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Inter- and Intra-Specific Differentiation of Natural Wine Strains of *Hanseniaspora* (*Kloeckera*) by Physiological and Molecular Methods

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Summary

Six different species and five different strains within one species of the *Hanseniaspora* (anamorph *Kloeckera*) were obtained from CBS Culture Collection, Delft, Netherlands, to analyze and compare with unidentified *Hanseniaspora* strains isolated from juice and fermenting wine. Identification and differentiation were done using physiological and molecular methods. When defining the species of the genera *Hanseniaspora* (*Kloeckera*) by phenotypic characteristics, misidentification occurred for growth at 37 °C, for the assimilation of sucrose and 2-keto-D-gluconate. For specific and reliable genus, species, and strain identification we evaluated both amplification of ITS1–5.8S-ITS4 rDNA, cut with various restriction enzymes, and the application of random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) using microsatellite and oligonucleotide (10-mers) primers. All the different primers (microsatellite, RAPD) worked properly and identically at both species and strain discrimination. The procedures were repeated several times and the techniques were found to be accurate and dependable.

Key words: wine yeast, *Hanseniaspora* (*Kloeckera*), RAPD-PCR, RFLP (restriction fragment length polymorphism), yeast identification

Introduction

The conversion of grape juice into wine is a complex process that is carried out by a succession of various yeasts *(1-3)*. The fermentation of must or juice is often initiated by indigenous yeasts, mostly non-*Saccharomyces* strains (*Hanseniaspora*/*Kloeckera*, *Rhodotorula*, *Candida*, *Debaryomyces*, *Pichia*). Growth of these yeasts is influenced by various factors such as temperature *(4,5)*, pH *(6)*, starter culture *(7,8)*, SO_2, treatments *(9–11)*, grape variety *(12,13)*, grape maturity *(14)*, climate *(15)* and geographical location *(16)*. Strains of *Saccharomyces cerevisiae* are typically present in very small numbers at the beginning of fermentation. At favorable temperatures and with SO_2 present, *Saccharomyces* yeasts grow rapidly and increase the alcohol content. *Hanseniaspora* (*Kloeckera*) and other non-*Saccharomyces* yeasts are suppressed. During the middle and final stages of fermentation strains of *Saccharomyces cerevisiae* predominate the population *(17)*.

Interest in whether *Hanseniaspora* (*Kloeckera*) produce positive or negative flavors in wine is increasing.

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Several reports speculate on the influence of apiculate yeasts during fermentation, their effect on growth of other yeasts and how much they contribute to the flavor of wine *(18–25)*. However, there are other reports, which consider these species potent spoilage microorganisms. That should be eliminated according to them, because of their ability to produce large amounts of acetic acid, fatty acids and esters, which can adversely affect the growth of *Saccharomyces cerevisiae* during the fermentation of wine, thus resulting in stuck fermentations and wines with flavor defects *(26,27)*.

In order to determine the impact of these yeasts on wine quality, it is necessary to define the size of their population in wine and to know how long potentially harmful strains can persist during wine fermentation. We also need to know more about differences between strains in the production of possibly detrimental or beneficial flavoractive compounds. Some researchers have already made trial fermentations to study the aromatic compounds generated by *Hanseniaspora* (*Kloeckera*) *(21, 23,24)*. Additionally, there are other areas such as the production of wine vinegar and apple cider, where the strains of genera *Hanseniaspora* (*Kloeckera*) are under investigation for the production of substances like acetic acid, D-sorbitol, xylitol, formaldehyde, formate, hydrogen peroxide, dihydroxyacetone, ATP, FAD, benzaldehyde, benzyl alcohol and benzoic acid *(28-33)*. For the tracking of individual yeasts during fermentation, it is important to have a reliable technique that contains an appropriate isolation method for strains of *Hanseniaspora* (*Kloeckera*) followed by an accurate identification at species and strain levels.

Traditional methods using morphological, physiological and biochemical tests for the isolation and identification of species within different yeast genera are described *(34,35)*. These techniques are labor-intensive and can give ambiguous results. Another disadvantage of these methods is that they are not able to discriminate well among strains within a species. There are other, faster methods that are based on certain physiological tests (*i.e.* API 20C AUX system, API ATB 32C system), but since these are inapplicable to the complete range of the six different species within genera *Hanseniaspora* (*Kloeckera*), they are not useful for the study of these yeasts.

Several studies show that various molecular techniques are useful in identifying wine yeast species and strains *(2,36,37)*. The most widely used techniques in this category are the nDNA/nDNA homology method *(38)*, restriction fragment length polymorphism (RFLP) *(39)*, sequencing of DNA and ribosomal RNA *(40)*, molecular karyotyping with pulsed field gel electrophoresis *(41,42)*, restriction enzyme analysis (REA) of genomic and mitochondrial DNA *(43)*, and random amplified polymorphic DNA (RAPD)-PCR analysis *(44)*.

There are researchers who have already published results of differentiation of species of genera *Hanseniaspora* (*Kloeckera*) with rDNA RFLPs and arbitrarily primed (AP)-PCR *(45,46)*. The objectives of our project were to use alternative methods comparing traditional and molecular identifications of *Hanseniaspora* (*Kloeckera*), and to describe accurate and reliable procedures for isolation and identification of species and strains within the genera.

Materials and Methods

Yeast strains

The yeast strains used in this study were isolated in various countries listed in Table 1. Yeasts were grown on Phytone^{TM} yeast extract agar plates at 25 °C (72 g L^{-1}; Becton Dickinson, Cockeyville, MD, USA) and stored at 5 °C. Type strains of *Hanseniaspora* (*Kloeckera*) were purchased from Centraalbureau voor Schimmelcultures (CBS Yeast Division, Delft, The Netherlands) and used as reference strains. Yeast strains from each species of *H. guilliermondii*, *H. occidentalis*, *H. osmophila*, *H. valbyensis*, *H. vineae* and five strains from *H. uvarum* were included in this study as shown in Table 1.

