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rDNA RFLPs and AP-PCR Fingerprinting of Type Strains and Grape-Must Isolates of *Hanseniaspora (Kloeckera)* Yeasts

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Summary

Molecular analyses of nucleic acid polymorphisms have proved to be very helpful in avoiding taxonomic ambiguities and simplifying yeast identification schemes. PCR mediated typing assays have the advantage of a rapid amplification of yeast target DNA in the laboratory thus enabling rapid analysis of DNA polymorphisms on different levels. We used two approaches – restriction analysis of amplified rDNA fragments (RFLPs of 2550 bp long 18S-ITS1-5.8S-ITS2 and 3350 bp long 25S rDNA amplicons) and AP-PCR fingerprinting of yeast DNA with six non-specific oligonucleotide primers for the characterization of Hanseniaspora (anamorph Kloeckera) yeasts. Species-specific restriction patterns were derived from type strains analysis. Two restriction analyses of 18S-ITS1-5.8S-ITS2 rDNA and/or 25S rDNA fragment were sufficient for species delineation among Hanseniaspora/Kloeckera yeasts. They were used for rapid species identification of thirthy-three Hanseniaspora/Kloeckera strains isolated from grapes and musts at the start of spontaneous fermentation in two geographically distinct wine-producing subregions in Slovenia. All identified strains were H. uvarum/K. apiculata. AP-PCR fingerprinting revealed great heterogeneity of the isolates at the intraspecies level. No correlation between genetic similarity, calculated from AP-PCR fingerprints, and the geographical origin of H. uvarum/K. apiculata strains was evidenced.

Keywords: rDNA-RFLP, AP-PCR, molecular identification, *Hanseniaspora (Kloeckera)*, wine yeast

Introduction

Conventional yeast identification schemes still rely on phenotypic characterization, such as morphological and physiological tests *(1–3)*. However, these methods are time-consuming, tedious and not always reliable. In the case of industrial yeasts identification they fail to discriminate among phenotypically very similar species, if miniaturized identification schemes with a reduced number of tests are involved. Furthermore, physiological tests do not allow yeast discrimination at the intraspecific level *(4)*.

Recently, new techniques based on yeast DNA analysis have been applied for identification and typing of industrially important yeasts from many different genera. Genus-, species- or strain-specific characteristics were found on the basis of polymorphism of chromosomal DNA (*i.e.* electrophoretic karyotypes – patterns of chromosomal bands separated by pulsed field gel electrophoresis, PFGE) *(5,6)*, restriction and hybridization analysis of mitochondrial DNA *(7,8)* or more specialized regions of the yeast chromosomal DNA (*i.e.* nuclear ribosomal RNA genes and more heterogeneous internal, external transcribed and/or non-transcribed spacer regions) *(9–12)*. In recent years, because of the possibility of simple and rapid *in-vivo* amplification of DNA fragments, new PCR-based DNA fingerprinting techniques have been introduced for the rapid identification and typing of yeasts (*i.e.* AP-PCR, arbitrarily primed polymerase chain reaction) *(13,14)*.

A great majority of published data are collected for identification of the industrially most important *Saccharomyces* yeasts. Much fewer data are available about other, »non-*Saccharomyces*« yeasts, whose application as industrial yeasts is increasing. This study, using restriction analysis of PCR-amplified 18S-ITS1-5.8S-ITS2 and 25S rDNA fragments and AP-PCR fingerprinting, was created to develop a simple and rapid protocol for *Hanseniaspora* (anamorph *Kloeckera*) yeasts identification at a species and strain level. A combination of both tech-

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niques was applied for identification and evaluation of intraspecies genetic similarity of apiculate yeast strains, isolated from the surface of grape berries and musts in early stages of fermentation. The samples were collected in geographically distinct wine producing subregions in Slovenia.

Materials and Methods

Strains: The origin of *Hanseniaspora* and *Kloeckera* yeasts used in this study is indicated in Table 1. They are maintained in the Culture Collection of Industrial Microorganisms (ZIM), Ljubljana. Initial screening of the yeast isolates was performed on the basis of the morphological features of vegetative cells and colonies on a solid medium and some physiological tests *(2,3)*. On the basis of limited number of phenotypic characters tested, it was not possible to distinguish among species of the genus *Hanseniaspora/Kloeckera*.

DNA isolation: Growth conditions for biomass cultivation and a small-scale preparation of yeast DNA have been described previously *(12)*.

