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Microsatellite analysis of commercial wine yeast strains

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The character of wines is strongly influenced by the fermentation process. Metabolism of individual yeasts will result in specific wines. Therefore identification of yeast strains is an important task for the wine industry. Each supplier of yeasts wants his strains to be unique on the market. Due to the steady development of new and improved yeast strains, it is of increasing importance to have a good tool for identification. The verification of the uniqueness of new selected yeast strains is possible with the help of microsatellite PCR. In this work 38 different commercial wine yeast strains were characterized by the use of microsatellite markers. Four SSR loci were sufficient to identify all tested yeasts and to assess their genetic relationship. Microsatellite analysis will be an appropriate tool for the identification of new selected yeast strains.
Keywords: wine, yeast, SSR marker, PCR, fragment analysis

Mikrosatelliten-Analyse von kommerziellen Weinhefe-Stämmen. *Der Charakter von Weinen wird stark durch den Fermentationsprozess beeinflusst. Verschiedene Hefen produzieren unterschiedliche Stoffwechselprodukte und dadurch unterscheidbare, typische Weine. Die Identifikation der Hefestämme ist eine wichtige Aufgabe für die Weinindustrie, da jeder Hefeproduzent möchte, dass seine Stämme einzigartig auf dem Markt sind. Durch die stetige Entwicklung von neuen verbesserten Hefen ist es von wachsender Notwendigkeit, ein gutes Werkzeug zur Identifizierung zu haben. Der Nachweis der Einzigartigkeit von neu selektionierten Hefen ist mit Hilfe der Mikrosatelliten-Analyse möglich. In dieser Arbeit wurden 38 verschiedene, käuflich erwerbbare Hefestämme charakterisiert. Vier SSR (Single Sequence Repeats)-Loci waren ausreichend, um alle getesteten Hefen zu identifizieren und ihre genetische Verwandtschaft zu ermitteln. Die Mikrosatelliten-Analyse erwies sich als ein adäquates Werkzeug für die Identifikation von neu selektionierten Hefestämmen.*
Schlagwörter: Wein, Hefe, SSR-Marker, PCR, Fragmentanalyse

Analyse de microsatellites de souches de levure de vin en vente dans le commerce. *Le caractère des vins est fortement influencé par le processus de fermentation. Les différentes levures produisent des produits du métabolisme différents et donc des vins typiques distinguables. L'identification des souches de levure est une tâche importante de l'industrie du vin, étant donné que chaque producteur de levure souhaite que ses souches soient uniques sur le marché. En raison du développement permanent de levures nouvelles et améliorées, il est de plus en plus important de disposer d'un outil d'identification efficace. L'analyse de microsatellites permet de fournir la preuve du caractère unique de levures nouvellement sélectionnées. 38 souches de levure en vente sur le marché ont été caractérisées dans le présent travail. Quatre loci SSR (Single Sequence Repeats) ont suffi pour identifier toutes les levures testées et pour déterminer leur parenté génétique. L'analyse de microsatellites s'est révélée être un outil adéquat d'identification de souches de levure nouvellement sélectionnées.*
Mots clés : vin, levure, marqueurs SSR, PCR, analyse de fragments

Naturally grape juice is metabolized to wine by spontaneous alcoholic fermentation due to the activity of native yeasts. Particular vineyard sites favour the establishment of individual yeast populations. Nevertheless yeasts of *Saccharomyces cerevisiae* are the most important species, as the development of cell densities and

their accelerated propagation rate dominates the fermentation process (FUGELSANG, 1997). But the disadvantage of spontaneous alcoholic fermentation is the lack of sufficient control. Sometimes fermentation ends up in sluggish and stuck fermentation. Another risk is that microorganisms, like *Hanseniaspora*, *Brettanomyces/Dekkera,* lactic acid bacteria or acetic acid bacteria produce undesired metabolites spoiling the wine. Therefore nowadays most wineries add yeasts of a selected *Saccharomyces* strain in a pure culture. This procedure guarantees a safe and complete fermentation. For production of specific wines it seems important that differentiation of yeasts at the strain level is feasible so that individual new yeast strains can be selected. These new strains must be analysed for their uniqueness, to check if they will be useful for commercial application. Another reason is that identification at strain level ensures that the commercialized yeasts are identical with the originally selected ones (LOPEZ, 2001).