Morphological and physiological characterization

All the yeast strains were first grown on lysine agar [20 g L^{-1} wort agar (Difco Laboratories, Detroit, MI, USA); 11.75 g L^{-1} Yeast Carbon Base (Difco), 2.5 g L^{-1} L-lysine·HCl (Sigma Chemical Co., St. Louis, MO, USA)]. A loopful of yeast from each culture was streaked on WL selective agar (75 g L^{-1}; Oxoid Ltd, Basingstoke, Hampshire, UK). *Hanseniaspora* (*Kloeckera*) yeasts develop flat colonies with intense green color on this medium *(47)*. This allows easy discrimination from other genera. Next, strains of *Hanseniaspora* (*Kloeckera*) were identified on species level by different physiological tests. All the media used in the identification were made as published before *(35)*. The physiological tests used in the identification procedure are shown in Table 2 *(34)*. Each identification was accompanied by microscopic examination.

DNA extraction

The DNA extraction was performed as previously described *(37)*.

Amplification conditions for RAPD-PCR

The 10-mer arbitrary primers were purchased from Operon Technologies, Alameda, CA, USA. Initially, twenty-seven RAPD primers were screened at random. Those presented in Table 3 were used for identification of species and strains. The PCR reactions were performed in 25 μL reaction mixtures containing 50 ng μL^{-1} of DNA template, 18.05 μL of distilled water, 0.1 μL of *Taq* DNA polymerase (5 U; Fisher Scientific, Pittsburgh, PA, USA), 0.1 μL of 100 μM primer, 2.5 μL of 10× Assay Buffer B (Fisher) [100 mM Tris-HCl, pH = 8.3 (at 25 °C); 500 mM KCl], 2 μL of 25 mM $MgCl_2$ and 1.25 μL of 4 mM deoxynucleoside triphosphate (dNTP) mixture.

The reaction mixtures were covered with 17 μL of liquid wax (MJ Research, Watertown, MA, USA). Stratagene® Robocycler Gradient 40 (La Jolla, CA, USA) was used for DNA amplification with the following conditions: 94 °C for 1 min; 45 cycles at 94 °C for 1 min, 36 °C for 1 min, and 72 °C for 2 min; then one final extension at 72 °C for 8 min. After that, 2.5 μL of 10× loading dye (25 % Ficoll 400; 0.2 % bromophenolblue; 0.2 M EDTA, pH = 8.0) was added to each reaction tube and loaded onto a 1.5 % agarose gel (Molecular Biology Certified, Bio-Rad Laboratories, Hercules, CA, USA) containing 3 μL of ethidium bromide (5 μg mL^{-1}). The DNA

Table 1. Reference and unknown strains of genera *Hanseniaspora* (*Kloeckera*) used in this study

CBS 106*	*Hanseniaspora osmophila*	København, Denmark
CBS 279*	*Hanseniaspora uvarum*	Tokyo, Japan
CBS 314*	*Hanseniaspora uvarum*	Crimea, Ukraine
CBS 480*	*Hanseniaspora valbyensis*	Klöcker's Culture Collection
CBS 2570*	*Hanseniaspora uvarum*	Brazil
CBS 2589*	*Hanseniaspora uvarum*	Lucera, Italy
CBS 2591*	*Hanseniaspora guilliermondii*	France
CBS 2592*	*Hanseniaspora occidentalis*	St. Croix, West Indies
CBS 5073*	*Hanseniaspora uvarum*	Chile
CBS 8031*	*Hanseniaspora vineae*	Ontario, Canada
FL562	*Hanseniaspora* (*Kloeckera*)	Finger Lakes Region, USA[4]
CE114	*Hanseniaspora* (*Kloeckera*)	Lallemand, Canada[2]
HUS2	*Hanseniaspora* (*Kloeckera*)	Wädenswil, Switzerland[3]
E6-III/5	*Hanseniaspora* (*Kloeckera*)	Basilicata, Italy[1]
C-315	*Hanseniaspora* (*Kloeckera*)	Emilia-Romagna, Italy[1]
HUS4	*Hanseniaspora* (*Kloeckera*)	Wädenswil, Switzerland[3]
S6–16	*Hanseniaspora* (*Kloeckera*)	Sicily, Italy[1]
V5–230	*Hanseniaspora* (*Kloeckera*)	Napa Valley, USA[1]
C-131	*Hanseniaspora* (*Kloeckera*)	Emilia-Romagna, Italy[1]
E19-II/1	*Hanseniaspora* (*Kloeckera*)	Basilicata, Italy[1]
CE80	*Hanseniaspora* (*Kloeckera*)	Cornell University, USA[4]
E2-I/5	*Hanseniaspora* (*Kloeckera*)	Basilicata, Italy[1]
C-257	*Hanseniaspora* (*Kloeckera*)	Emilia-Romagna, Italy[1]
S2–6	*Hanseniaspora* (*Kloeckera*)	Sicily, Italy[1]
S5–9	*Hanseniaspora* (*Kloeckera*)	Sicily, Italy[1]
S7–14	*Hanseniaspora* (*Kloeckera*)	Sicily, Italy[1]
V7–237	*Hanseniaspora* (*Kloeckera*)	Napa Valley, USA[1]
C1–172	*Hanseniaspora* (*Kloeckera*)	Napa Valley, USA[1]

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bands were measured with DNA Marker (100 bp), (Promega, Madison, WI, USA) and separated in an electrophoresis chamber (Bio-Rad) containing 1× TAE (40 mM Tris acetate, 2 mM EDTA) buffer with 3 μL of ethidium bromide (5 μg mL^{-1}) by 2 h electrophoresis at 80 V. DNA bands were visualized under UV light (302 nm) and digitally photographed with a Gel Doc 1000 system (Bio-Rad).

