisiae* have a high frequency, in both Lucera and Ascoli Satriano vineyard. These evidence might be addressable to the presence of damaged grape berries that may be very rich depositories of *S. cerevisiae* (e.g. Nisiotou et al. 2007; Barata et al. 2012).

The majority of the strains isolated at the beginning of AF belong to *Hanseniaspora* spp., in particular *H. uvarum*. Other yeast well represented on grape juice at the beginning of AF are *Candida spp.* Among *Candida spp.* the species most important identified is *C. zemplinina.* Several yeast ecology studies demonstrated the frequent presence of this species in wine fermentations (e.g. Nisiotou et al. 2007; Urso et al. 2008; Zott et al. 2008, 2012; Tofalo et al. 2009), is a typical contaminant of botrytized juice fermentations but its presence is also common onto healthy grapes (Barata et al. 2012). In terms of yeast natural biodiversity, strains collected from grape juice are similar to those found in other wine-producing areas. Several authors reported the predominance of *Candida* and *Hanseniaspora* genera at the beginning of spontaneous AF (Bezerra-Bussoli et al. 2013; Garofalo et al. 2015), nevertheless Cordero-Bueso et al. (2012) suggested that other non-

297 *Saccharomyces* yeast such as *Lachancea*, *Wickerhamomyces* and *Torulaspora* can be present as
298 dominant species.
299 Among the species belonging to the *Hanseniaspora* genera, our results suggest the dominance of *H.*
300 *uvarum*, confirming those reported by Ocón et al. (2010). Contrariwise, Garofalo and coworkers
301 (2015) reported major frequency of *H. guilliermondii* analyzing Apulian regional wines.
302 Yeast isolated from wine, at the end of AF, show the predominance of *Saccharomyces* spp. (i.e. *S.*
303 *cerevisiae*). Our findings confirming those reported by other authors (e.g. Tristezza et al. 2009), that
304 suggested the rapidity, reproducibility and sensibility of this method.
305 Several studies suggested the important role of indigenous non-*Saccharomyces* and *Saccharomyces*
306 yeast on wine quality. For this reason, multi-starter cultures designed using autochthonous
307 microbial resources has been suggested as a tool to take advantage of natural biodiversity,
308 enhancing the complexity and specific characteristics of wine (Romano et al. 2003b; Ciani et al.
309 2006; Ciani et al., 2010; Jolly et al. 2014; Garofalo et al. 2015).
310 This is the first report on the population dynamics of 'cultivable' microbiota diversity of "Uva di
311 Troia" cultivar from the grape to the corresponding wine ("Nero di Troia"), and more general for
312 Southern Italian oenological productions. We also select possible candidates for the design of
313 mixed/multi-strains autochthonous starter cultures for typical Apulian wines, in order to obtain a
314 final product characterized by unique peculiarities as result of the autochthonous virtuous microbial
315 biodiversity. A certain geographical-dependent variability has been reported, suggesting the need of
316 local based formulation for tailored starter cultures for typical wines, especially in the proportion of
317 the different species/strains in the conceiving of mixed microbial preparations.
318
319 **Acknowledgements**
320 This research was supported by the Apulian Region in a grant from Project 6N7AD82
321 "Autochthonous2Autochthonous: risorse microbiologiche per vini in purezza da vitigni autoctoni (e

322 per produzioni biologiche" and by the framework of the "OenoMicroManagement" project (PIF no.
323 94750304571). Vittorio Capozzi is supported by a grant by the Apulian Region in the framework of
324 'FutureInResearch' program (practice code 9OJ4W81).
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Captions to figures

Figure 1. Geographical localization of the two sampled vineyards.

Figure 2. Cluster analysis of the profiles obtained by PCR inter-delta region from 146 *Saccharomyces cerevisiae* strains (92% of similarity).

Captions to Supplementary Figures.

Figure S1. Frequency (%) of different yeasts species isolated from grape berries of “Uva di Troia”, during vintages 2012. Open bars, Ascoli Satriano vineyards; black bars, Lucera vineyards; light grey bars, total yeast identified. Different letters in superscript bars indicate statistical significance (One-way ANOVA, Turkey test $P < 0.005$).

Figure S2. Frequency (%) of different yeasts species isolated from spontaneous fermentation of grape juice of “Uva di Troia”, during vintages 2012. Open bars, Ascoli Satriano vineyards; black bars, Lucera vineyards; light grey bars, total yeast identified. Different letters in superscript bars indicate statistical significance (One-way ANOVA, Turkey test $P < 0.005$).