Table 1: Yeast strains used in this study

Strain	Company	Country of origin	Saccharomyces cerevisiae var.
Oenoferm Klosterneuburg	Erbsloeh	Austria	cerevisiae
Oenoferm Rouge	Erbsloeh	Austria	cerevisiae
Oenoferm Bouquet	Erbsloeh	France	bayanus
Oenoferm Freddo	Erbsloeh	Australia	bayanus
Oenoferm Interdry	Erbsloeh	Australia	bayanus
Oenoferm Tipico	Erbsloeh	Australia	cerevisiae
Oenoferm Color	Erbsloeh	France	bayanus
Coll. Cépage Cab. Sauvignon	DSM	France	cerevisiae
Noble.ferm	Chr. Hansen	unknown	cerevisiae
Ruby.ferm	Chr. Hansen	unknown	cerevisiae
Zymaflore VL1	Lallemand	France	cerevisiae
Enoferm T306	Lallemand	Australia	cerevisiae
Enoferm M2	Lallemand	South Africa	bayanus
Uvaferm CS2	Lallemand	France	cerevisiae
Lalvin RC212	Lallemand	France	cerevisiae
Lalvin AMH	Lallemand	Germany	cerevisiae
Lalvin R-HST	Lallemand	Austria	cerevisiae
Lalvin 71B	Lallemand	France	cerevisiae
Lalvin L2056	Lallemand	France	cerevisiae
Lalvin L1033	Lallemand	South Africa	cerevisiae
GIST LW05	GIST Brocades*	unknown	cerevisiae
Fermiblanc Arom	DSM	France	cerevisiae
Coll. Cépage Merlot	DSM	France	cerevisiae
Coll. Cépage Chardonnay	DSM	unknown	cerevisiae
Fermicru Primeur	DSM	France	cerevisiae
Oenoferm GV	Erbsloeh	unknown	bayanus
Oenoferm Pinot Type	Erbsloeh	Austria	cerevisiae
Hefix 2000	Erbsloeh	unknown	bayanus
Oenoferm Rosé	Erbsloeh	France	bayanus
Lalvin EC1118	Lallemand	France	bayanus
Lalvin ICV-D47	Lallemand	France	cerevisiae
Lalvin EGH2 Pannonia	Lallemand	Austria	cerevisiae
Lalvin ICV-K1 (V1116)	Lallemand	France	cerevisiae
Fermicru LS2	DSM	France	bayanus
Fermivin	DSM	France	cerevisiae
Fermicru VB1	DSM	France	bayanus
Maurivin AWRI 35	Mauri	Australia	bayanus
Maurivin AWRI R2	Mauri	France	bayanus

*GIST Brocades is now DSM and the strain LW05 is now available as Collection Cépage Chardonnay

The unequivocal identification of wine yeast strains was impossible with traditional microbiological methods (LAVALLÉE et al., 1994). Hence several new methods based on DNA analysis including RFLP (Restriction Length Fragment Polymorphism) of genomic and mitochondrial DNA (DEGRÉ et al., 1989; LAVALLÉE et al., 1994; QUEROL et al., 1992), DNA hybridization with probe sequences (DEGRÉ et al., 1989), chromosome karyotyping (YAMAMOTO et al., 1991; VAN DER WESTHUIZEN and PRETORIUS, 1992), RAPD (Randomly Amplified Polymorphic DNA) (LAVALLÉE et al., 1994; QUESADA et al., 1995) and AFLP (Amplified Fragment Length Polymorphism) (DE BARROS LOPES et al., 1999) were developed. PCR fingerprinting with microsatellites (BALEIRAS COUTO et al., 1996; GALLEGO et al.; 1998) could also be used for yeast identification and differentiation. FIELD and WILLS (1998) characterized twenty microsatellite loci of *Saccharomyces cerevisiae* by sequencing. Three of them were verified by GONZALEZ TECHERA et al. (2001). These loci seem to be very useful for routine discrimination of wine yeast strains. Other studies (HENNEQUIN et al.; 2001; PÉREZ et al., 2001; HOWELL et al., 2004; LEGRAS et al., 2005) demonstrate the advantages of applying microsatellite analysis for identification

Table 2: PCR primer pairs used for amplification of loci containing microsatellites