Amplification conditions for ITS-PCR

Primers ITS1 and ITS4 were used to amplify the 5.8S rDNA and two Internal Transcribed Spacers flanking it *(48,49)*. These regions of the DNA are highly conserved and can be different among fungal species *(50)*. The ITS primers were made by BioResource Center, Cornell University, Ithaca, NY, USA. Reaction mixture of 25 μL was prepared with 50 ng μL^{-1} of DNA template, 0.1 μL of *Taq* DNA polymerase (5 U; Promega), 2.5 μL of 10× reaction buffer (Promega) (50 mM KCl; 10 mM Tris-HCl, pH = 9.0; 0.1 % Triton x–100), 2 μL of 25 mL $MgCl_2$, 0.1 μL 100 μM each of primers ITS1 and ITS4, 1.25 μL of 4 mM deoxynucleoside triphosphate mixture and 17.95 μL of distilled water. Amplification reactions were performed with RoboCycler® Gradient 40 Temperature Cycler under the following conditions: 95 °C for 1 min; 30 cycles at 95 °C for 1 min, 61 °C for 2 min, and 72 °C for 1 min; and one final extension at 72 °C for 5 min. After that the procedure was the same as described for RAPD-PCR. The purification of DNA was achieved with sodium acetate when it was demanded *(51)*.

Restriction enzyme digestion

Restriction endonucleases *Rsa* I, *Tru9* I, *Hinf* I, *Sau*3A I, *Hsp*92 II, *Hpa* II, *Cfo* I, *Alu* I, *Taq* I, *Dde* I, and *Sfi* I (Promega) were used to digest DNA fragments of yeasts amplified with ITS-PCR. In the reaction mixture all the enzymes were used in concentration of 10 units μL^{-1} except for *Dde* I which was used at 12 units μL^{-1}. The amount of digesting mixture was 30 μL containing 5 μL of DNA template, 3 μL of 10× buffer (enzyme type dependent), 1.5 μL of BSA, 0.5 μL of restriction enzyme (3–5 units μL^{-1}) and 20 μL of distilled water. The reaction mixture was placed on Thermolyne Dri-Bath (Sybron Corporation, Dubuque, IA, USA) at 37 °C for 2 h, then 3 μL of loading dye (10×) were added to the tubes and pipetted into 2 % agarose gel (NuSieve 3:1, FMC, Rockland, ME, USA) containing 3 μL ethidium bromide (5 μg mL^{-1}). The procedure described above was then used.

Microsatellite-PCR analysis

In the amplification reactions, primers (M13, RM13) used listed in Table 3 were made by BioResource Center, Cornell University, Ithaca, NY, USA; according to Mycology Reference Laboratory, Bristol Public Health Laboratory, Bristol, UK. The reaction mixture contained 1 μL 50 ng μL^{-1} of DNA template, 0.1 μL of *Taq* DNA polymerase (5 U; Promega), 2.5 μL of 10× reaction buffer (Promega) (50 mM KCl; 10 mM Tris-HCl, pH = 9,0; 0.1 % Triton x–100), 2 μL of 25 mM $MgCl_2$, 0.1 μL of 100 μM primer, 1.25 μL of 4 mM deoxynucleoside triphosphate mixture and 18.05 μL of distilled water. The am-

Table 2. Physiological tests applied for identification and discrimination of the six species: *Hanseniaspora guilliermondii*, *Hanseniaspora occidentalis*, *Hanseniaspora osmophila*, *Hanseniaspora uvarum*, *Hanseniaspora valbyensis* and *Hanseniaspora vineae* of genera *Hanseniaspora* (*Kloeckera*). Strains were purchased from Centraalbureau voor Schimmelcultures (CBS), Baarn & Delft, The Netherlands and isolated at different sources

Isolate Number	Tests for identification					Experimental Identification
	Sucrose	Assimilation of Maltose	2-Keto-D-gluconate	Growth at 37 °C	Cycloheximide Resistance	
CBS 106*	—	+	—	—	—	*H. osmophila*
CBS 279*	—	—	+	—	+	*H. uvarum*
CBS 314*	—	—	+	—	+	*H. uvarum*
CBS 480*	—	—	—	—	+	*H. valbyensis*
CBS 2570*	—	—	+	—	+	*H. uvarum*
CBS 2589*	—	—	+	—	+	*H. uvarum*
CBS 2591*	—	—	+	+	+	*H. guilliermondii*
CBS 2592*	+	—	—	—	—	*H. occidentalis*
CBS 5073*	—	—	+	—	+	*H. uvarum*
CBS 8031*	—	—	—	—	—	*H. vineae*
FL562	—	—	+	—	+	*H. uvarum*
CE114	—	—	+	—	+	*H. uvarum*
HUS2	—	—	+	—	+	*H. uvarum*
E6-III/5	—	—	+	—	+	*H. uvarum*
C-315	—	—	+	—	+	*H. uvarum*
HUS4	—	—	+	—	+	*H. uvarum*
S6–16	—	—	+	—	+	*H. uvarum*
V5–230	—	—	+	—	+	*H. uvarum*
C-131	—	—	+	—	+	*H. uvarum*
E19-II/1	—	—	+	—	+	*H. uvarum*
CE-80	—	—	+	—	+	*H. uvarum*
E2-I/5	—	—	+	—	+	*H. uvarum*
C-257	—	—	+	—	+	*H. uvarum*
S2–6	—	—	+	+	+	*H. guilliermondii*
S5–9	—	—	+	+	+	*H. guilliermondii*
S7–14	—	—	+	+	+	*H. guilliermondii*
V7–237	—	—	+	+	+	*H. guilliermondii*
C1–172	—	—	+	+	+	*H. guilliermondii*

* Reference strains identified by Centraalbureau voor Schimmelcultures (CBS), Baarn & Delft, The Netherlands

Table 3. Primers used for the identification of species and differentiation of strains within species of genera *Hanseniaspora* (*Kloeckera*) using RAPD-, Microsatellite- and ITS-PCR