Table 1. Identification of yeasts isolated from grape berries obtained by ITS-RFLP and comparative analysis of their ITS1-5.8S-ITS4 region. (*) The accession numbers correspond to two ITS1-ITS4 PCR products randomly selected and sequenced for each obtained pattern to confirm the specie assignment.

Species [1]	ITS	Restriction fragments				N° isolates	Origin	Accession numbers
		CfoI	*HaeIII*	*HinfI*	*DdeI*			
Saccharomyces cerevisiae	880	385+365	320+230+180+150	365+155	/	3	Lucera	KT029756-KT029757
Issatchenkia orientalis	500	185+ 170+ 69+ 56	370+ 90	225+ 160+ 145	/	1	Lucera	KT029781
Metchinkowia pulcherrima	400	205+100+95	280+100	200+190	/	28	Lucera	KT029783-KT029784
Hanseniaspora uvarum	750	320+310+105	750	350+200+180	300+180+95+90+85	5	Lucera	KT029770-KT029771
Candida zemplinina	475	215+110+80+60	475	235+235	/	7	Lucera	KT029748-KT029749
Issatchenkia terricola	450	130+100+90+85+45	290+125	240+105+105	/	2	Lucera	KT029791-KT029792
Kluyveromyces thermotolerans	700	315+285+95	310+215+90+90	355+354	/	9	Lucera	KT029796-KT029797
Torulaspora delbrueckii	800	330+220+150+100	800	410+380	/	2	Lucera	KT029800-KT029801
Metchinkowia chrysoperlae	360	200+90+80	350+110	180+160	/	1	Lucera	KT029765
Pichia fermentans	450	170+100+100+80	340+80+30	250+200	/	4	Lucera	KT029804-KT029805
Hanseniaspora opuntiae	750	320+310+120	750	340+190+170+60	360+180+180+85+70+50	1	Lucera	KT029778
Metchinkowia pulcherrima	400	205+100+95	280+100	200+190	/	19	Ascoli Satriano	KT029785-KT029786
Hanseniaspora uvarum	750	320+310+105	750	350+200+180	300+180+95+90+85	25	Ascoli Satriano	KT029772-KT029773
Candida zemplinina	475	215+110+80+60	475	235+235	/	6	Ascoli Satriano	KT029750-KT029751
Issatchenkia terricola	450	130+100+90+85+45	290+125	240+105+105	/	1	Ascoli Satriano	KT029793
Hanseniaspora guilliermondii	750	320+310+105	750	350+200+180	380+180+95+80	18	Ascoli Satriano	KT029766-KT029767

[1] Species assignation according to Esteve-Zarzoso *et al*., (1999)

Table 2. Identification of yeast isolated from grape juice, sampled at the beginning of the alcoholic fermentation, profiles obtained by ITS-RFLP and comparative analysis of their ITS1-5.8S-ITS4 region. (*) The accession numbers correspond to two ITS1-ITS4 PCR products randomly selected and sequenced for each obtained pattern to confirm the specie assignment.

		Restriction fragments				N°		
Species [1]	**ITS**	***CfoI***	***HaeIII***	***HinfI***	***DdeI***	**isolates**	**Origin**	**Accession numbers (*)**
Saccharomyces cerevisiae	880	385+365	320+230+180+150	365+155	/	2	Lucera	KT029758- KT029759
Issatchenkia orientalis	500	185+ 170+ 69+ 56	370+ 90	225+ 160+ 145	/	1	Lucera	KT029782
Metchinkowia pulcherrima	400	205+100+95	280+100	200+190	/	9	Lucera	KT029787-KT029788
Hanseniaspora uvarum	750	320+310+105	750	350+200+180	300+180+95+90+85	18	Lucera	KT029774-KT029775
Candida zemplinina	475	215+110+80+60	475	235+235	/	4	Lucera	KT029752-KT029753
Issatchenkia terricola	450	130+100+90+85+45	290+125	240+105+105	/	1	Lucera	KT029794
Kluyveromyces thermotolerans	700	315+285+95	310+215+90+90	355+354	/	3	Lucera	KT029798
Torulaspora delbrueckii	800	330+220+150+100	800	410+380	/	2	Lucera	KT029802-KT029803
Pichia fermentans	450	170+100+100+80	340+80+30	250+200	/	1	Lucera	KT029806
Hanseniaspora opuntiae	750	320+310+120	750	340+190+170+60	360+180+180+85+70+50	2	Lucera	KT029779-KT029780
Hanseniaspora guilliermondii	750	320+310+105	750	350+200+180	380+180+95+80	2	Lucera	KT029768-KT029769
Saccharomyces cerevisiae	880	385+365	320+230+180+150	365+155	/	9	Ascoli Satriano	KT029760-KT029761
Metchinkowia pulcherrima	400	205+100+95	280+100	200+190	/	3	Ascoli Satriano	KT029789-KT029790
Hanseniaspora uvarum	750	320+310+105	750	350+200+180	300+180+95+90+85	29	Ascoli Satriano	KT029776-KT02977
Candida zemplinina	475	215+110+80+60	475	235+235	/	29	Ascoli Satriano	KT029754-KT029755
Issatchenkia terricola	450	130+100+90+85+45	290+125	240+105+105	/	1	Ascoli Satriano	KT029795
Kluyveromyces thermotolerans	700	315+285+95	310+215+90+90	355+354	/	/	Ascoli Satriano	KT029799