Amplification of 18S-ITS-5.8S-ITS2 and 25S rDNA fragments: The target DNA was adjusted to a concentration of 1–5 μg mL^{-1}. PCR amplifications were carried out in 25 μL reaction volume containing 5–15 ng yeast DNA, 10 mmol L^{-1} Tris-HCl (pH = 8.3), 50 mmol L^{-1} KCl, 200 μmol L^{-1} each of dATP, dGTP, dCTP and dTTP, 2 mmol L^{-1} $MgCl_2$ (Perkin Elmer), 50 pmol L^{-1} each of primer and 0.5 U of Perkin Elmer AmpliTaq DNA polymerase. Two primer pairs were used to separately amplify the 18S rDNA with 5.8S and both internal transcribed spacers (ITS1, ITS2) *(15)* and 25S rDNA *(11)* with the following sequences:
NS1/ITS4: 5' GTAGTCATATGCTTGTCTC 3'/ 5' TCC TCCGCTTATTGATATGC 3'
25S rDNA1/2: 5' GGTAGGAATACCCGCTG 3'/ 5' CAA GGCTACTCTACTGCTTAC 3'

Amplifications were performed in a Perkin Elmer Gene Amp PCR System 2400 with preliminary denaturation of DNA at 95 °C for 2 min, followed by 35 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 3 min and a final step at 72 °C for 7 min. Purity and concentration of PCR products were checked on a gel.

Restriction analysis of amplified rDNA fragments: According to the DNA concentration, 1–3 μL of the amplification mixtures with 18S-ITS1-5.8S-ITS2 or 25S rDNA amplified fragment of type strains were digested directly with the four and five base cutting restriction enzymes: *Hae*III, *Scr*FI, *Msp*I, *Cfo*I and *Rsa*I, according to the instructions of the supplier (Boehringer, Mannheim). For RFLP analysis of grape-must yeast isolates, the endonucleases, previously found to give species-specific restriction patterns have been used. Restriction fragments were electrophoresed on 1.5% agarose gels in 1 × TAE, stained with ethidium bromide and documented on Polaroid 667 films.

AP-PCR fingerprinting: Four decamer oligonucleotide primers with 50 – 80% GC content and two »microsatellite« oligonucleotide primers were used for the amplification of yeast DNA:
5' GCA AGT AGC T 3' (50% GC, $t_{anneal.}$ = 36 °C),
5' CGG TCA CTG T 3' (60% GC, $t_{anneal.}$ = 38 °C),
5' AGC GGG CGT A 3' (70% GC, $t_{anneal.}$ = 38 °C),
5' CGC GTG CCC A 3' (80% GC, $t_{anneal.}$ = 42 °C),
$(GACA)_{4x}$, $t_{anneal.}$ = 51°C, $(GTG)_{5x}$, $t_{anneal.}$ = 52 °C.
Preliminary denaturation of DNA was performed at 94 °C for 5 min followed by 35 cycles at 94 °C for 30 s, $t_{anneal.}$ for 45 s and at 72 °C for 45 s. A final step of 7 min at 72 °C was followed by a 4 °C soak until recovery. Amplification products were analysed by electrophoresis in 1.5% agarose gels in 1 × TAE.

Data analysis: Binary data matrices were generated on the basis of DNA fragments from rDNA restriction patterns and AP-PCR fingerprints. A genetic similarity

Table 1. Type strains and other yeast isolates examined (T means type strain)

Species	ZIM	Origin
Hanseniaspora guilliermondii Pijper	0233	NCAIM Y.00915
H. occidentalis^T Smith	0252	NRRL Y-7946
H. osmophila^T (Niehaus) Phaff *et al.*	0116	NRRL Y-1613
H. uvarum^T (Niehaus) Shelata *et al.*	0117	NRRL Y-1614
H. valbyensis^T (Kloecker)	0250	NRRL Y-1626
H. vineae^T van der Walt & Tscheuschner	0268	NRRL Y-17529
Kloeckera africana^T(Kloecker) Janke	0113	NRRL Y-1274
K. apiculata^T (Reess) Janke	0115	NRRL Y-1573
K. apis Lavie ex Smith *et al.*	0300	NCAIM Y.00482
K. corticis^T (Kloecker) Janke	0114	NRRL Y-1381
Hansenniaspora/Kloeckera yeast isolates from	1042–1045	Subregion Koper, location I (1994)
grape berries and early stages of must	1024, 1031	Subregion Koper, location II (1994)
fermentations (Refošk, Kraški teran)	1081–1083, 1093	Subregion Kras, location I (1994)
	1055, 1066, 1071	Subregion Kras, location II (1994)
	1458–1462	Subregion Koper, location I (1995)
	1313, 1319	Subregion Koper, location II (1995)
	1622–1631, 1633, 1647	Subregion Kras, location I (1995)
	1575, 1578, 1579	Subregion Kras, location II (1995)

NCAIM, National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary;
NRRL, Northern Regional Research Center, Peoria, IL, USA;
ZIM, Culture Collection of Industrial Microorganisms, Ljubljana, Slovenia.