Primer	Sequence	Application
OPA-01	5'CAGGCCCTTC3'	RAPD-PCR[1]
OPA-03	5'AGTCAGCCAC3'	RAPD-PCR[1]
OPA-09	5'GGGTAACGCC3'	RAPD-PCR[1]
OPD-08	5'GTGTGCCCCA3'	RAPD-PCR[1]
M13	5'GAGGGTGGXGGXTCT3'*	Microsatellite-PCR[2]
RM13	5'AGAXCCXCCACCCTC3'*	Microsatellite-PCR[2]
ITS1	5'TCCGTAGGTGAACCTGCGG3'	Internal Transcribed Spacer[3]
ITS4	5'TCCTCCGCTTATTGATATGC3'	Internal Transcribed Spacer[3]

* X: equal amounts of dATP, dCTP, dGTP and dTTP

[1] Operon Technologies, Alameda, CA, USA

[2] BioResource Center, Cornell University, Ithaca, NY, USA, according to Mycology Reference Laboratory, Bristol, UK

[3] BioResource Center, Cornell University, Ithaca, NY, USA

plification of DNA was performed in a RoboCycler® Gradient 40 Temperature Cycler with the following amplification conditions: 94 °C for 3 min, 40 cycles of 94 °C for 30 s, 45 °C for 1 min 20 s, 72 °C for 2 min 20 s, and a final extension at 72 °C for 7 min. The procedure used was the same as that used for RAPD-PCR.

Results

Isolation and experimental identification by traditional methods

Six different species of *Hanseniaspora* (*Kloeckera*) and five different strains within *Hanseniaspora uvarum* were obtained from CBS Culture Collection for comparison with other *Hanseniaspora* strains isolated from different countries (Table 1). Isolation and physiological tests are summarized in the identification scheme shown in Fig. 1. In the first step, the yeast cultures presumed to be *Hanseniaspora* were placed on lysine agar which inhibits the growth of certain organisms such as *Saccharomyces cerevisiae*, *Candida glabrata*, *Pichia mucosa* and *Rhodotorula bacarum*. Cycloheximide was not used as a selective agent, because some species of *Hanseniaspora* (*Kloeckera*) are inhibited by it. Those yeasts that grew on lysine agar were transferred to WL agar, which provided an easy separation between *Hanseniaspora* and other yeast strains. *Hanseniaspora* yeasts form characteristic flat, green colonies *(47)*. Preselection on lysine agar is important. If *Saccharomyces* yeasts are not excluded they can be misidentified as strains of *Hanseniaspora* because both form a very similar, greenish colony on WL agar. The result of lysine and WL agar should be confirmed microscopically.

For yeast identified as *Hanseniaspora* further identification was achieved by using different physiological tests (Fig. 1). Specific tests that allow for differentiation of species within *Hanseniaspora* (*Kloeckera*) were based on data published before *(34)*. The results of the identification of the samples obtained in different countries are shown in Table 2. The CBS strains were also subjected to these procedures. These techniques were effective for identifying and differentiating isolated cultures as species *H. uvarum* or *H. guilliermondii*. The techniques also confirmed the identification of the reference strains classified by CBS Culture Collection. Ambiguous results occurred frequently for the growth of *H. occidentalis* (CBS 2592) and *H. vineae* (CBS 8031) at 37 °C, for the assimilation of sucrose at *H. osmophila* (CBS 106) and for the assimilation of 2-keto-D-gluconate at *H. guilliermondii* strains (C1–172, V7–237) which required several repetitions of the experiments. To save time and to have more accurate identification it is important to have a more reliable method for identifying species of *Hanseniaspora* (*Kloeckera*).

ITS-PCR and RFLP analysis

Six different species of genera *Hanseniaspora* (*Kloeckera*) and five different strains within *H. uvarum* from CBS Culture Collection as well as species from other sources were used to test the ability of ITS-PCR discriminating

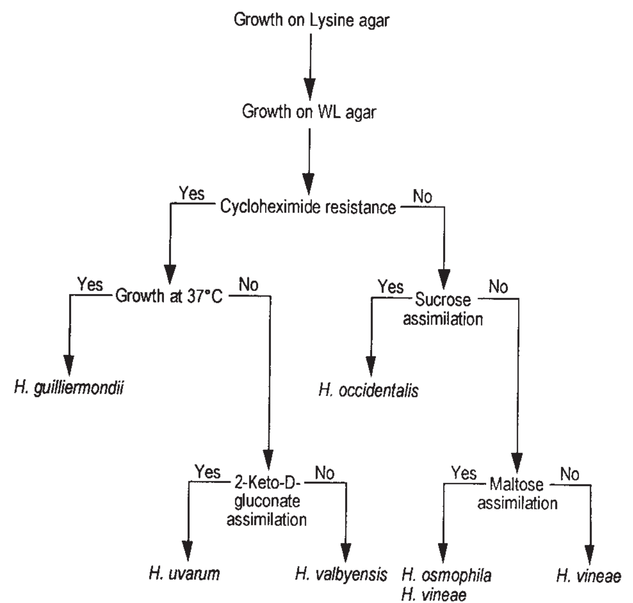

Fig. 1. Simplified identification scheme for the six species: *Hanseniaspora guilliermondii, Hanseniaspora occidentalis, Hanseniaspora osmophila, Hanseniaspora uvarum, Hanseniaspora valbyensis* and *Hanseniaspora vineae* of genera *Hanseniaspora* (*Kloeckera*) was made according to that published before *(34,35)*

