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Characterization of *Hanseniaspora* (*Kloeckera*) Strains Isolated in Finger Lakes Wineries Using Physiological and Molecular Techniques

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Summary

Hanseniaspora (anamorph *Kloeckera*) yeast strains were isolated from several wineries in the Finger Lakes region of New York State, USA. These cultures were discriminated on species level by traditional methods. Experimental physiological tests could separate the species, and most of the strains were identified as species *H. uvarum*. However, there was a dissimilarity between the reference strain *H. osmophila* and an isolated strain in the assimilation of maltose. Molecular methods were applied to corroborate the results of physiological tests employing random amplified polymorphic DNA (RAPD)-, restriction fragment length polymorphism (RFLP)-, and microsatellite-polymerase chain reaction (PCR) techniques. These procedures were reliable and gave unambiguous results on species level. RAPD- and Microsatellite-PCR were also used to differentiate strains belonging to species *H. uvarum*. These provided a reliable tool for identification of specific strain.

Key words: molecular techniques, *Hanseniaspora* (*Kloeckera*), RAPD-PCR, RFLP, wine yeast

Introduction

The Finger Lakes area is one of the most remarkable wine regions in the United States. It is the second largest vine growing territory in the country. The region is located in New York State and was formed by Ice Age glaciers, which carved parallel lakes appearing like a print of human fingers from above. The climate is predominantly continental, with a long, cold winter and an abbreviated growing season. The lakes, however, moderate the temperature by warming the cold air on the slopes. Grape cultivation dates back to the 1820s when the 'foxy' taste of Native American grapes (*Vitis labrusca*) characterized that region (*1*,*2*). Later, French-American hybrids and several European types (*Vitis vinifera*) were acclimatized making for extensive and fascinating wine diversity in the area. More than 35 different grape varieties are grown in the Finger Lakes today. The main varieties are Niagara, Seyval, Chardonel, Chardonnay, Pinot Noir, Riesling and Cabernet Sauvignon, which are grown at about 60 wineries in the region (*1*).

It is well known that a succession of different yeast cultures follow each other during wine fermentation (*3*). In the preliminary stages of fermentation different genera such as *Metschnikowia*, *Rhodotorula*, *Candida*, *Debaryomyces*, *Pichia*, and the lemon-shaped apiculate yeasts

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Hanseniaspora (*Kloeckera*) are predominant. As the volume fraction of alcohol increases above 5–6 %, most of these strains are suppressed. Then, the more tolerant *Saccharomyces cerevisiae* strains take over and finish the process at approximately 11–12 % alcohol (*3–11*).

It is important to better understand the apiculate yeasts since they can cause great problems for wine-makers, though there is also a possibility that this genus could contribute to wines producing desirable flavors. There are different methods based on conventional physiological identifications, *e.g.* the API 20C AUX system, API ATB 32C system, simplified identification key, microtitre tray and Biolog systems (*12–15*), which utilize mostly assimilation of diverse carbon and nitrogen compounds for characterization and discrimination of species and genera of different yeasts (*16,17*). Using these procedures, the results can be easily misinterpreted, in addition to being labor-intensive and time-consuming, and they are not specific for each species within genera (*18–23*). Furthermore, there are limitations in the identification of strains within species if, for instance, it is necessary to identify a strain for further examination.

However, other techniques employ the genetic information of microorganisms for characterization, applying different molecular methods developed over the last decade (*i.e.* karyotyping, nDNA/nDNA homology, sequencing). These techniques are useful for the identification and classification of yeasts and other microorganisms (*24–31*). Most commonly used among these methods is the random amplified polymorphic DNA (RAPD)-polymerase chain reaction (PCR) technique (*23,32–35*). The restriction fragment length polymorphism (RFLP) of rDNA amplified with PCR is often applied as well (*34, 36–39*).

Only a few researchers have effectively used these methods for the identification and discrimination of yeasts within genera *Hanseniaspora* (*Kloeckera*) (*40–43*), although these methods are well established, and considered fast, accurate and dependable tools.

The aims of this research were *(i)* to isolate *Hanseniaspora* (*Kloeckera*) strains from uninoculated fermenting juice in different wineries in the Finger Lakes region using traditional methods, and *(ii)* to characterize them by physiological tests and different molecular techniques such as RFLP and RAPD-PCR. For this study, the simplified identification scheme, developed earlier for the separation and identification of strains of *Hanseniaspora* (*Kloeckera*), was used (*44*).

Materials and Methods

Yeast strains

The 17 yeast strains used in this study were isolated from 11 different wineries in the Finger Lakes region, NY, USA, and are listed in Table 1. Yeasts were grown on Phytone™ 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 uvarum* and *H. osmophila* were purchased from Centraalbureau voor Schimmelcultures (CBS Yeast Division, Baarn & Delft, The Netherlands) and used as reference strains (Table 1).