Table 2. Number of total and polymorphic restriction fragments revealed by different restriction enzymes after digestion of 18S-ITS1-5.8S-ITS2 and 25S rDNA of *Hanseniaspora/Kloeckera* type strains.

	18S-ITS1-5.8S-ITS2 rDNA		25S rDNA	
Restriction endonuclease	all fragments*	polymorphic fragments	all fragments	polymorphic fragments
*Cfo*I	5	3	4	2
*Hae*III	11	10	7	3
*Msp*I	5	4	8	5
*Rsa*I	6	2	7	1
*Scr*FI	7	6	n.d.	n.d.
TOTAL	34	25	26	11

* only fragments longer than 200 bp were monitored
n.d.: not determined

was estimated between all pairs of strains using the Nei equation *(16)* and unweighted pair-group arithmetic average (UPGMA) analysis with the software package NTSYS-pc. Based on the calculated percent similarities, dendrograms were drawn by the simple linkage method *(17)*.

Results

Restriction analysis of amplified rDNA fragments of Hanseniaspora/Kloeckera type strains: With the primer pairs NS1/ITS4 and 25S rDNA1/2, described in Materials and Methods, we amplified the entire rDNA repeat in two parts. The first included 18S and 5.8S with both ITS regions and the second 25S rDNA. Both amplicons were invariant in length for all tested yeasts and had approximately 2550 bp and 3350 bp, respectively. We tested different restriction enzymes to find species-specific restriction patterns of *Hanseniaspora/Kloeckera* yeasts, thus offering a simple and reliable test for the rapid identification of apiculate yeast strains, collected from grapes and must fermentations in different wine-producing sub-regions in Slovenia in two subsequent years. The number of total and polymorphic (*i.e.* different for the strains tested) restriction fragments derived from digestions of both amplified rDNA fragments depended on the enzyme used (Table 2, Fig. 1). In general, heterogeneity, *i.e.* a proportion of polymorphic restriction fragments, was higher during the 18S-ITS1-5.8S-ITS2 rDNA analysis. Comparing these results with the published data of rDNA RFLPs of other yeasts *(11,18)*, it is evident that the reason is the highly variable ITS region. However, for *Hanseniaspora/Kloeckera* yeasts, it was found that the digestion of a 25S rDNA fragment with *Msp*I discriminated between closely related species *H. uvarum/K. apiculata* and *H. guilliermondii/K. apis*, (Fig. 2), which was not achieved by any of the enzymes applied on shorter rDNA amplicon.

All polymorphic restriction fragments, presented in Table 2, were included in further analysis of the genetic similarity among yeasts tested with NTSYS-pc. On the basis of calculated similarity coefficients a dendrogram was constructed (Fig. 3). It is worth noting that this presentation of the relationships among *Hanseniaspora/Kloeckera* yeasts fully corresponds to the phylogeny derived from the sequences of different parts of 18S and 25S rRNA/DNA which have already been published *(20,21)*. A dichotomy of the genus (see also Fig. 1A) and close relationship among pairs *H. vineae/K. africana* – *H. osmophila/K. corticis* (93% similarity based on rDNA RFLPs studied) and *H. uvarum/K. apiculata* – *H. guilliermondii/K. apis* (86% similarity based on rDNA RFLPs studied) are very similar to those derived from rDNA/ rRNA sequencing, although a relatively low number of restriction enzymes, *i.e.* polymorphic restriction patterns were analysed.

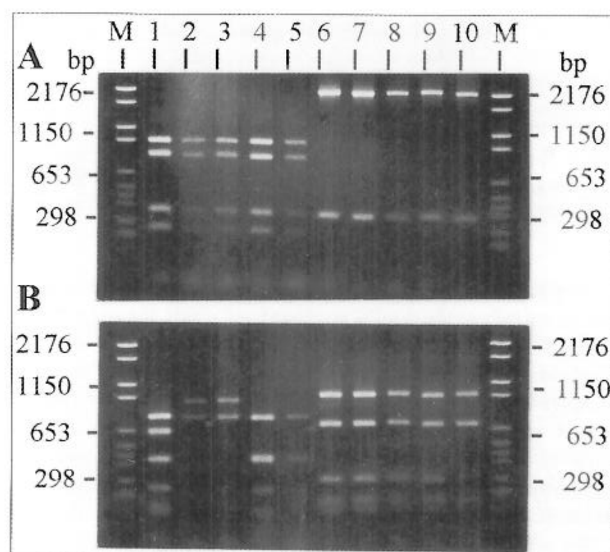

Fig. 1. Restriction patterns of 18S-ITS1-5.8S-ITS2 rDNA of *Hanseniaspora/Kloeckera* type strains, digested with (A) *Msp*I and (B) *Hae*III. Lanes: 1: *H. occidentalis*^{T}; 2: *H. vineae*^{T}; 3: *K. africana*^{T}; 4: *H. osmophila*^{T}; 5: *K. corticis* ^{T}; 6: *H. uvarum*^{T}; 7: *K. apiculata*^{T}; 8: *H. guilliermondii*; 9: *K. apis*; 10: *H. valbyensis*^{T}; M: Molecular weight marker Boehringer VI