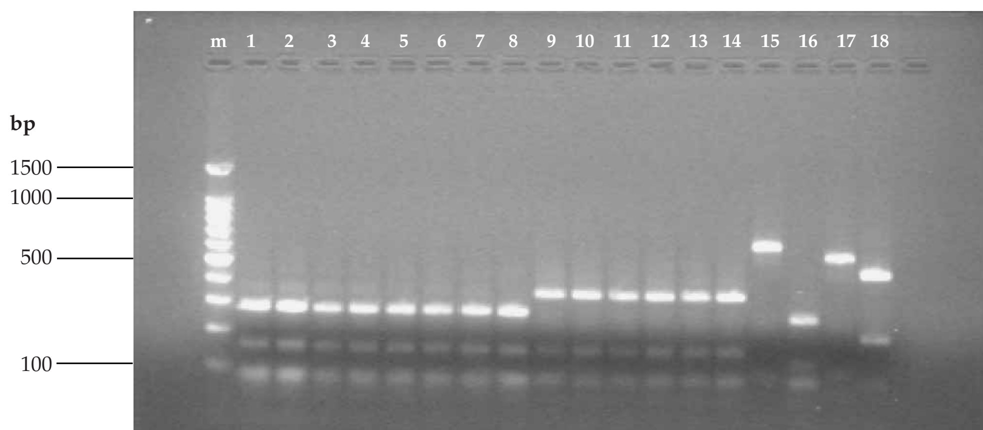

Fig. 2. Restriction fragment length polymorphism of the ITS1–5.8S-ITS2 region in rDNA amplified by PCR for species identification of *Hanseniaspora* (*Kloeckera*) revealed by digestion with *Dde* I restriction endonuclease; lane m, DNA marker; lane 1, *Hanseniaspora uvarum*, 279 (CBS); lane 2, *H. uvarum*, 314 (CBS); lane 3, *H. uvarum*, 2570 (CBS); lane 4, *H. uvarum*, 2589 (CBS); lane 5, *H. uvarum*, 5073 (CBS); lane 6, *H. uvarum*, FL562; lane 7, *H. uvarum*, CE114; lane 8, *H. uvarum*, HUS2; lane 9, *Hanseniaspora guilliermondii*, 2591 (CBS); lane 10, *H. guilliermondii*, S2–6; lane 11, *H. guilliermondii*, S5–9; lane 12, *H. guilliermondii*, S7–14; lane 13, *H. guilliermondii*, V7–237; lane 14, *H. guilliermondii*, C1–172; lane 15, *Hanseniaspora osmophila*, 106 (CBS); lane 16, *Hanseniaspora valbyensis*, 480 (CBS); lane 17, *Hanseniaspora occidentalis*, 2592 (CBS); lane 18, *Hanseniaspora vineae*, 8031 (CBS)

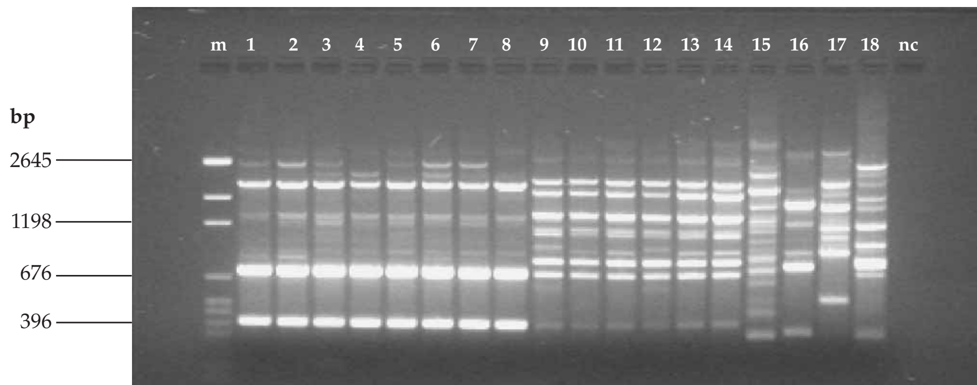

Fig. 3. PCR fingerprints with microsatellite primer M13 for the differentiation of the species of *Hanseniaspora* (*Kloeckera*); lane m, DNA marker; lane 1, *Hanseniaspora uvarum*, 279 (CBS); lane 2, *H. uvarum*, 314 (CBS); lane 3, *H. uvarum*, 2570 (CBS); lane 4, *H. uvarum*, 2589 (CBS); lane 5, *H. uvarum*, 5073 (CBS); lane 6, *H. uvarum*, FL562; lane 7, *H. uvarum*, CE114; lane 8, *H. uvarum*, HUS2; lane 9, *Hanseniaspora guilliermondii*, 2591 (CBS); lane 10, *H. guilliermondii*, S2–6; lane 11, *H. guilliermondii*, S5–9; lane 12, *H. guilliermondii*, S7–14; lane 13, *H. guilliermondii*, V7–237; lane 14, *H. guilliermondii*, C1–172; lane 15, *Hanseniaspora osmophila*, 106 (CBS); lane 16, *Hanseniaspora valbyensis*, 480 (CBS); lane 17, *Hanseniaspora occidentalis*, 2592 (CBS); lane 18, *Hanseniaspora vineae*, 8031 (CBS); lane nc, negative control