Morphological and physiological characteristics

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

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

Strain	Species, genera	Origin
CBS 279*	*Hanseniaspora uvarum*	Inst. Brewing, Tokyo, Japan
FL73	*Hanseniaspora* (*Kloeckera*)	Delaware, Wagner Vineyards[1]
FL194	*Hanseniaspora* (*Kloeckera*)	Chardonnay, Wagner Vineyards[1]
FL200	*Hanseniaspora* (*Kloeckera*)	Riesling, Wagner Vineyards[1]
FL519	*Hanseniaspora* (*Kloeckera*)	Gewurztraminer, Wagner Vineyards[1]
FL529	*Hanseniaspora* (*Kloeckera*)	Seyval, Wagner Vineyards[1]
FL592	*Hanseniaspora* (*Kloeckera*)	Ravat, Wagner Vineyards[1]
FL176	*Hanseniaspora* (*Kloeckera*)	Ives, Lakewood Vineyards[1]
FL355	*Hanseniaspora* (*Kloeckera*)	Riesling, Hermann J. Wiemer Vineyard[1]
FL436	*Hanseniaspora* (*Kloeckera*)	Chardonnay, Dr. Frank's Wine Cellars[1]
FL562	*Hanseniaspora* (*Kloeckera*)	Gewurztraminer, Lameroux Landing Wine Cellars[1]
FL599	*Hanseniaspora* (*Kloeckera*)	Chardonnay, Prejean Winery[1]
FL602	*Hanseniaspora* (*Kloeckera*)	Ravat, Wagner Vineyards[1]
FL722	*Hanseniaspora* (*Kloeckera*)	Riesling, Glenora Wine Cellars[1]
FL775	*Hanseniaspora* (*Kloeckera*)	Vignoles, Hunt Country Vineyards[1]
FL843	*Hanseniaspora* (*Kloeckera*)	Riesling, Standing Stone Vineyards[1]
FL871	*Hanseniaspora* (*Kloeckera*)	Cabernet Franc, Swedish Hill Vineyard[1]
CBS 106*	*Hanseniaspora osmophila*	Bark of tree, København, Denmark
FL392	*Hanseniaspora* (*Kloeckera*)	Baco Noir, Fulkerson Vinery[1]

* Centraalbureau voor Schimmelcultures (CBS), Baarn & Delft, The Netherlands

[1] Isolated in Finger Lakes region, NY, USA; belongs to Cornell University, New York State Agricultural Experiment Station, Wine Research Program, USA

selective agar (75 g L^{-1}; Oxoid Ltd, Basingstoke, Hampshire, UK). *Hanseniaspora* (*Kloeckera*) yeast develops flat colonies with intense green color on this medium (*45*). Strains of *Hanseniaspora* (*Kloeckera*) were identified on species level by different physiological tests. All the media used in the identification were made as previously described (*16*). The physiological tests used in the identification procedure are shown in Table 2 (*17*). Identification included microscopic examination.

DNA extraction

DNA extraction was performed according to the method published earlier (*23*).

Amplification conditions for RAPD-PCR

The 10-mer arbitrary primers were purchased from Operon Technologies (Alameda, CA, USA) and are presented in Table 3. 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 10x 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 10x loading dye (25 % Ficoll 400; 0.2 % bromophenolblue; 0.2 M EDTA, pH=8) was added to each reaction tube and loaded onto 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 bands were measured with DNA Marker (100 bp), (Promega, Madison, WI, USA) and separated in an electrophoresis chamber (Bio-Rad) containing 1x 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 with two Internal Transcribed Spacers flanking it (*36,39*). 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 10x Reaction Buffer (Promega) (50 mM KCl; 10 mM Tris-HCl, pH = 9.0; 0.1 % Triton x-100),

Table 2. Physiological tests applied for identification and discrimination of yeast strains isolated in different wineries at Finger Lakes region, NY, USA

Isolate number	Tests for identification					Experimental identification
	Sucrose	Assimilation of maltose	2-keto-D-gluconate	Growth at 37 °C	Cycloheximide resistance	
CBS 279*	—	—	+	—	+	*H. uvarum*
FL73, FL194, FL200	—	—	+	—	+	*H. uvarum*
FL519, FL529, FL592	—	—	+	—	+	*H. uvarum*
FL176, FL355, FL436	—	—	+	—	+	*H. uvarum*
FL562, FL599, FL602	—	—	+	—	+	*H. uvarum*
FL722, FL775, FL843	—	—	+	—	+	*H. uvarum*
FL871	—	—	+	—	+	*H. uvarum*
CBS 106*	—	+	—	—	—	*H. osmophila*
FL392	—	—	—	—	—	*H. osmophila*

* 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]
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: one of dATP, dCTP, dGTP and dTTP added each in equal amount to the mixture

[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

2 μL of 25 mM $MgCl_2$, 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. DNA purification was done where necessary (*46*).

Restriction enzyme digestion

Restriction endonuclease *Dde* I (Promega) was used to digest DNA fragments of yeasts amplified with ITS-PCR. In the reaction mixture the enzyme was used in concentration of 12 units μL^{-1}. The amount of digesting mixture was 30 μL containing 5 μL of DNA template, 3 μL of 10x 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 (10x) 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

In the amplification reactions, the microsatellite oligonucleotide 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 10x 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 amplification 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.

Assessment of the relationship between H. uvarum strains

Presence or absence of the corresponding bands was recorded for each of the 17 yeast strains, and a Jaccardian similarity matrix was calculated between any two strains (*47*). Mutual presence of a band is scored as unity, while presence in one strain and absence in the other is scored as zero. Mutual absence is ignored. Summation of these scores and division by the number of scored bands results in Jaccard's coefficient of similarity between two strains.

Results

Characterization by physiological tests

At the outset several yeast strains were isolated from different Finger Lakes wineries by the previously developed technique of simplified identification (*44*). Strains belonging to genera *Hanseniaspora* (*Kloeckera*) were isolated without difficulty using lysine and WL agar. Since there was no information previously available on *Hanseniaspora* (*Kloeckera*) strains originating from that region, it was decided to isolate yeasts from the first stages of fermentation of grape juice uninoculated with yeast strains. The goal was to characterize *Hanseniaspora* (*Kloeckera*) strains with experimental physiological tests and to compare these results with genotypic identification. Only one strain was selected from each winery, along with several different strains from spontaneously fermented grape juices obtained at Wagner Vineyards (Table 1). The identification was accomplished first by traditional physiological characterization as described above. The results are presented in Table 2. As can be seen, most of the isolated strains belonged to the species *H. uvarum* within genera *Hanseniaspora* (*Kloeckera*). There was no significant difference on the species level among yeast strains. Only one exception occurred with strain FL392, isolated at Fulkerson Winery from Baco Noir grape juice, that proved to be similar, but not identical to *H. osmophila* (CBS 106) type strain.