Primer	Sequence	References
SCYOR267C-fw	5'- tac taa cgt caa cac tgc tgc caa – 3'	(LEGRAS et al., 2005)
SCYOR267-rv	5'- gga tct act tgc agt ata cgg g – 3'	(GONZÁLEZ TECHERA et al., 2001)
C5fw	5'- tga cac aat agc aat ggc ctt ca – 3'	(LEGRAS et al., 2005)
C5rv	5'- gca agc gac tag aac aac aat cac a – 3'	(LEGRAS et al., 2005)
C11fw	5'- ttc cat cat aac cgt cgt gga tt – 3'	(LEGRAS et al., 2005)
C11rv	5'- tgc ctt ttt ctt aga tgg gct ttc – 3'	(LEGRAS et al., 2005)
SC8132X-fw	5'- ctg ctc aac ttg tga tgg gtt ttg g – 3'	(GONZÁLEZ TECHERA et al., 2001)
SC8132X-rv	5'- cct cgt tac tat cgt ctt cat ctt gc – 3'	(GONZÁLEZ TECHERA et al., 2001)

approaches.
In this paper the differentiation of 38 commercial available yeast strains by four SSR (Single Sequence Repeats) loci is presented. Furthermore reference strains of *Saccharomyces cerevisiae* and *Saccharomyces bayanus* were involved in the study and the relationship of the strains was calculated.

Material and methods

Strains and media

38 commercial available yeast strains (Table 1) and two reference strains were used for this study. The reference strains were *Saccharomyces cerevisiae* HA227^T and *Saccharomyces bayanus* HA326^T from the ACBR Collection (Austrian Center of Biological Resources and Applied Mycology, Vienna). All strains were grown on YM-medium (3 g/l yeast extract, 3 g/l malt extract, 5 g/l peptone, 10 g/l glucose, 20 g/l agar) for DNA isolation and stored at 4 °C.

DNA preparation

The DNA was extracted according to the following method. The yeasts were grown in 5 ml YM-medium overnight at 30 °C. Yeast cells were pelleted and washed with H_2O. The pellet was resuspended in 500 µl Sorbitol-buffer (1 M sorbitol, 0,1 M EDTA pH 7,5) and 200 µl of Lyticase (25KU/mg) was added. After 1 h at 37 °C, the suspension was centrifuged at 13000 rpm for 5 min. The pellet was resuspended in 500 µl Tris/EDTA-buffer (50 mM Tris pH 7,5, 20 mM EDTA) and 50 µl SDS (10%). After incubation for 30 min at 65 °C, 200 µl 5 M potassium acetate was added and the mixture was put on ice for 1 h. After centrifugation at 13000 rpm for 10 min the supernatant was precipitated with 1.5 volumes of 2-propanol for 15 min and then collected by centrifugation at 13000 rpm for 10 min. The pellet was resuspended in 300 µl TE at 65 °C. Afterwards 20 µl RNase A (2.5 mg/l) were added and a digest at 37 °C for 1 h followed. The DNA was precipitated with 500 µl 2-propanol, pelleted by centrifugation at 13000 rpm for 10 min, rinsed with 70% EtOH, dried and resuspended in 35 µl TE. A 1:10 dilution was prepared and 2 µl were used for a 25 µl PCR reaction.

Oligonucleotide primers

For fragment analysis all forward primers were labelled on the 5' end with different fluorescent dyes: 6-FAM (6-carboxyfluorescein), TET (tetrachlorofluorescein), HEX (hexachloro-fluorescein).

PCR amplification

The amplifications were carried out in 25 µl reactions consisting of 2 µl 1:10 diluted DNA and 23 µl reaction mix, containing 20 pmol each of forward and reverse primer, dNTP, 2.5 units Biotherm^{TM} DNA Polymerase (GeneCraft, Germany) and 2.5 µl 10x reaction buffer (Biotherm, GeneCraft, Germany). The reactions were performed in a GeneAmp PCR System 2400 (Perkin-Elmer, USA). For the amplification of the loci SCYOR267C, C5 and C11 the following protocol was used: 94 °C/4 min - (94 °C/30 sec - 55 °C/30 sec - 72 °C/1 min) x 35 - 72 °C/7min. The program for the locus SC8132X was: 94 °C/4 min - (94 °C/30 sec - 64,4 °C/30 sec - 72 °C/1 min) x35 - 72 °C/7min. After amplification the DNA samples were stored at 4 °C.