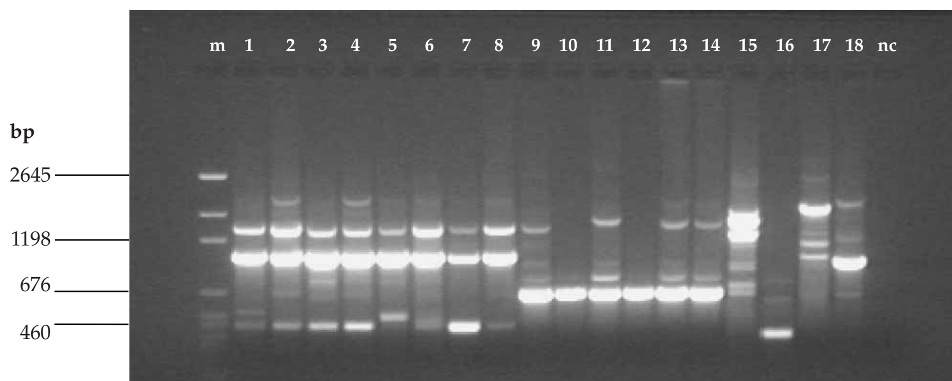

Fig. 4. RAPD-PCR fingerprints of the species of genera *Hanseniaspora* (*Kloeckera*) amplified with primer OPD-08; lane m, DNA marker; lane 1, *Hanseniaspora uvarum*, 279 (CBS); lane 2, *H. uvarum*, 314 (CBS); lane 3, *H. uvarum*, 2570 (CBS); lane 4, *H. uvarum*, 2589 (CBS); lane 5, *H. uvarum*, 5073 (CBS); lane 6, *H. uvarum*, FL562; lane 7, *H. uvarum*, CE114; lane 8, *H. uvarum*, HUS2; lane 9, *Hanseniaspora guilliermondii*, 2591 (CBS); lane 10, *H. guilliermondii*, S2–6; lane 11, *H. guilliermondii*, S5–9; lane 12, *H. guilliermondii*, S7–14; lane 13, *H. guilliermondii*, V7–237; lane 14, *H. guilliermondii*, C1–172; lane 15, *Hanseniaspora osmophila*, 106 (CBS); lane 16, *Hanseniaspora valbyensis*, 480 (CBS); lane 17, *Hanseniaspora occidentalis*, 2592 (CBS); lane 18, *Hanseniaspora vineae*, 8031 (CBS); lane nc, negative control

yeast strains *(50)*. One size of band occurred which was approximately 800 bp for each species after amplifying the rDNA thus showing that ITS1 and ITS4 primers are not adequate for making efficient discrimination even at species level. For further examination of these species, the same region of rDNA was amplified and digested with different restriction enzymes. Among the tested 11 restriction endonucleases only *Dde* I was useful for the overall discrimination. Fig. 2 shows the separation of the different species of *Hanseniaspora* after digestion using the *Dde* I restriction enzyme. In the case of species *H. uvarum* and *H. guilliermondii* more strains were tested in addition to the reference strains. These additional strains were isolated in different countries and had previously been identified by physiological test as *Hanseniaspora* (personal communication; J. Gafner, and P. Romano). For the remaining species only the reference strains were assayed by physiological tests. The band sizes for *H. uvarum* after digestion were approximately 80, 170 and 290 bp (lanes 1–10). *H. guilliermondii* had three different sizes of bands, approximately 90, 170 and 350 bp (lanes 11–14). *H. osmophila* had two bands, approximately 100 and 650 bp in size (lane 15). *H. valbyensis* had three bands, approximately 80, 130 and 260 bp in size (lane 16). *H. occidentalis* had two bands, approximately 100 and 550 bp in size (lane 17). *H. vineae* had three different sizes of bands, approximately 80, 190 and 450 bp (lane 18). The digestion was performed at different times obtaining identical sizes of products for the same species amplified by ITS-PCR. It was observed that the species of *Hanseniaspora* could be unambigously discriminated from each other by the digested products. Some difficulties occurred in the differentiation of *H. uvarum* and *H. valbyensis* when the smaller DNA bands were not sufficiently visible, since the largest bands are very close to each other in size, approximately 290 bp and 260 bp (lanes 1, 16) (Fig. 2). In this case DNA purification was performed *(51)*. However, with RFLP analysis we were not successful in further discriminating among strains of *H. uvarum* or *H. guilliermondii*. ITS-RFLP with enzyme *Dde* I is seemingly appropriate for differentiation only on species level.

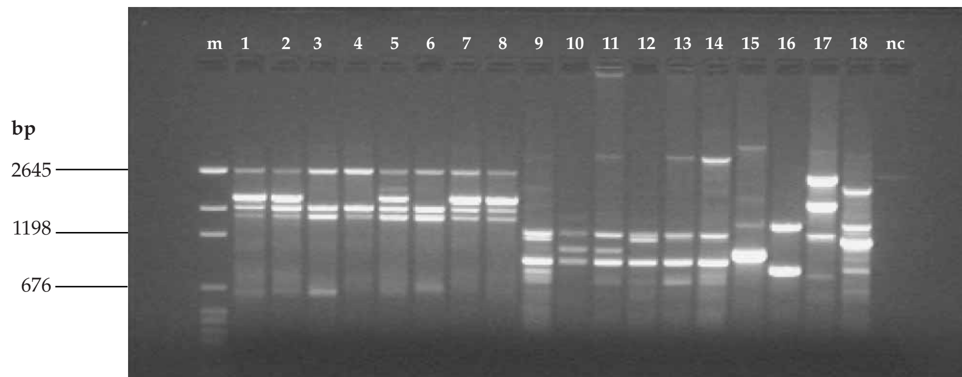

Fig. 5. PCR amplification of genomic DNA of genera *Hanseniaspora* (*Kloeckera*) strains primed with OPA-03; lane m, DNA marker; lane 1, *Hanseniaspora uvarum*, 279 (CBS); lane 2, *H. uvarum*, 314 (CBS); lane 3, *H. uvarum*, 2570 (CBS); lane 4, *H. uvarum*, 2589 (CBS); lane 5, *H. uvarum*, 5073 (CBS); lane 6, *H. uvarum*, FL562; lane 7, *H. uvarum*, CE114; lane 8, *H. uvarum*, HUS2; lane 9, *Hanseniaspora guilliermondii*, 2591 (CBS); lane 10, *H. guilliermondii*, S2–6; lane 11, *H. guilliermondii*, S5–9; lane 12, *H. guilliermondii*, S7–14; lane 13, *H. guilliermondii*, V7–237; lane 14, *H. guilliermondii*, C1–172; lane 15, *Hanseniaspora osmophila*, 106 (CBS); lane 16, *Hanseniaspora valbyensis*, 480 (CBS); lane 17, *Hanseniaspora occidentalis*, 2592 (CBS); lane 18, *Hanseniaspora vineae*, 8031 (CBS); lane nc, negative control