Molecular analysis of Hanseniaspora strains

Strains belonging to genera *Hanseniaspora* (*Kloeckera*) were subjected to diverse molecular methods for their characterization and to confirm the results of the classification completed by physiological experiments. The DNA extracted from these strains was first amplified by ITS-PCR using primers ITS1 and ITS4. It was then separated in agarose gel according to the fragments cut by restriction endonuclease *Dde* I (Fig. 1). This figure shows that the first 17 strains, including reference strain *H. uvarum* (CBS 279), had the same size bands, approximately 90 bp, 160 bp and 290 bp, distinguishable from the final two strains. In lane 18, the fragments of the reference strain *H. osmophila* (CBS 106) were separated, and in lane 19, the strain FL392 isolated at Fulkerson Winery, NY, USA.

In order to substantiate this result, genotypic identification and differentiation were also carried out by polymerase chain reaction (PCR) using microsatellite oligonucleotide primer M13 (Fig. 2). After the amplification of the DNA samples of the previous strains they were arranged on the agarose gel in the same order, so that comparisons with earlier results could be made. For the majority of the strains the same intense bands were recognized at sizes of approximately 396 bp, 690 bp and 1700 bp. For the two different strains DNA bands were identical. Strains were separated easily and unambiguously according to the size of their DNA fragments. They showed well-marked bands at sizes of approximately 700 bp, 1220 bp, 1500 bp and 1900 bp.

In order to verify the results of the previous experiment, the DNA of these strains was amplified by RAPD--PCR using primer OPA-03 (Fig. 3), which discriminated

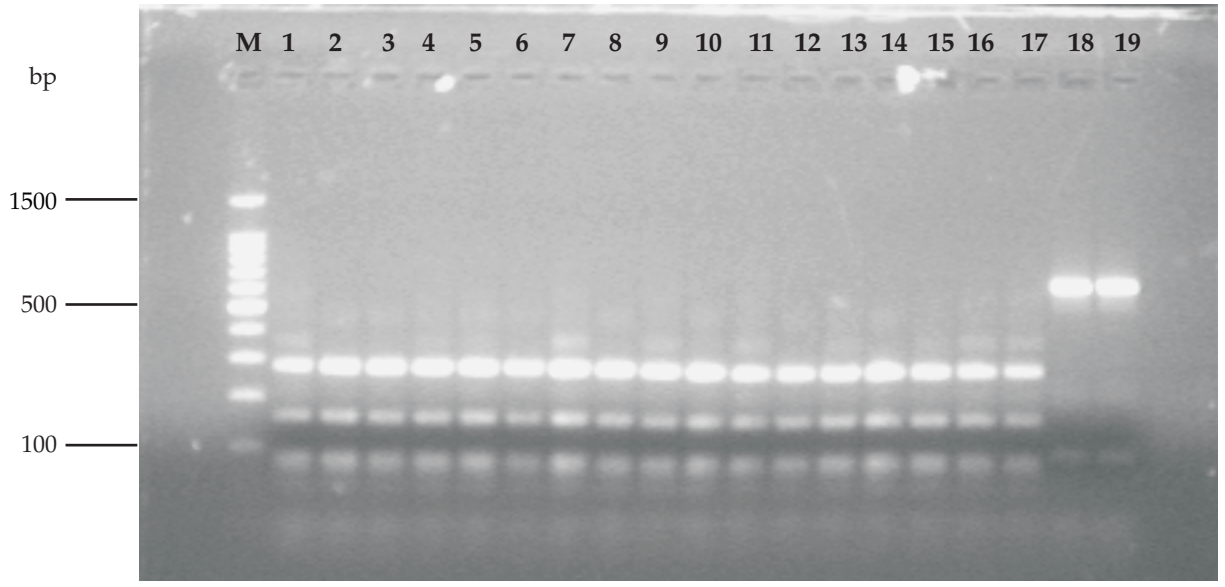

Fig. 1. Restriction fragment length polymorphism of the region ITS1-5.8S-ITS2 rDNA amplified by PCR for species identification of *Hanseniaspora* (*Kloeckera*) revealed by digestion with *Dde* I restriction endonuclease; reference strains CBS 106 and CBS 279 were purchased from Centraalbureau voor Schimmelcultures (CBS), Baarn & Delft, The Netherlands. Other strains were isolated in the Finger Lakes wine region, NY, USA. The strains that are underlined were isolated at the same winery (Wagner Vineyards, NY, USA). Lane M, DNA marker (100 bp); lane 1, *Hanseniaspora uvarum*, CBS 279; lane 2, *H. uvarum*, FL73; lane 3, *H. uvarum*, FL194; lane 4, *H. uvarum*, FL200; lane 5, *H. uvarum*, FL519; lane 6, *H. uvarum*, FL529; lane 7, *H. uvarum*, FL592; lane 8, *H. uvarum*, FL176; lane 9, *H. uvarum*, FL355; lane 10, *H. uvarum*, FL436; lane 11, *H. uvarum*, FL562; lane 12, *H. uvarum*, FL599; lane 13, *H. uvarum*, FL602; lane 14, *H. uvarum*, FL722; lane 15, *H. uvarum*, FL775, lane 16, *H. uvarum*, FL843; lane 17, *H. uvarum*, FL871; lane 18, *Hanseniaspora osmophila*, CBS 106; lane 19, *H. osmophila*, FL392