Identification of apiculate yeast isolates with RFLPs of amplified rDNA: After amplification of rDNA fragments and their digestion with the restriction enzymes, previously found to give species-specific patterns of *Hanseniaspora/Kloeckera* type strains, we could identify all yeast isolates except two as *H. uvarum/K. apiculata*. These yeasts are common members of the yeast microflora found on the surface of grapes and, consequently, in fresh musts *(19)*, from where the strains have been isolated. Exceptional were strains ZIM 1578 and 1629 (lane 6 and 10, Fig. 4), with the restriction patterns, non-specific for *Hanseniaspora/Kloeckera* yeasts.

AP-PCR fingerprinting of H. uvarum/K. apiculata strains: Since restriction analysis of amplified rDNA frag-

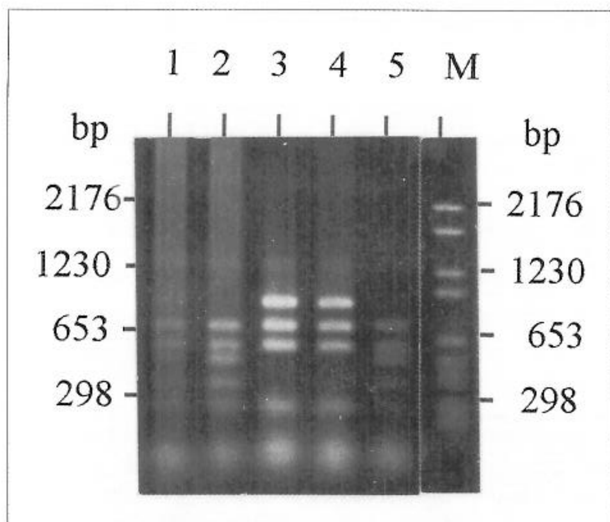

Fig. 2. Restriction patterns of 25S rDNA digested with *MspI*. Lanes: M: Molecular weight marker Boehringer VI, 1: *H. uvarum*^{T}; 2: *K. apiculata*^{T}; 3: *H. guilliermondii*; 4: *K. apis*; 5: *H. valbyensis*^{T}

ments can not be used for the discrimination of strains on an intraspecific level, we used AP-PCR fingerprinting with different non-specific oligonucleotide primers as a method of choice for strain delineation among *H.uvarum/ K. apiculata* yeasts. Several different strain-specific fingerprints were obtained (Fig. 5, 6). However, it is also possible to recognize a pattern, common for the majority of *H.uvarum/K.apiculata* strains and clearly distinguishable from another pattern (see lanes 20 in Fig. 5 and 6, ZIM 1578, which was not identified as *H. uvarum/K. apiculata*, see also lane 10 in Fig. 4). In this sense AP-PCR fingerprints confirmed the identification of strains based on restriction analysis of amplified rDNA fragments. The discrimination power of the method at the intraspecies level depended strongly on the primer and amplification conditions used. To obtain the most reliable results and highest distinction capacity, *i.e.* reliable discrimination of all strains from different origins, we used four different decamer and two »microsatellite« oligonucleotide primers to generate fingerprints of *H. uvarum/K. apiculata* strains. All polymorphic fragments were scored for further analysis of intraspecific similarity among isolates. A dendrogram (Fig. 7) shows some similarities among isolates from different subregions and locations, isolated in autumn 1995. It is evident from Fig. 7, that on the basis of AP-PCR fingerprinting, we could not confirm any specificity of the strains, isolated from geographically specific, wine producing subregions of Slovenia.