Gel electrophoresis and fragment analysis

An aliquot of the amplified DNA was separated for estimation of yield on a 3% (w/v) agarose gel (Conda, Madrid, Spain) in TBE buffer with a PCR 20 bp low ladder (Sigma, Steinheim, Germany) as size marker. The gels were stained with ethidium bromide and visualized under UV light and photographed. The estimated amount of nucleic acid was denatured by heating up with formamide and loaded together with a size standard (Genescan 350 Tamra, Applied Biosystems, USA) on a 6% polyacrylamide gel. Detection of the fragments labelled with 6-FAM, TET and HEX was performed by an automated sequencer (ABI 373, Perkin El-

mer, Vienna). Labelling with different fluorescent dyes allowed the application of multiplex PCR. Data analysis was carried out with GeneScan-Software (Applied Biosystems, USA). Statistical analysis of the yeast data was performed as hierarchical cluster analysis with the software package SPSS (SPSS Inc., Illinois, USA).

Results and discussion

Table 3 shows the results of the fragment analysis. The four loci, used in this study were selected because of their high polymorphism. The loci C5 and C11 are localized in the intergenic regions (LEGRAS et al., 2005), SCYOR267C is located on ORF2 and SC8132X on ORF3 (FIELD and WILLS, 1998).

The calculation for the dendrogram was done with SPSS and hierarchical cluster analysis chosen. This cluster analysis pools on each cluster level the structures according to their similarity to clusters. The used similarity was calculated on basis of square Euclidian distances. This divisive method starts in the first cluster level with one cluster which includes all objects (in our case the different yeast strains) and separates successively in each following cluster level groups of objects,

Table 3: Allele length of the different microsatellite loci

Yeast strain	Locus			
	C5	C11	SCYOR267C	SC8132X
Oenoferm Klosterneuburg	115, 153	215, 238	335, 342	210, 219
Oenoferm Rouge	115, 153	215, 238	335, 342	210, 219
Oenoferm Bouquet	130, 160, 165	191, 219	280, 288	210, 216
Oenoferm Freddo	120, 128	189, 215	305, 310	186, 198
Oenoferm Interdry bayanus	144, 184	215, 224	305, 310	195, 216
Oenoferm Tipico	120	222	304, 310	195
Oenoferm Color	139, 148	199, 217	291, 296	166, 216
Coll. Cépage® Cab. Sauvignon	159, 169	207, 213	274, 279	189, 216
Viniflora® Noble.ferm	139, 148	199, 217	291, 296	166, 216
Viniflora® Ruby.ferm	139	226	318, 324	186
Zymaflore VL1	116, 132	212, 214	285, 291	210, 213
Enoferm T306	169	226	274, 280	213, 216
Enoferm M2	120	220	310	195
Uvaferm CS2	163, 173	189, 210	305, 319	198
Lalvin AMH	153	191	315, 322	207, 210
Lalvin R-HST	132, 155	191, 216	318, 324	210
Lalvin 71B	115, 155	185, 189	274, 279	166, 210
Lalvin L2056	118, 157	199, 210	274, 279	158, 216
Lalvin 1033	120	222	304, 310	195, 216
Lalvin RC212	115	185, 218	310, 359	213
GIST LW05*	146	191	280	216
Fermiblanc® Arom	116	191, 219	280	210
Collection Cépage® Merlot	115, 141	218, 245	246, 322	210
Collection Cépage® Chardonnay	118, 146	191	213, 280	216
Fermicru® Primeur	125, 151	215, 243	213, 280	216
Oenoferm GV	120, 128	189, 214	310	186, 198
Oenoferm PinoType	153, 165	206, 218	277, 280	210
Hefix 2000	110, 120, 128	189, 214	268, 310	186, 198
Oenoferm Rosé	135, 153	206, 218	237, 276	209
Lalvin EC 1118	119, 128	189, 214	304, 310	186, 198
Lalvin ICV-D47	117, 185	191, 199	279	209
Lalvin EGH2 "Pannonia"	117	199, 210	279	152, 157, 216
Lalvin ICV-K1 (V1116)	117, 148	191, 199	279	166, 189
Fermicru® L52	119, 128	189, 215	304	186, 198
Fermivin®	129, 146	191, 216	279	168, 215
Fermicru® VB1	119, 128	189, 217	304, 310	186, 198
Maurivin AWRI 350	146, 187	214, 222	310	195, 215
Maurivin AWRI R2	161, 165	191, 218	279, 288	210, 216
HA 326 S. bayanus	115, 119, 128	189, 214	290, 304, 310, 347, 367	186, 198, 210, 216
HA 227 S. cervisiae	117	216	276, 342	145, 210