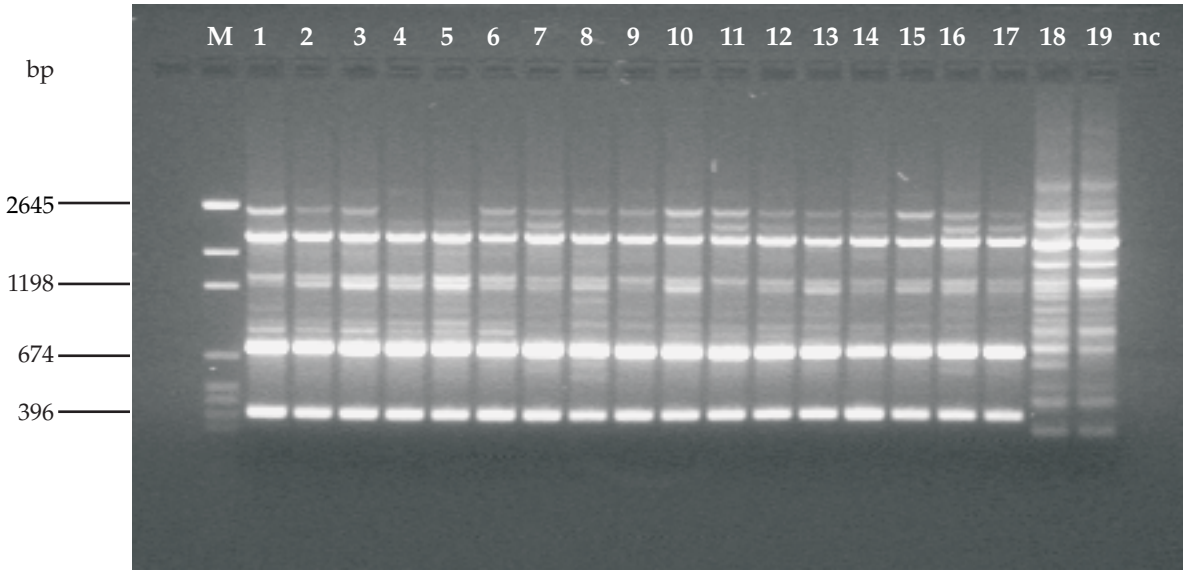

Fig. 2. Amplification of genomic DNA of genera *Hanseniaspora* (*Kloeckera*) strains isolated at Finger Lakes wineries by PCR reaction using microsatellite primer M13; reference strains CBS 106 and CBS 279 were purchased from Centraalbureau voor Schimmelcultures (CBS), Baarn & Delft, The Netherlands. The strains that are underlined were isolated at the same winery (Wagner Vineyards, NY, USA). Lane M, DNA marker; lane 1, *Hanseniaspora uvarum*, CBS 279; lane 2, *H. uvarum*, FL73; lane 3, *H. uvarum*, FL194; lane 4, *H. uvarum*, FL200; lane 5, *H. uvarum*, FL519; lane 6, *H. uvarum*, FL529; lane 7, *H. uvarum*, FL592; lane 8, *H. uvarum*, FL176; lane 9, *H. uvarum*, FL355; lane 10, *H. uvarum*, FL436; lane 11, *H. uvarum*, FL562; lane 12, *H. uvarum*, FL599; lane 13, *H. uvarum*, FL602; lane 14, *H. uvarum*, FL722; lane 15, *H. uvarum*, FL775, lane 16, *H. uvarum*, FL843; lane 17, *H. uvarum*, FL871; lane 18, *Hanseniaspora osmophila*, CBS 106; lane 19, *H. osmophila*, FL392; lane nc, negative control

between species *H. uvarum* and *H. osmophila* with the same result as the previous techniques, and also differentiated the strains within species *H. uvarum*. Bands were clearly recognizable and easily distinguished between cultures. In order to confirm the result of primer OPA-03 and to differentiate those strains classified under species *H. uvarum*, further examination was undertaken to determine if other primer(s) could achieve ac-