Discussion

Heterogeneity and regional specificity of yeast strains: Recently, several authors have applied molecular analysis of DNA polymorphism to study the intraspecific diversity of the indigenous yeast microflora involved in wine fermentations *(22–24)*. No correlation between the degree of genetic relatedness and the geographical vicinity was evidenced on the basis of mitochondrial DNA (mtDNA) restriction analysis of *Saccharomyces cerevisiae* strains isolated at different locations in the Charentes wine producing area, France *(23)*. On the basis of a similar analysis (UPGMA clustering based on mtDNA restriction analysis), red wine *Saccharomyces* strains from different Spanish wine producing regions were significantly grouped according to their geographic origin *(24)*. All investigations were focused on enological strains of *Sacch. cerevisiae* and other closely related species from the *Saccharomyces* sensu stricto group. Besides the evaluation of heterogeneity and regional specificity of *Sacch. cerevisiae* strains, we studied other, »non-*Saccharomyces*« yeasts, isolated in geographically different wine producing subregions in Slovenia and involved in the early stages of must fermentations. It is known that apiculate yeasts, which were the object of our study, are among the yeasts, most frequently found on grapes and also in the early stages of must fermentations. Although they are later overgrown with the alcohol more tolerant *Saccharomyces cerevisiae* yeasts, their metabolic activity at the start of fermentation could be reflected in the sensory quality specificities of the end products-wines *(19)*. Our strains were isolated from the grapes and musts of the »Refošk« vine variety in Koper and Kras region, where from the same wine variety musts two types of wine (»Refošk« and »Kraški Teran«) with very different chemical and sensory characteristics are produced. We have not confirmed any regional specificity of *Sacch. cerevisiae* strains which had started the spontaneous must fermentations studied at different locations *(25)*. On the basis of the application of two molecular typing methods, presented in this work, we could not confirm any regional or even local specificity of *Hanseniaspora/Kloeckera* strains either (Fig. 7). All strains isolated from grapes and in the first stages of fermentations studied in different wine-producing subregions were identifed as *H. uvarum/ K. apiculata*. However, great heterogeneity was revealed among isolates at an intraspecies level. Based on AP-PCR fingerprinting with six different non-specific primers, all the studied strains, isolated in 1995 could be differentiated (Fig. 7 shows only the strains which gave reliable fingerprints with all six non-specific oligonucleotide primers used). The impact of the differences among strains (revealed by molecular typing) on their technological characteristics, *i.e.* on their role during must fermentations, should be evalu-

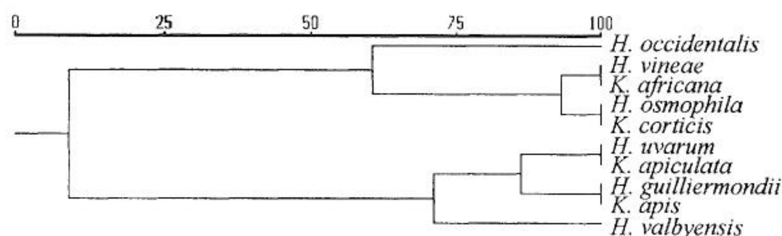

Fig. 3. Similarity dendrogram of *Hanseniaspora/Kloeckera* type strains constructed on the basis of polymorphic restriction fragments derived from digestions of rDNA amplicons with different endonucleases (Table 2)

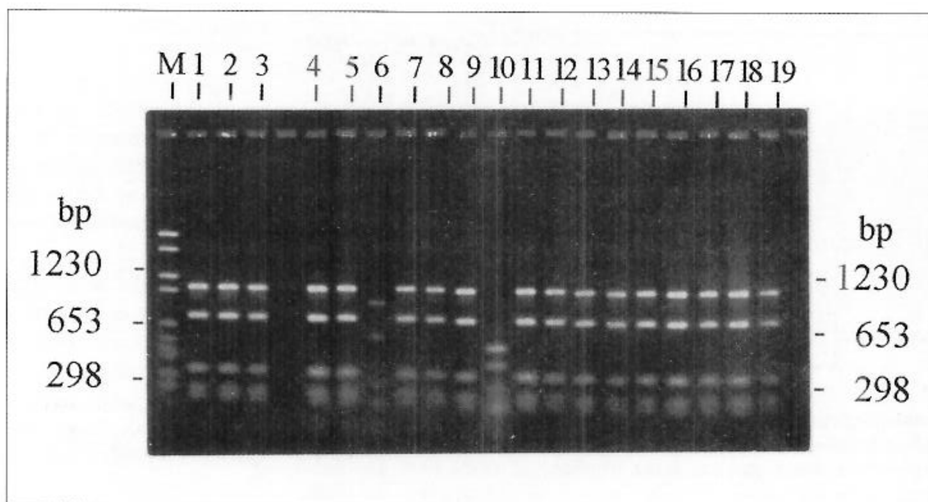

Fig. 4. Restriction patterns of 18S-ITS1-5.8S-ITS2 rDNA of *H. uvarum/K.apiculata* type strains and some other isolates digested with *Hae*III. Lanes: M: Molecular weight marker Boehringer VI, 1: *H. uvarum*^{T}; 2: *K. apiculata*^{T}, 3–5: ZIM 1623–1625; 6: ZIM 1629; 7: ZIM 1630; 8: ZIM 1631; 9: ZIM 1575; 10: ZIM 1578; 11: ZIM 1579 12–16: ZIM 1458–1462; 17: ZIM 1313; 18: ZIM 1319; 19: ZIM 1626