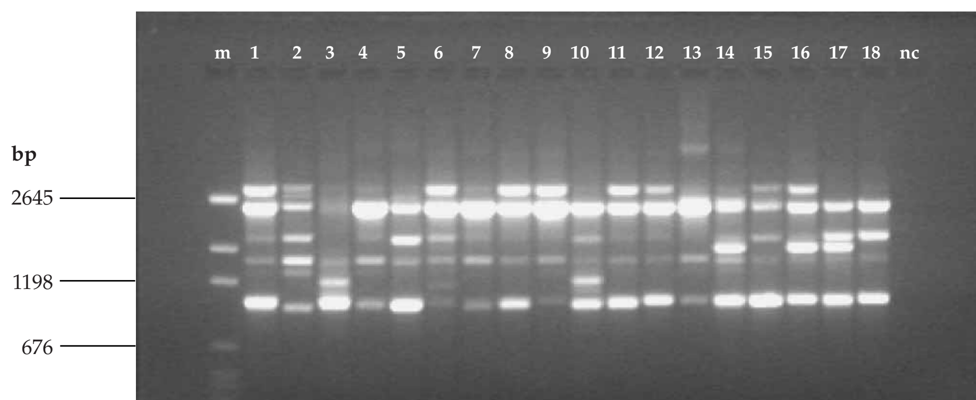

Fig. 6. RAPD-PCR band patterns of 18 strains of *Hanseniaspora uvarum* with microsatellite primer RM13; lane m, DNA marker; lane 1, 279 (CBS); lane 2, 314 (CBS); lane 3, 2570 (CBS); lane 4, 2589 (CBS); lane 5, 5073 (CBS); lane 6, FL562; lane 7, CE114; lane 8, HUS2; lane 9, E6-III/5; lane 10, C-315; lane 11, HUS4; lane 12, S6–16; lane 13, V5–230; lane 14, C-131; lane 15, E19-II/1; lane 16, CE80; lane 17, E2-I/5; lane 18, C-257; lane nc, negative control

RAPD- and Microsatellite-PCR analysis

The examination with different RAPD and microsatellite techniques was conducted to confirm the reliability of the discrimination of species with the ITS-RFLP technique described above and to search for suitable primer(s) for discrimination between strains within species. Previous results showed that different primers used by RAPD-PCR could be used for identification at species and strain level *(25,37)*. Two microsatellite primers, M13 and RM13, were examined at species level (Fig. 3). For the primer M13, the number of bands ranged from 3–7 among the species. Twenty-seven RAPD primers were tested for different species and two of them (OPD-08 and OPA-03) turned out to be apparently capable of distinguishing among the species of *Hanseniaspora* (Fig. 4,5). The RAPD primer, OPD-08 gave dependable results consisting of 3–4 bands and allowed for easy separation. The primer OPA-03 (Fig. 5) was competent in discerning six different species within the same genus and to distinguish strains of *H. uvarum* and *H. guilliermondii*.

All the RAPD primers previously used in this work and microsatellite primer RM13 were analyzed further for ability to differentiate within species *H. uvarum*, since this species occurs most frequently in juices and at the beginning of the fermentation of wine. Microsatellite primer RM13 was found to be applicable in discriminating among strains within species of *H. uvarum* (Fig. 6). OPA-01 and OPA-09 were also efficient in differentiating strains within *H. uvarum* (Figs. 7 and 8).

Discussion

This work was done to find a procedure for discriminating between species and strains within the genera *Hanseniaspora* (*Kloeckera*). The experiment was conducted using physiological tests, since this is the traditional way by which species are identified and differentiated. The data described above for the classification of *Hanseniaspora* were compared for the six species and simplified using only the discriminatory tests *(34)*. Even after precise monitoring it was concluded that species of *H. osmophila* and *H. vineae* could not be discrimi-

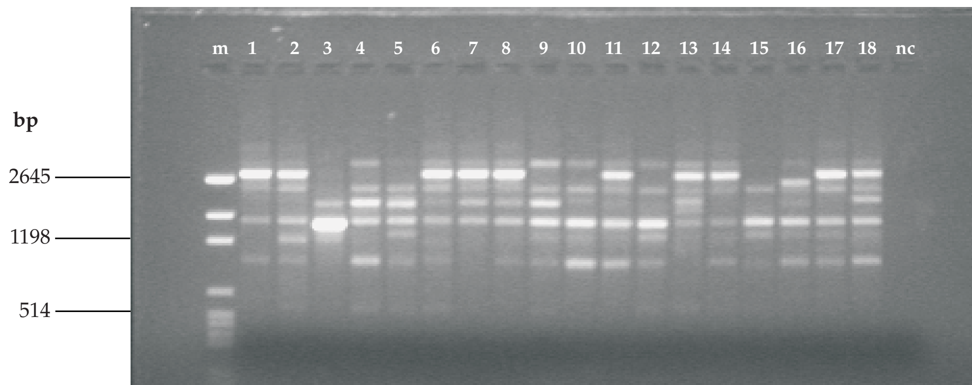

Fig. 7. RAPD-PCR amplified genomic DNA from 18 strains of *Hanseniaspora uvarum* primed with OPA-01; lane m, DNA marker; lane 1, 279 (CBS); lane 2, 314 (CBS); lane 3, 2570 (CBS); lane 4, 2589 (CBS); lane 5, 5073 (CBS); lane 6, FL562; lane 7, CE114; lane 8, HUS2; lane 9, E6-III/5; lane 10, C-315; lane 11, HUS4; lane 12, S6–16; lane 13, V5–230; lane 14, C-131; lane 15, E19-II/1; lane 16, CE80; lane 17, E2-I/5; lane 18, C-257; lane nc, negative control