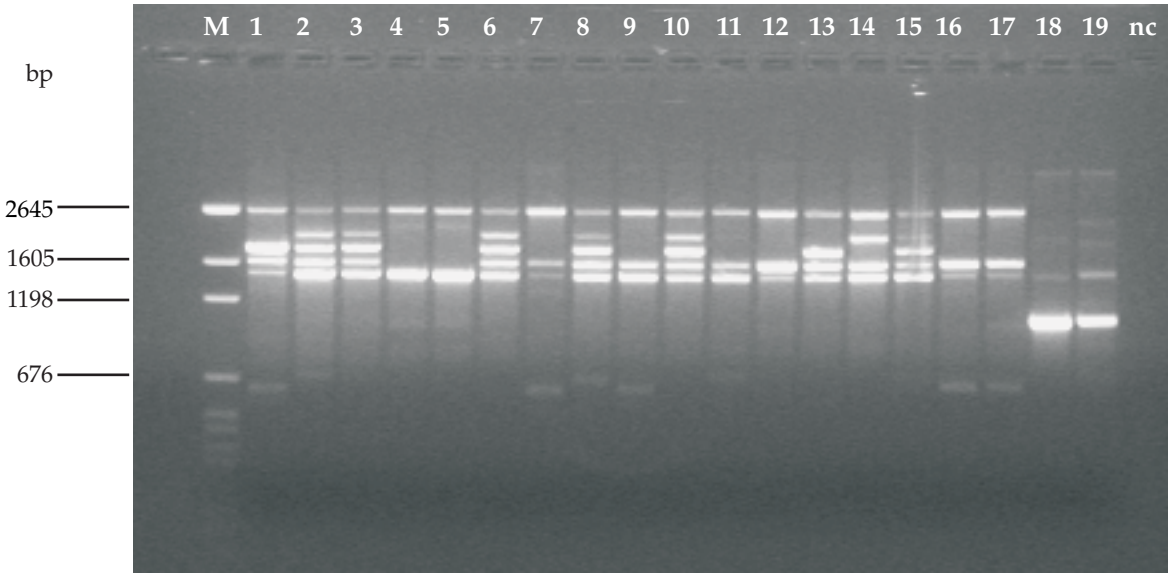

Fig. 3. PCR fingerprints with primer OPA-03 for the differentiation of the species of *Hanseniaspora* (*Kloeckera*); reference strains CBS 106 and CBS 279 were purchased from Centraalbureau voor Schimmelcultures (CBS), Baarn & Delft, The Netherlands. The additional strains were isolated at Finger Lakes region, NY, USA. The strains that are underlined were isolated at the same winery (Wagner Vineyards, NY, USA). Lane M, DNA marker; lane 1, *Hanseniaspora uvarum*, CBS 279; lane 2, *H. uvarum*, FL73; lane 3, *H. uvarum*, FL194; lane 4, *H. uvarum*, FL200; lane 5, *H. uvarum*, FL519; lane 6, *H. uvarum*, FL529; lane 7, *H. uvarum*, FL592; lane 8, *H. uvarum*, FL176; lane 9, *H. uvarum*, FL355; lane 10, *H. uvarum*, FL436; lane 11, *H. uvarum*, FL562; lane 12, *H. uvarum*, FL599; lane 13, *H. uvarum*, FL602; lane 14, *H. uvarum*, FL722; lane 15, *H. uvarum*, FL775; lane 16, *H. uvarum*, FL843; lane 17, *H. uvarum*, FL871; lane 18, *Hanseniaspora osmophila*, CBS 106; lane 19, *H. osmophila*, FL392; lane nc, negative control

ceptable separation. To this end, the isolated DNA of strains of *H. uvarum* was amplified by microsatellite primer RM13 (Fig. 4). Bands were intense, clearly recognizable and easily distinguished from each other. In addition, DNA of *H. uvarum* was amplified by RAPD-PCR using primer OPA-01 (Fig. 5), which provided another kind of differentiation of strains of *H. uvarum*. The bands were clear, intense, and distinctly separated, making it possible to continue further research on a particular strain belonging to the same species where necessary.

The resulting relationships between the different *H. uvarum* strains based on the patterns of RAPD-PCR amplified with primer OPA-01 are displayed in the dendrogram using average linkage clustering (Fig. 6). It is remarkable that even within one-winery strains belonging to species *H. uvarum* there was a considerable level of diversity among the fragments amplified by primer OPA-01.

Discussion

During this research several yeast strains were isolated from wineries surrounding the Finger Lakes in New York State, USA. Strains of *Hanseniaspora* (*Kloeckera*) randomly picked at a given winery, and several at Wagner Vineyards from a variety of uninoculated fermenting grape juices were monitored by diverse techniques. 17 strains of *Hanseniaspora* (*Kloeckera*) from 11 different wineries in the Finger Lakes region were isolated and identified using the method described earlier (*44*), which was developed to isolate and identify the species of *Hanseniaspora* (*Kloeckera*) from fermenting must and juice by traditional and molecular techniques. All of the strains were identified as *H. uvarum* except one, which was identified as *H. osmophila*, which could not be identified unambiguously by physiological tests. Other researchers have already noted the unreliability of traditional methods applied solely for identification (*18*,*23*). There was dissimilarity between the reference strain and the isolated strain belonging to the same species. Reference strain CBS 106 could utilize maltose, but the isolated strain FL392 could not. Thus, results of molecular techniques had also to be considered. Searching the database of CBS type strains for *H. osmophila*, no strain with negative assimilation of maltose was discovered, nor was there any reference to this feature in relevant sources. However, this result was confirmed by different fingerprinting techniques. Therefore it is recommended for consideration that maltose assimilation within *H. osmophila* could be variable. More strains belonging to species *H. osmophila* must be scrutinized by both physiological and different molecular methods in order to confirm this interpretation. Regarding these conclusions: it is notable that the strains of *H. uvarum* within genera *Hanseniaspora* (*Kloeckera*) were prevalent in samples isolated randomly in the Finger Lakes region.

These strains identified by physiological tests were also subjected to several genotypic methods. Certain molecular techniques were applied earlier for the genomic identification and differentiation of *Hanseniaspora* (*Kloeckera*) strains (*44*). Since there was difficulty in the identification of strain FL392 by physiological tests, it was necessary to employ different molecular methods. Another reason for using these methods was to verify the results of this experimental characterization. The results obtained by restriction enzyme *Dde* I matched