*now Coll. Cépage Chardonnay

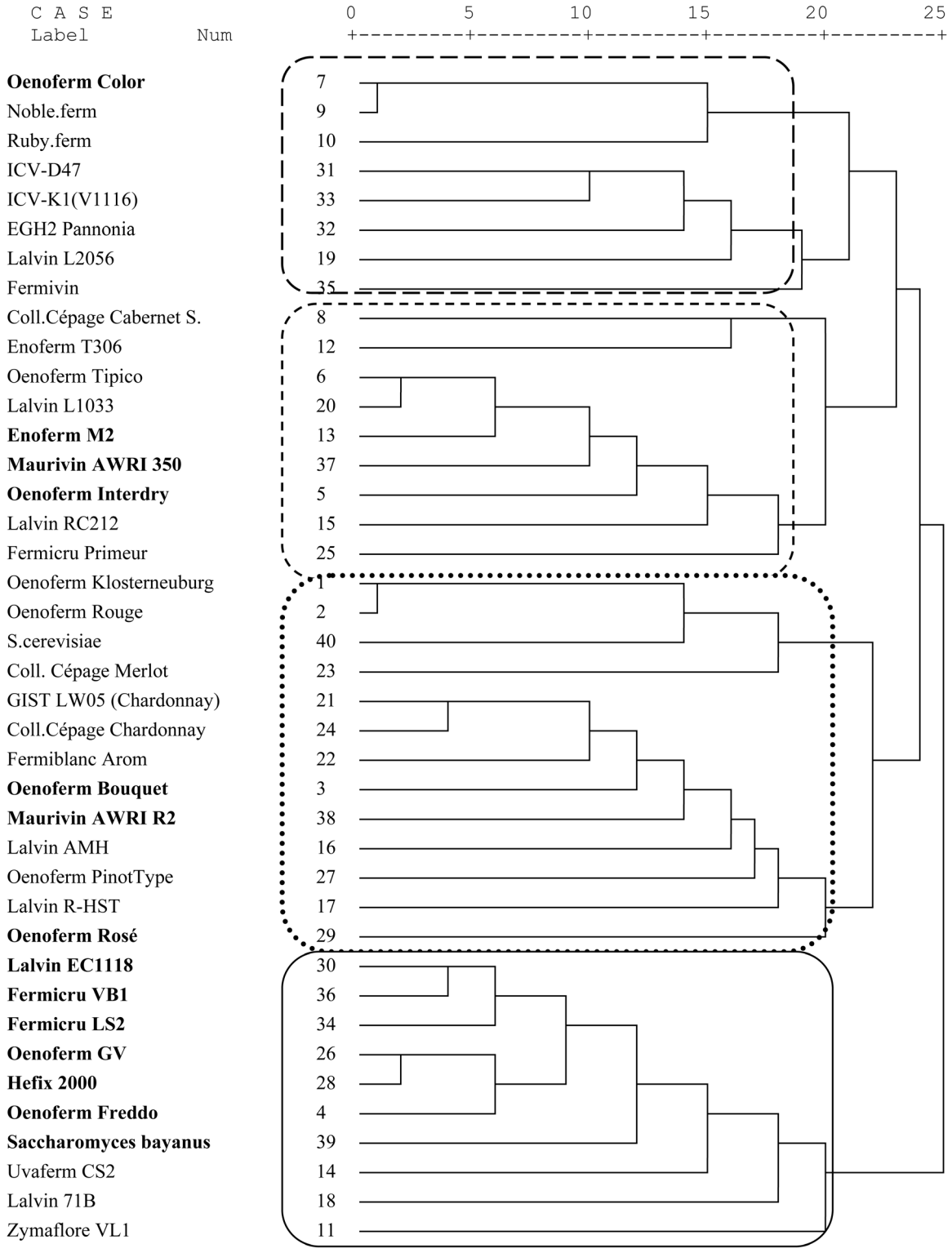

Fig.1: Dendrogram which shows the clustering of the used commercial yeast strains and the two type strains.
— — — Cluster 1 - - - - Cluster 2 ••••• Cluster 3 ——— Cluster 4