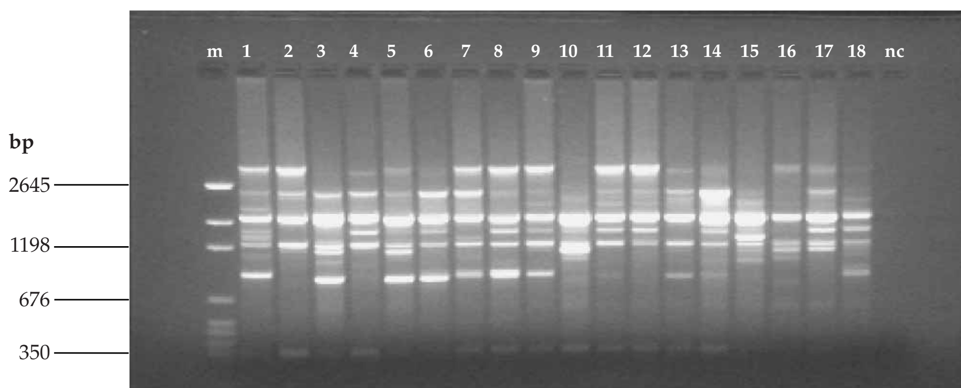

Fig. 8. DNA bands polymorphism for 18 strains of *Hanseniaspora uvarum* primed with primer OPA-09; lane m, DNA marker; lane 1, 279 (CBS); lane 2, 314 (CBS); lane 3, 2570 (CBS); lane 4, 2589 (CBS); lane 5, 5073 (CBS); lane 6, FL562; lane 7, CE114; lane 8, HUS2; lane 9, E6-III/5; lane 10, C-315; lane 11, HUS4; lane 12, S6–16; lane 13, V5–230; lane 14, C-131; lane 15, E19-II/1; lane 16, CE80; lane 17, E2-I/5; lane 18, C-257; lane nc, negative control

nated solely by physiological tests, since the assimilation of maltose, for example, can be positive or negative for *H. vineae*, while only positive for *H. osmophila*. The difficulties regarding the discrimination between species *H. osmophila* and *H. vineae* were confirmed also by Maudy Th. Smith, CBS Yeast Division, Identification Service, Delft, the Netherlands (personal communication). Although this laboratory identified and differentiated several strains belonging to these species at 34 °C, they would not consider it a dependable method due to the analyses made. For this reason additional research focused on several molecular techniques.

The ITS-PCR amplified the 5.8S rDNA and the two Internal Transcribed Spacers flanking it *(49)*. This method produced approximately 800 bp bands in size for each species. However, the differentiation was not possible even at species level with these primers (ITS1 and ITS4) for the strains of *Hanseniaspora* (*Kloeckera*). Therefore, restriction enzymes were used to cut the amplified products. One of the restriction enzymes, *Dde* I was seemingly suitable for providing reliable separation of bands for species within genera *Hanseniaspora* (*Kloeckera*). To verify the reliability of these results, other methods had to be tested for the same species. Microsatellite primer, M13 was also useful for successful discrimination and confirmed the results of ITS-RFLP. RAPD-PCR was tested with 27 primers. Two of those primers, OPD-08 and OPA-3 gave discerning results for identification using the same strains. Although RAPD primer OPA-03 was seemingly appropriate for discriminating within the species *H. uvarum*, the search was continued to obtain more primers, which are able to distinguish strains within that species. Microsatellite primer RM13 was competent in distinguishing the strains in that species. After screening of the same 27 primers, OPA-01 and OPA-09 were found effective for strain differentiation within *H. uvarum* as well.

Some researchers have already discriminated the species of *Hanseniaspora* with RFLPs of 18S-ITS1-5.8S-ITS2 and 25S rDNA respectively, though they applied several restriction enzymes in order to separate the six species within this genus *(45)*. They discriminated the closely related species *H. uvarum*/*K. apiculata* and *H. guilliermondii*/*K. apis* by the digestion of 25S rDNA which is approximately four times longer than the 800 bp ITS-PCR fragment amplified by primers ITS1 and ITS4. Nevertheless, in contrast to our experience, they did not distinguish species within these genera by phys-

iological tests. They used four different arbitrary, and two microsatellite primers to discriminate between strains within *H. uvarum*. In our work, three different primers were used for the differentiation of strains within *H. uvarum*.

The outcomes of the identifications for reference strains, as well as for unknown *Hanseniaspora* (*Kloeckera*) strains isolated from different sources and identified by physiological tests were reinforced with results produced using diverse molecular techniques. It can be concluded that the molecular techniques are adequate to provide the desired separation at certain levels, making the isolation and identification of strains within genera *Hanseniaspora* (*Kloeckera*) easier, faster and more accurate.

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Inter- i intraspecifično razlikovanje prirodnih vinskih sojeva *Hanseniaspora* (*Kloeckera*) fiziološkim i molekularnim postupcima

Sažetak

Šest različitih vrsta i pet različitih sojeva unutar jedne vrste *Hanseniaspora* (*Kloeckera*) dobiveni su od CBS Culture Collection, Delft, Nizozemska, kako bi se analizirali i usporedili neutvrđeni sojevi *Hanseniaspora* izolirani iz mošta i prevrelog vina. Identifikacija i razlikovanje provedeno je fiziološkim i molekularnim postupcima. Prilikom utvrđivanja vrste rodova *Hanseniaspora* (*Kloeckera*) na osnovi fenotipskih značajki, došlo je do pogrešne identifikacije za rast pri 37 °C, te pri asimilaciji saharoze i 2-keto-D-glukonata. Za specifičnu identifikaciju roda, vrste i soja koristili smo rezultate amplifikacije ITS1–5,8S-ITS4 rDNA, pocijepane različitim restrikcijskim enzimima, te primjenu lančane reakcije s nasumce amplificiranom polimorfnom DNA-polimerazom (RAPD-PCR), koristeći mikrosatelite i oligonukleotidne (dekamerne) klice (primers). Sve klice iako različite (mikrosateliti, RAPD) djelovali su ispravno i identično pri razlikovanju vrsta i sojeva. Postupci su ponavljani nekoliko puta, a način izvedbe bio je točan i pouzdan.