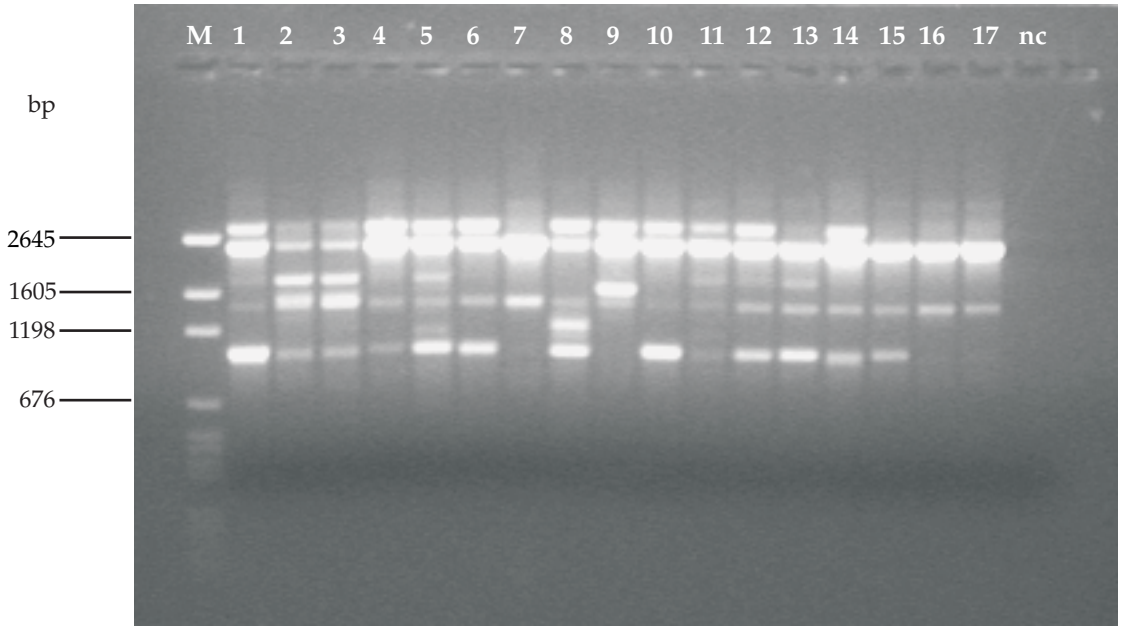

Fig. 4. RAPD-PCR band patterns of strains of *Hanseniaspora uvarum* were isolated at Finger Lakes wineries using microsatellite primer RM13; reference strain CBS 279 was purchased from Centraalbureau voor Schimmelcultures (CBS), Baarn & Delft, The Netherlands. The strains that are underlined were isolated at the same winery (Wagner Vineyards, NY, USA). Lane M, DNA marker; lane 1, *Hanseniaspora uvarum*, CBS 279; lane 2, *H. uvarum*, FL73; lane 3, *H. uvarum*, FL194; lane 4, *H. uvarum*, FL200; lane 5, *H. uvarum*, FL519; lane 6, *H. uvarum*, FL529; lane 7, *H. uvarum*, FL592; lane 8, *H. uvarum*, FL176; lane 9, *H. uvarum*, FL355; lane 10, *H. uvarum*, FL436; lane 11, *H. uvarum*, FL562; lane 12, *H. uvarum*, FL599; lane 13, *H. uvarum*, FL602; lane 14, *H. uvarum*, FL722; lane 15, *H. uvarum*, FL775; lane 16, *H. uvarum*, FL843; lane 17, *H. uvarum*, FL871, lane nc, negative control

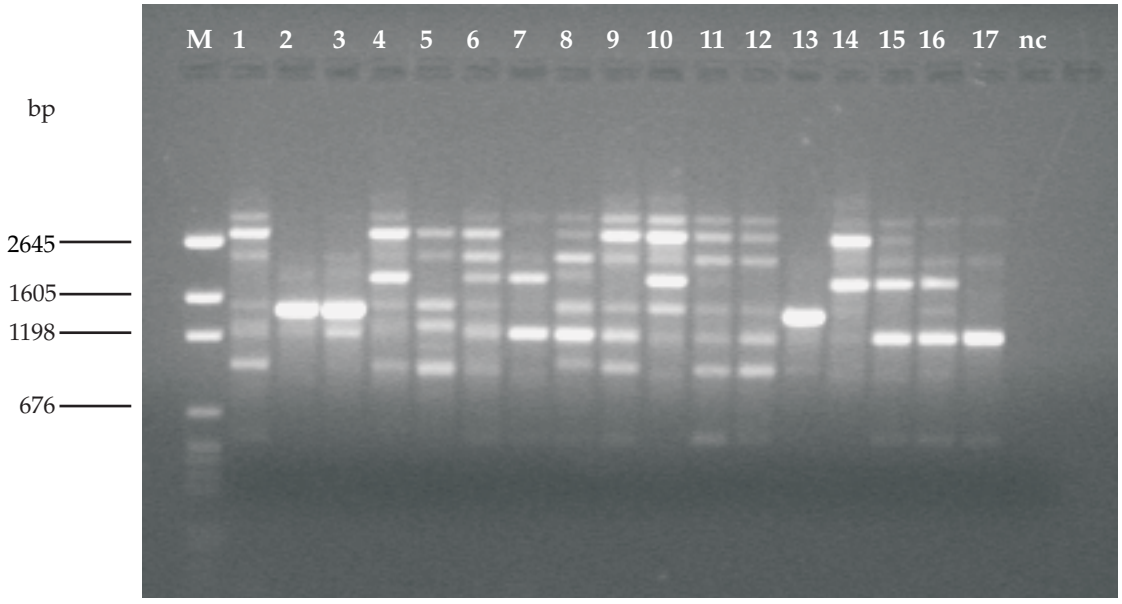

Fig. 5. DNA bands polymorphism for strains of *Hanseniaspora uvarum* isolated at Finger Lakes region, NY, USA, primed with OPA-01; reference strain CBS 279 was purchased from Centraalbureau voor Schimmelcultures (CBS), Baarn & Delft, The Netherlands. The strains that are underlined were isolated at the same winery (Wagner Vineyards, NY, USA). Lane M, DNA marker, lane 1, *Hanseniaspora uvarum*, CBS 279; lane 2, *H. uvarum*, FL73; lane 3, *H. uvarum*, FL194; lane 4, *H. uvarum*, FL200; lane 5, *H. uvarum*, FL519; lane 6, *H. uvarum*, FL529; lane 7, *H. uvarum*, FL592; lane 8, *H. uvarum*, FL176; lane 9, *H. uvarum*, FL355; lane 10, *H. uvarum*, FL436; lane 11, *H. uvarum*, FL562; lane 12, *H. uvarum*, FL599; lane 13, *H. uvarum*, FL602; lane 14, *H. uvarum*, FL722; lane 15, *H. uvarum*, FL775; lane 16, *H. uvarum*, FL843, lane 17, *H. uvarum*, FL871; lane nc, negative control