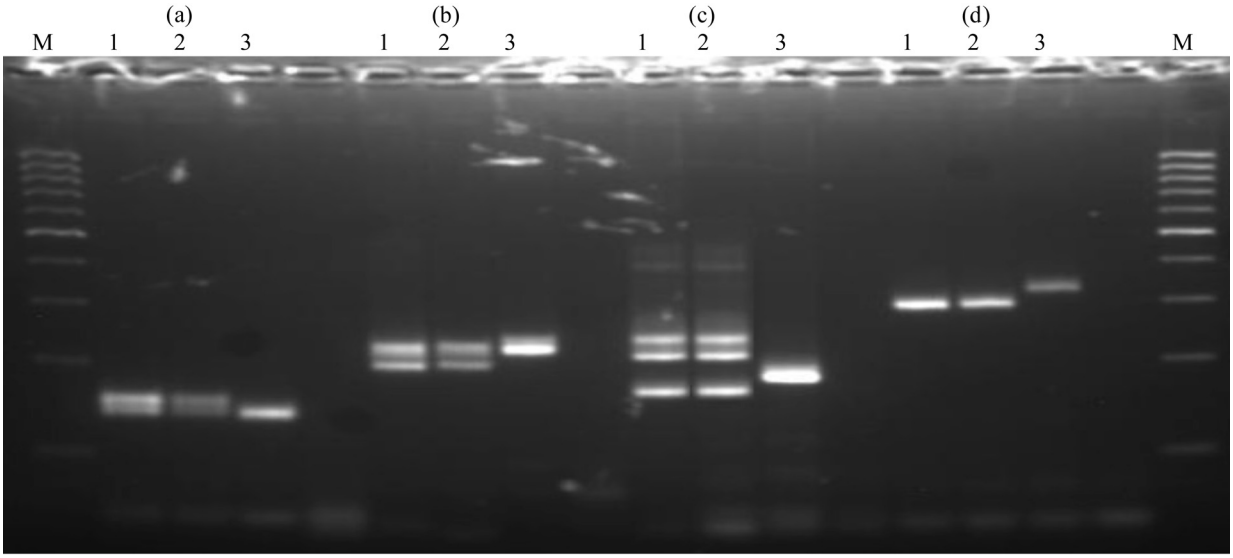

Fig. 2: PCR with the four microsatellite primerpairs. Lane 1: Oenoferm Color, Lane 2: Noble.ferm, Lane 3: Ruby.ferm, M: 100 bp DNA ladder (New England Biolabs). (a) locus C5, (b) locus C11, (c) locus SC8132X and (d) locus SCYOR267C

until each object belongs to an own cluster. This hierarchical grouping is shown in the dendrogram (Fig. 1).
The dendrogram shows that the commercial yeast strains can be easily distinguished from each other. Most strains from cluster 2 (Fig. 1) were from the southern hemisphere. Exceptions are strains 8, 15 and 25. These strains were selected in France. In cluster 4 (Fig. 1) most strains were isolated in France, only the strains from Erbsloeh are from Australia, the second small cluster in this grouping.
Those strains supposed to be related, reflect their relationship in the SSR profile. So it was found that the genetic profiles of the yeast strains Oenoferm Klosterneuburg and Oenoferm Rouge are identical. In fact Oenoferm Rouge was selected out of Oenoferm Klosterneuburg, they differ only in some characteristics during fermentation but no genetical differences are detectable by SSR analysis. Other high homologous strains are Oenoferm GV, Hefix 2000 and Oenoferm Freddo from Erbsloeh (Geisenheim, Germany). These strains are closely related to each other in the dendrogram and form a specific small cluster. According to Erbsloeh Oenoferm GV and Hefix 2000 derive from the same mother cell. Nevertheless they are not identical, due to different behavior in nutrition, in reproduction and drying conditions.
Also the yeast strains Oenoferm Color and Noble.ferm seem to be identical (Fig. 2). This result was not expected, because according to information from the manufacturer Oenoferm Color is a *Saccharomyces bayanus* and Noble.ferm a *Saccharomyces cerevisiae* strain. Possible explanations for their identical SSR-profiles are that there is a gap in one's knowledge regarding the selection of the yeast or that the applied SSR method is not sensitive enough to detect the difference between these two yeasts.
Nevertheless SSR-analysis of DNA seems to be really a promising method for the differentiation of commercial and new selected yeast strains. New selected strains can be easily checked against a database of commercial available yeast strains if they are really new ones or if this yeast already exists on the market. For more specific results of the strains it will be necessary to repeat this analysis with more than four microsatellite loci, so that finally a clear differentiation and clustering of the strains with sharper cluster formation will be achieved. Especially it is necessary to find efficient locations of origin to separate the two species *Saccharomyces cerevisiae* and *Saccharomyces bayanus*, because with regard to presented data a clear discrimination of these two species is still lacking.

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