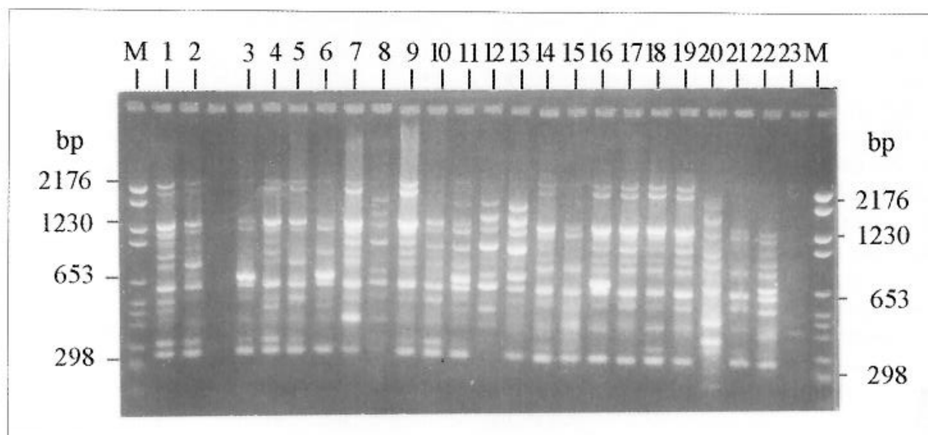

Fig. 5. AP-PCR fingerprints of *H. uvarum/K. apiculata* strains generated with non-specific oligonucleotide primer with 80% GC content (5' CGC GTG CCC A 3'). Lanes: 1: *H. uvarum*^{T}; 2: *K. apiculata*^{T}; 3–6: ZIM 1622–1625; 7: ZIM 1647; 8–10: ZIM 1626–1628; 11: ZIM 1630; 12: ZIM 1631; 13: ZIM 1633; 14: ZIM 1575; 15–19: ZIM 1458–1462; 20: ZIM 1578; 21: ZIM 1313; 22: ZIM 1319; 23: negative control; M: Molecular weight marker Boehringer VI

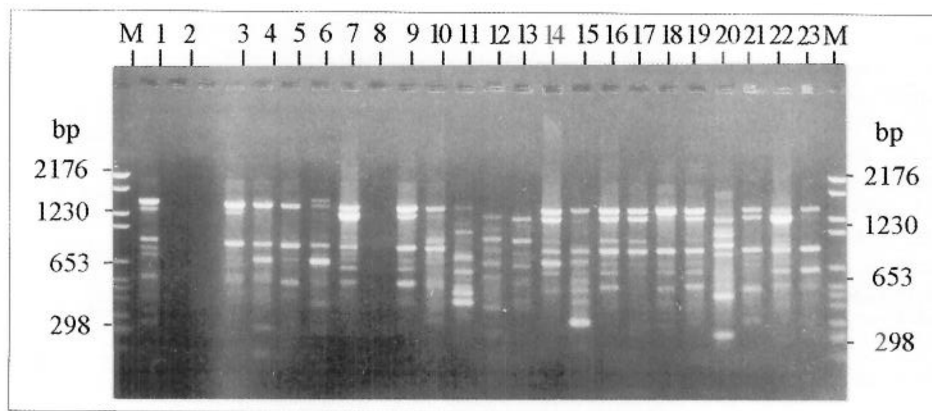

Fig. 6. AP-PCR fingerprints of *H. uvarum/K. apiculata* strains generated with non-specific oligonucleotide primer with 70% GC content (5' CGC GTG CCC A 3'). Lanes are the same order as in Fig. 5, with the exception, that type strain *K. apiculata*^{T} is in lane 23 instead of lane 2