those of other researchers (*43*), although they did not report all the differences for the six species within these genera using the same enzyme. There were, however, other scientists who were able to discriminate these species by restriction enzymes, although they employed different ones (*41,42*). The primers used to separate at intra-species level turned out to be very reliable, and useful for the discrimination of those strains. Repeating

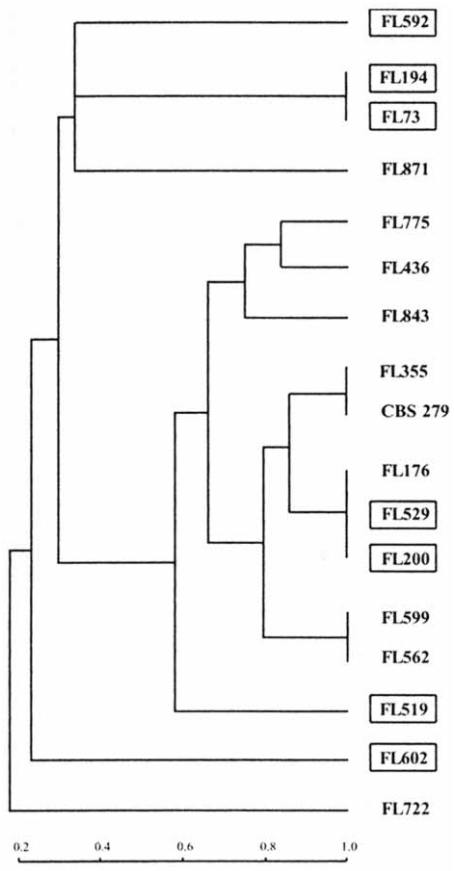

Fig. 6. Dendrogram based on the linkage clustering of Jaccardian similarity matrix constructed by the pattern of RAPD--PCR amplified with primer OPA-01; reference strain, *Hanseniaspora uvarum*, coded CBS 279 was purchased from Centraalbureau voor Schimmelcultures (CBS), Baarn & Delft, The Netherlands. The rest of the strains were isolated at Finger Lakes wineries and classified to species *Hanseniaspora uvarum* with physiological tests, RFLP and RAPD-PCR at Cornell University, New York State Agricultural Experiment Station, Wine Research Program, USA. The strains that are framed were isolated at the same winery (Wagner Vineyards, NY, USA) from different fermenting juice samples (Table 1)

the separation DNA fingerprinting profiles showed uniformity. Hence, this result provided the capability to differentiate a particular strain within *H. uvarum* by using physiological and molecular biological methods combined where required.

In conclusion, it was found that while results of physiological tests for experimental identification of species within *Hanseniaspora* (*Kloeckera*) were inconsistent, molecular techniques allowed for reliable discrimination on both species and strain levels.

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References

1. J. Hugh: North America. In: *The World Atlas of Wine*, Simon & Schuster, New York (1994) pp. 250–275.
2. J. Robinson: New York. In: *The Oxford Companion to Wine*, Oxford University Press, Oxford (1994) pp. 668–669.
3. G. H. Fleet, G. M. Heard: Yeasts-Growth During Fermentation. In: *Wine Microbiology and Biotechnology*, G. H. Fleet (Ed.), Harwood Academic Publishers, Chur (1993) pp. 27–54.
4. M. A. Amerine, M. S. Berg, R. E. Kunkee, C. S. Ough, V. L. Singleton, A. D. Webb: *The Technology of Wine Making*, AVI Publishing Company, Westport (1980).
5. S. Lafon-Lafourcade: Wine and Brandy. In: *Biotechnology*, H. H. Rehm, G. Reed (Eds.), Verlag Chemie, Weinheim (1983) pp. 81–163.
6. G. Rosini, *J. Gen. Appl. Microbiol. 30* (1984) 249.
7. I. Pardo, M. J. García, M. Zúñiga, F. Uruburu, *Appl. Environ. Microbiol. 55* (1989) 539.
8. A. Martini, A. Vaughan-Martini: Grape Must Fermentation: Past and Present. In: *Yeast Technology*, J. F. T. Spencer, D. M. Spencer (Eds.), Springer-Verlag, Berlin (1990) pp. 105–123.
9. J. Gafner, M. Schütz (Eds.), *Proceedings of the 23rd Annual New York Wine Industry Workshop*, Geneva, USA (1994) pp. 9–18.
10. A. Querol, E. Barrio, D. Ramón, *Int. J. Food Microbiol. 21* (1994) 315.
11. T. Henick-Kling, W. Edinger, P. Monk, *J. Appl. Microbiol. 84* (1998) 865.
12. T. Deák, L. R. Beuchat, *J. Food Prot. 50* (1987) 243.
13. G. M. Heard, G. H. Fleet, *J. Appl. Bacteriol. 68* (1990) 447.
14. T. Deák, *Int. J. Food Microbiol. 19* (1993) 15.
15. W. Praphailong, M. Van Gestel, G. H. Fleet, G. M. Heard, *Lett. Appl. Microbiol. 24* (1997) 455.
16. N. J. W. Kreger van Rij: *The Yeasts: A Taxonomic Study*, Elsevier Science Publishers, Amsterdam (1984).
17. J. A. Barnett, R. W. Payne, D. Yarrow: *Yeasts: Characteristics and Identification*, Cambridge University Press, Cambridge (1983).
18. H. Rohm, F. Lechner, *Appl. Environ. Microbiol. 56* (1990) 1290.
19. H. Rohm, F. Lechner, M. Lehner, *Int. J. Food Microbiol. 11* (1990) 215.
20. T. Török, A. D. King, *Appl. Environ. Microbiol. 57* (1991) 1207.
21. M. M. Baleiras Couto, J. M. B. M. van der Vossen, H. Hofstra, J. H. J. Huis in't Veld, *Int. J. Food Microbiol. 24* (1994) 249.
22. C. M. Egli, W. D. Edinger, C. M. Mitrakul, T. Henick-Kling, *J. Appl. Microbiol. 85* (1998) 779.
23. C. M. Mitrakul, T. Henick-Kling, C. M. Egli, *Food Microbiol. 16* (1999) 3.
24. G. Rosini, F. Federici, A. E. Vaughan, A. Martini, *Eur. J. Appl. Microbiol. Biotechnol. 15* (1982) 188.
25. A. Vaughan Martini, C. P. Kurtzman, *Int. J. Syst. Bacteriol. 35* (1985) 508.
26. A. V. Martini, *Syst. Appl. Microbiol. 12* (1989) 179.
27. M. Schütz, J. Gafner, *J. Appl. Bacteriol. 75* (1993) 551.
28. T. Török, D. Rockhold, A. D. King, *Int. J. Food Microbiol. 19* (1993) 63.
29. T. Boekhout, C. P. Kurtzman, K. O'Donnell, M. T. Smith, *Int. J. Syst. Bacteriol. 44* (1994) 781.
30. G. Cardinali, A. Martini, *Int. J. Syst. Bacteriol. 44* (1994) 791.
31. M. Schütz, J. Gafner, *Lett. Appl. Microbiol. 19* (1994) 253.
32. E. Lieckfeldt, W. Meyer, T. Börner, *J. Basic Microbiol. 33* (1993) 413.