Table 3. Diversity indices of the two yeast populations present in must produced with grape samples collected from Lucera and Ascoli Satriano

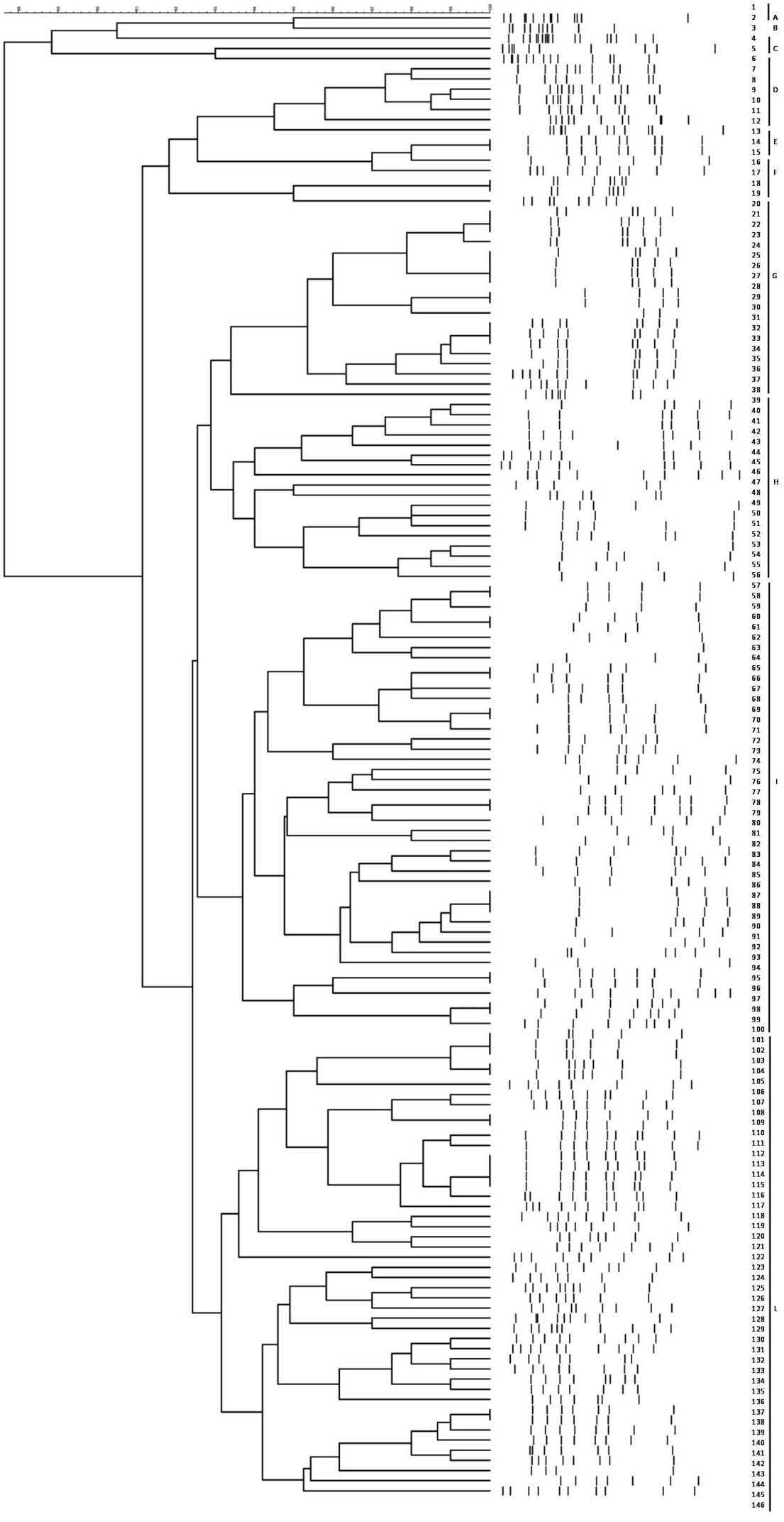
	Lucera	Ascoli Satriano
Species richness (S)	12	6
Dominance (D)	0.2363	0.2515*
Simpson (1-D)	0.7637	0.7485
Shannon (H)	1.793	1.512*
Evenness (e^H/S)	0.5005	0.7559*