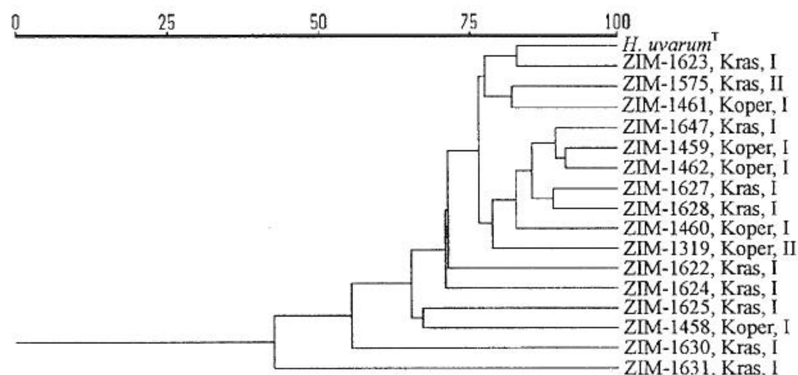

Fig. 7. Similarity dendrogram of *Hanseniaspora/Kloeckera* grape-must isolates from Koper and Kras wine producing subregions and different locations (a comment after ZIM number describes the origin, e.g. Kras, I means subregion Kras, location I, see Table 1). Dendrogram was constructed on the basis of polymorphic AP-PCR fingerprints generated with all oligonucleotide primers mentioned in Materials and Methods.

ated in further experiments. Controlled pilot fermentations enhanced with selected yeast strains are currently under study.

Comparative analysis of PCR-typing methods used in study: Many different molecular typing methods have been applied in studying the role of yeasts in spontaneous and starter enhanced must-wine fermentations. Among them, mtDNA restriction analysis and electrophoretic karyotyping revealed by pulsed field gel electrophoresis (PFGE) were most frequently used *(5–7, 22–26)*. However, both techniques require the more or less demanding, time- and material-consuming isolation of the target DNA (mitochondrial, intact chromosomal DNA, respectively) from the yeast cells. PCR mediated typing assays have the advantage of a rapid amplification of the target yeast DNA in the laboratory, *i.e.* they enable quick and simple analysis of yeast DNA polymorphisms at different levels. A rapid and simple small-scale preparation of total yeast DNA could be used for the isolation of target DNA for different amplifications. We found in this study a combined use of the amplification of rRNA genes and internal transcribed spacers with subsequent restriction analysis of amplified fragments and amplification of yeast DNA with non-specific oligonucleotide primers as a suitable methodology for the characterization of *Hanseniapora/Kloeckera* yeasts. Species-specific »restriction« patterns of *Hanseniapora/Kloeckera* type strains were used for quick identification of isolates at a species level. A combination of maximally two restriction analyses of 18S-ITS1-5.8S-ITS2 and/or 25S rDNA fragment were sufficient for species delineation among *Hanseniapora/Kloeckera* yeasts. In addition, AP-PCR fingerprints, generated with different non-specific oligonucleotide primers, enabled a discrimination and the evaluation of genetic similarity among yeast strains originating from geographically different wine-producing subregions of Slovenia.

Conclusions

A combination of two PCR mediated typing methods, RFLPs of amplified rDNA fragments and AP-PCR fingerprinting, is a useful tool for quick identification of *Hanseniapora/Kloeckera* yeasts from the genus to the subspecies level. The first approach is crucial for the determination of the isolate's taxonomic position at the genus and species level, but the latter contributes to the rate of discrimination at the subspecies level.

Apiculate yeast strains isolated from the samples of grapes and musts at the start of fermentation in different wine producing subregions of Slovenia in two subsequent years were very homogenous at the species level (only *H. uvarum/ K. apiculata* species was determined), but very heterogeneous at the strain level. On the basis of AP-PCR fingerprinting, no correlation between genetic similarity among strains and their geographical origin has been evidenced.

Acknowledgement

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References

1. *The Yeasts, a Taxonomic Study*, 3rd ed., N. J. W. Kreger van Rij (Ed.), Elsevier Science Pub., Amsterdam (1984).
2. J. A. Barnett, R. W. Payne, D. Yarrow: *Yeasts: Characterization and Identification*, Cambridge University Press, Cambridge (1990).
3. J. A. Barnett, R.W. Payne, D. Yarrow: *Yeast Identification PC Program*, version 4, Cambridge University Press, Cambridge (1996).
4. A. Querol, E. Barrio, D. Ramon, *Syst. Appl. Microbiol. 15* (1992) 439.
5. F. Vezinhet, B. Blondin, J. N. Hallet, *Appl. Microbiol. Biotechnol. 32* (1990) 568.
6. G. Naumov, E. Naumova, C. Gaillardin, *Syst. Appl. Microbiol. 16* (1993) 274.
7. J. M. Guillamon, E. Barrio, T. Huerta, A. Querol, *Int. J. Syst. Bacteriol. 44* (1994) 708.
8. J. Piškur, S. Smole Možina, J. Stenderup, M. B. Pedersen, *Appl. Environ. Microbiol. 61* (1995) 2780.
9. F. I. Molina, T. Inoue, S. C. Jong, *Int. J. Syst. Bacteriol. 42* (1992) 499.
10. F. I. Molina, S. C. Jong, J. L. Huffman, *FEMS Microbiol. Lett. 108* (1993) 259.
11. R. Messner, H. Prillinger, *Antonie van Leeuwenhoek, 67* (1995) 363.
12. S. Smole Možina, D. Dlauchy, T. Deak, P. Raspor, *Lett. Appl. Microbiol. 24* (1997) 311.
13. E. Lieckfeldt, W. Meyer, T. Börner, *J. Basic Microbiol. 33* (1993) 413.
14. O. Molnar, R. Messner, H. Prillinger, U. Stahl, E. Slavikova, *Syst. Appl. Microbiol. 18* (1995) 136.