33. O. Molnár, R. Messner, H. Prillinger, U. Stahl, E. Slavikova, *Syst. Appl. Microbiol. 18* (1995) 136.
34. D. Paffetti, C. Barberio, E. Casalone, D. Cavalieri, R. Fani, G. Fia, E. Mori, M. Polsinelli, *Res. Microbiol. 146* (1995) 587.
35. M. P. Quesada, J. L. Cenis, *Am. J. Enol. Vitic. 46* (1995) 204.
36. F. I. Molina, T. Inoue, S. C. Jong, *Curr. Microbiol. 25* (1992) 251.
37. M. M. Baleiras Couto, B. Eijsma, H. Hofstra, J. H. J. Huis in't Veld, J. M. B. M. van der Vossen, *Appl. Environ. Microbiol. 62* (1996) 41.
38. I. Masneuf, M. Aigle, D. Dubourdieu, *FEMS Microbiol. Lett. 138* (1996) 239.
39. M. T. Wyder, Z. Puhan, *Milchwissenschaft*, *52* (1997) 327.
40. A. Versavaud, J. N. Hallet, *Syst. Appl. Microbiol. 18* (1995) 303.
41. S. S. Možina, N. Čadež, P. Raspor, *Food Technol. Biotechnol. 36* (1998) 37.
42. D. Dlauchy, J. Tornai-Lehoczki, G. Péter, *Syst. Appl. Microbiol. 22* (1999) 445.
43. B. Esteve-Zarzoso, C. Belloch, F. Uruburu, A. Querol, *Int. J. Syst. Bacteriol. 49* (1999) 329.
44. G. Bujdosó, C. M. Egli, T. Henick-Kling, *Food Technol. Biotechnol. 39* (2001) 19.
45. A. Cavazza, M. S. Grando, C. Zini, *Vignevini*, *19* (1992) 17.
46. J. Sambrook, E. F. Fritsch, T. Maniatis: *Molecular Cloning: A Laboratory Manual*, C. Nolan (Ed.), Cold Spring Harbor Laboratory Press, New York (1989).
47. Genstat Committee: *Genstat 5 Release 3 Reference Manual*, Clarendon Press, Oxford (1995).

Karakterizacija sojeva *Hanseniaspora (Kloeckera)* izoliranih u Finger Lakes vinarijama koristeći fiziološke i molekularne postupke

Sažetak

Sojevi kvasca *Hanseniaspora (Kloeckera)* izolirani su iz nekoliko vinarija u regiji Finger Lakes, država New York, SAD. Te su kulture bile determinirane na razini sojeva tradicionalnim postupcima. Eksperimentalni fiziološki testovi mogu razdvajati sojeve, a najčešći identificirani sojevi bili su vrste *H. uvarum.* Međutim, postojala je razlika između referentnog soja *H. osmophila* i izoliranog soja u asimilaciji maltoze. Da bi se potvrdili rezultati fizioloških testova, upotrijebljeni su molekularni postupci primjenom amplificirane polimorfne DNA (RAPD)-, duljinski polimorfizam restrikcijskog fragmenta (RFLP)- i mikrosatelitska polimerazna lančana reakcija (PCR). Ti su postupci bili pouzdani i dali su nedvojbene rezultate na razini sojeva. Za razlikovanje sojeva *H. uvarum* primijenjen je postupak RAPD-mikrosatelitski PCR. Time je postignut pouzdan način identifikacije specifičnog soja.