Asterisk indicate statistically significant differences ($p<0.05$) of values in the same row

455 **Table 4.** Intraspecific diversity of the two *S. cerevisiae* populations

	Individuals	Clusters	Dominance (D)	Simpson (1-D)	Shannon (H)	Evenness (e^H/S)
Lucera	69	7	0.2596	0.7486	1.528	0.6582
Ascoli Satriano	77	9	0.2596	0.7404	1.640	0.5726


Lucera
Ascoli Satriano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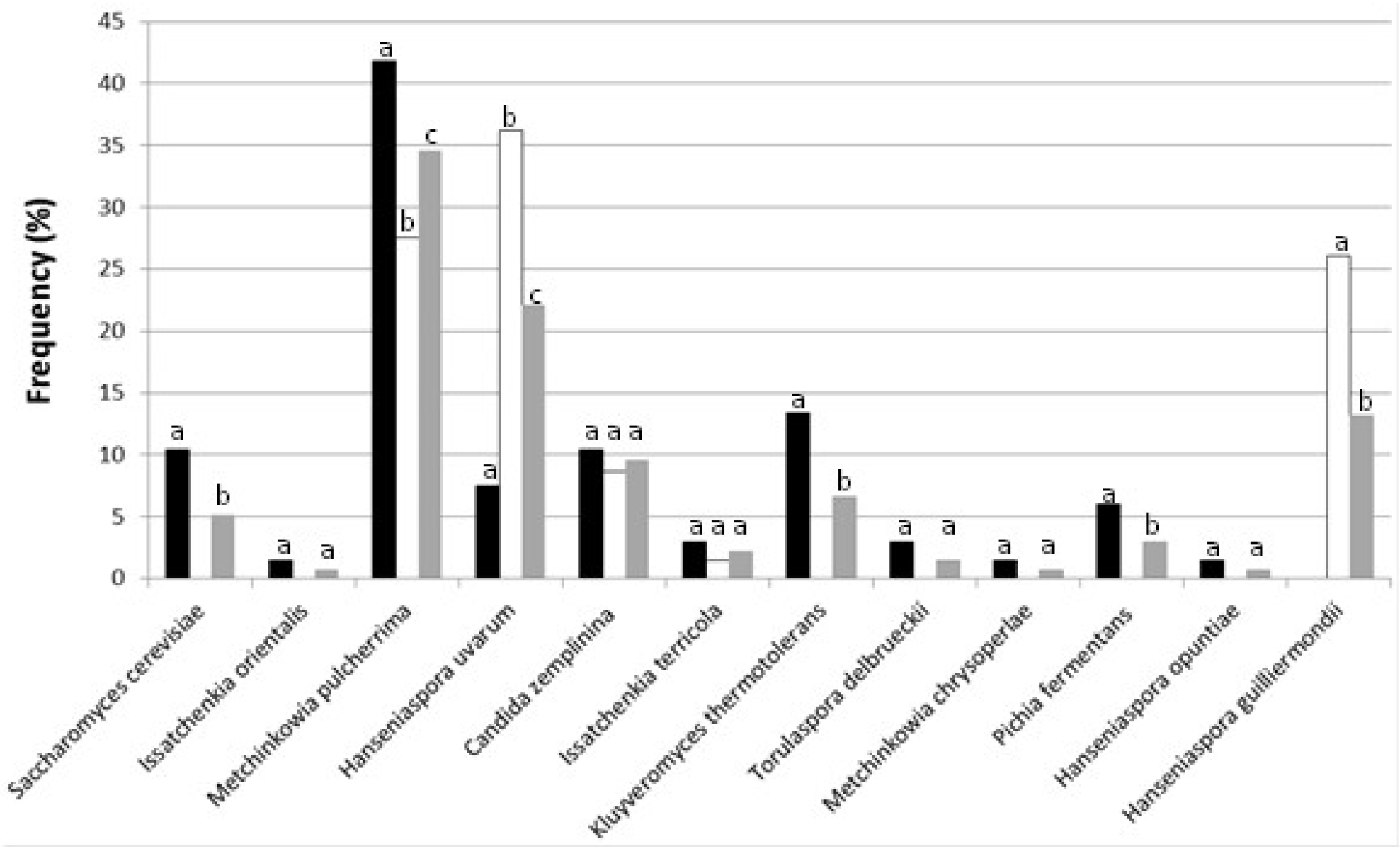
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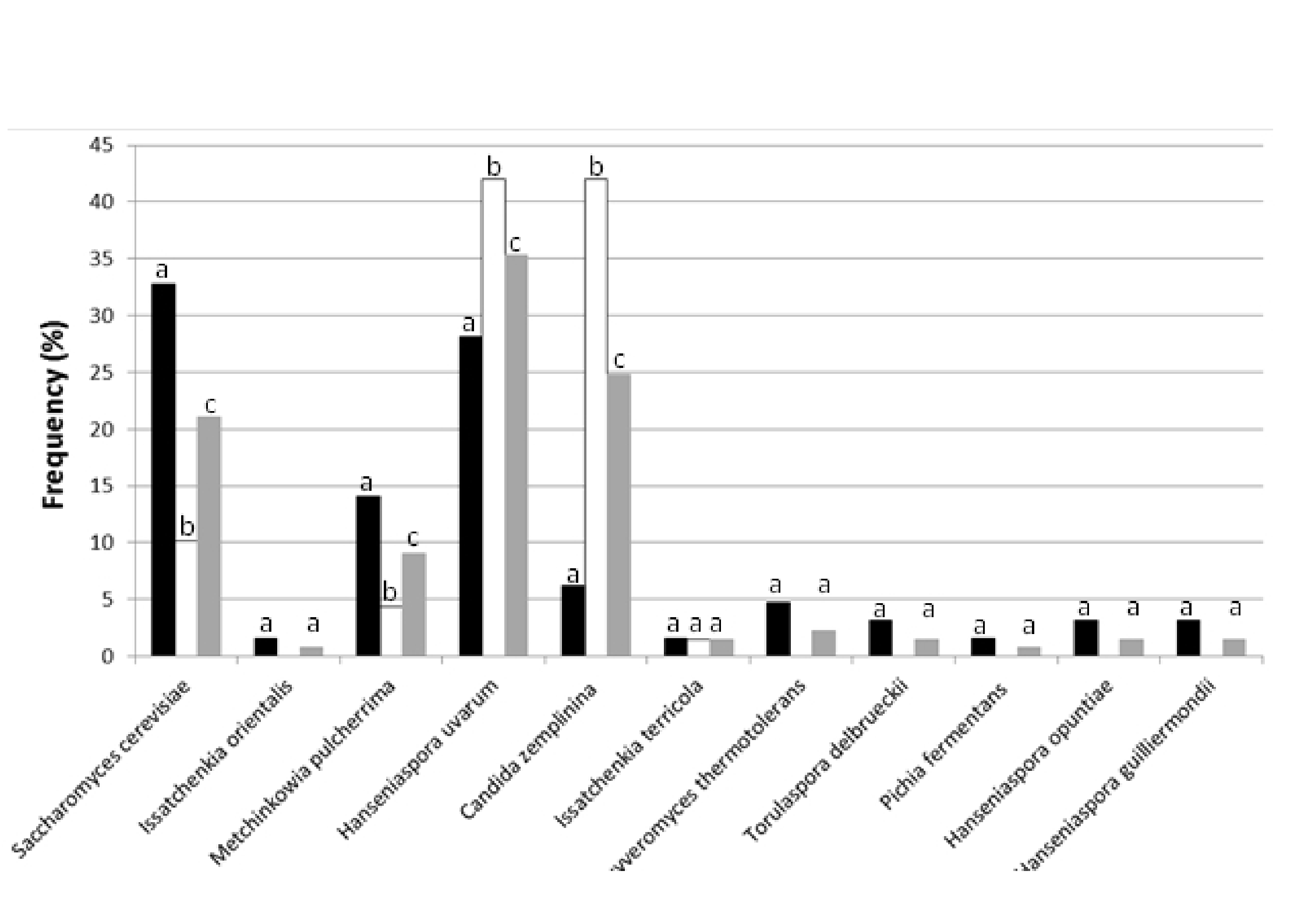
Saccharomyces cerevisiae
Issatchenkia orientalis
Metchinkowia pulcherrima
Hanseniaspora uvarum
Candida zemplinina
Issatchenkia terricola
Kluyveromyces thermotolerans
Torulaspora delbrueckii
Metchinkowia chrysoperlae
Pichia fermentans
Hanseniaspora opuntiae
Hanseniaspora guilliermondii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Saccharomyces cerevisiae
Issatchenkia orientalis
Metchinkowia pulcherrima
Hanseniaspora uvarum
Candida zemplinina
Issatchenkia terricola
Torulaspora delbrueckii
Pichia fermentans
Hanseniaspora opuntiae