15. T. J. White, T. Bruns, S. Lee, J. Taylor, Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. In: *PCR Protocols: A Guide to Methods and Applications*, M. A. Innis, D. H. Gelfand, J. J. Sninsky, T. J. White (Eds.), Academic Press, London (1990) pp. 315–322.
16. M. Nei, W. H. Li, *Proc. Natl. Acad. Sci. USA, 76* (1979) 5269.
17. F. J. Rohlf: *NTSYS – pc Numerical Taxonomy and Multivariate Analysis System*, version 1.80. Exeter Software, Applied Biostatistics Inc., Setauket, New York (1993).
18. K. Shin, S. Hong, K. S. Bae, *J. Gen. Appl. Microbiol. 42* (1996) 481.
19. G. H. Fleet, The Microorganisms of Winemaking-Isolation, Enumeration and Identification. In: *Wine Microbiology and Biotechnology*, G. H. Fleet (Ed.), Harwood Academic Publishers, Camberwell (1993) pp. 1–26.
20. Y. Yamada, K. Maeda, I. Banno, *J. Gen. Appl. Microbiol. 38* (1992) 585.
21. T. Boekhout, C. P. Kurtzman, K. O'Donnel, M. T. Smith, *Int. J. Syst. Bacteriol. 44* (1994) 781.
22. F. Vezinhet, J. N. Hallet, M. Valade, A. Poulard, *Am. J. Enol. Vitic. 43* (1992) 83.
23. A. Varsavaud, P. Courcoux, C. Roulland, L. Dulau, J. N. Hallet, *Appl. Environ. Microbiol. 61* (1995) 3521.
24. J. M. Guillamon, E. Barrio, A. Querol, *Syst. Appl. Microbiol.* 19 (1996) 122.
25. P. Raspor, S Smole Možina, J Podjavoršek, K. Jemec-Povhe, V. Režić-Dereani, M. Pečjak, *Food Technol. Biotechnol.* (submitted 1998).
26. D. Nadal, B. Colomer, B. Pina, *Appl. Environ. Microbiol. 62* (1996) 1944.

RFLP i AP-PCR postupak »fingerprinting« provedeni su s rDNA kvasaca *Hanseniaspora (Kloeckera)* iz tipičnih sojeva te izolata iz grožđa i mošta

Sažetak

Molekularne analize polimorfizama nukleinske kiseline pokazale su se vrlo korisnim, kako bi se izbjegle taksonomske nejasnoće i pojednostavio postupak identifikacije kvasaca. PCR-om posredovani način tipizacije ima prednost u brzoj amplifikaciji DNA određenog kvasca omogućujući tako brzu analizu DNA polimorfizama. U radu su primijenjena dva pristupa: restrikcijska analiza amplificiranih rDNA fragmenata (RFLP-i 2.550 bp dugog fragmenta 18S-ITS1-5,8S-ITS2 i 3.350 bp dugih 25S rDNA amplikona) te AP-PCR postupak »fingerprinting« kvaščeve DNA sa šest nespecifičnih oligonukleotidnih klica (eng. primer) za karakterizaciju Hanseniaspora (anamorfni Kloeckera) kvasaca. Za vrstu specifičan restrikcijski uzorak dobiven je analizom tipičnih sojeva. Dvije restrikcijske analize fragmenta 18S-ITS1-5,8S-ITS2 i/ili 25S fragmenta rDNA bile su dostatne za karakterizaciju vrste između kvasaca Hanseniaspora (Kloeckera). Tim je postupcima ubrzana identifikacija 33 vrste soja Hanseniaspora (Kloeckera) izoliranih iz grožđa i mošta na početku spontane fermentacije u dvije geografski određene vinorodne subregije Slovenije. Svi su identificirani sojevi pripadali H. uvarum/K. apiculata. AP-PCR »fingerprinting« otkrio je veliku heterogenost izolata na razini međusojeva. Nije utvrđena neka korelacija između genetske sličnosti i geografskog podrijetla H. uvarum/K. apiculata prema rezultatima postupka AP-PCR »fingerprinting«.