DIFFERENTIATION OF COMMERCIAL WINE YEAST STRAINS BY MOLECULAR MARKERS

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In recent years an increasing trend towards terroir-emphasizing yeasts has emerged, and with that a need to distinguish local strains from commercial available wine yeasts. Therefore, in the present study, a database was set up with the genetic profiles of commercial wine yeasts used in Austria. This is an important tool for the selection and verification of autochthonous wine yeasts from vineyards, wineries or must productions. Three different molecular markers, namely microsatellites, interdelta analysis and M13 fingerprinting, were tested for their ability to differentiate 75 commercial wine yeast strains in a single approach and in combination. It turned out that at least two of the methods should be used for a meaningful comparison and distinct discrimination. Four autochthonous yeasts could be differentiated from their commercially propagated relatives. If a quick initial assessment has to be done, then microsatellite analysis is certainly the favored method.
Keywords: SSR marker, PCR, interdelta, M13

Differenzierung von kommerziellen Weinhefestämmen mittels molekularer Marker. In den letzten Jahren hat sich ein zunehmender Trend zu terroir-betonten Hefen herausgebildet, mit dem die Notwendigkeit verbunden ist, lokale Stämme von kommerziell erhältlichen Weinhefen zu unterscheiden. In der vorliegenden Studie wurde daher eine Datenbank mit den genetischen Profilen der in Österreich verwendeten kommerziellen Weinhefen erstellt. Dies ist ein wichtiges Instrument für die Auswahl und Überprüfung von autochthonen Weinhefen aus Weinbergen, Weingütern oder Mostproduktionen. Drei verschiedene molekulare Marker, nämlich Mikrosatelliten, Interdelta-Analyse und M13-Fingerprinting, wurden auf ihre Fähigkeit getestet, 75 kommerzielle Weinhefestämme in einem einzigen Ansatz und in Kombination zu unterscheiden. Es stellte sich heraus, dass mindestens zwei der Methoden für einen aussagekräftigen Vergleich und eine eindeutige Unterscheidung verwendet werden sollten. Vier autochthone Hefen konnten von ihren kommerziell vermehrten Verwandten unterschieden werden. Wenn eine schnelle Ersteinschätzung durchgeführt werden soll, ist die Mikrosatellitenanalyse mit Sicherheit die zu bevorzugende Methode.
Schlagwörter: SSR Marker, PCR, Interdelta, M13

Yeasts are the most important additive component in winemaking. Yeasts of the *Saccharomyces* sensu stricto complex play the main role, their dominating species being *Saccharomyces cerevisiae*, *S. uvarum* and hybrids like *S. uvarum* × *S. eubayanus* (formerly: *S. bayanus*) or *S. cerevisiae* × *S. kudriavzevii*. Currently about 500 wine yeasts are registered as agents for wine production in Austria (Liste der Weinbehandlungsmittel, 2019). For the development of new yeast strains, it is necessary to identify them, compare certain individuals and produce pure yeast cultures. A quick and easy tool for identification is the use of molecular techniques. In contrast, morphological and physiological characterization is time-consuming and depends on environmental influences, and a clear distinction of the yeasts was not possible with the traditional microbiological methods (Lavallée et al., 1994).

Microsatellite analysis for the discrimination of *S. cerevisiae* strains has been a well-established method for over 20 years (Gallego et al., 1998; Hennequin et al., 2001; Howell et al., 2004). Due to the possibility of Multiplex PCR of microsatellite loci with a high degree of polymorphism a large number of yeast strains can be differentiated in short time (Legras et al., 2005). Another advantage of this method is its robustness, the high discriminatory power and that it even can be applied to raw DNA templates.

Microsatellites are simple sequence repeats (SSR or Short Tandem Repeats = STR) consisting of 2 to 7 base pairs arranged up to 50 times. The allele length varies between different individuals, making them good markers for high resolution discrimination of individuals. Typically, for identification several profiles of microsatellite loci are compared. Despite the fact that the loci are polymorphic, it is possible that different strains own the same profile at the used loci (Legras et al., 2005). Analysis by microsatellite loci is one of the most efficient marker methods and therefore used in studying genetic diversity, genotyping, construction of genetic maps, population analysis and clarifying phylogenetic relationships at the species/subspecies/genus level of many organisms as humans, plants, animals and microorganisms (Weising et al., 1995; Webster and Reichart, 2005; Carneiro Vieira et al., 2016).

Delta analysis is also a well-established method for the differentiation of yeasts (Lavallée et al., 1994; Fernández-Espinar et al., 2001; Legras and Karst, 2003) The used primers are homologous with the delta sequence of the Ty transposon (Ness et al., 1993). The delta elements are so-called LTRs (Long Terminal Repeats) which are present on retrotransposons as flanking 'direct repeats', but are also distributed as individual LTRs in the genome (Lesage and Todeschini, 2005). The number and position of the delta elements are different for each yeast strain, allowing discrimination (Ness et al., 1993).

PCR fingerprinting with primer M13 is a method that has already long been used for the differentiation of yeasts (Lieckfeldt et al., 1993; Andrighetto et al., 2000; Urso et al., 2008). The primer corresponds to the nuclear sequence of the phage M13 (Huey and Hall, 1989), which is specific to minisatellite DNA sequences in the yeast genome (Lieckfeldt et al., 1993).

The use of yeasts to underline the regional typicity plays an increasing role in wine and apple/pear must production. In order to find these yeasts, identify them and be able to clearly define them as autochthonous, it is necessary to compare them with existing data from commercial yeasts. This study was done to establish a database of genetic profiles from commercial available yeasts for Austria. It is the first time that such a tool is available for the quick discrimination of supposedly new selected yeast strains.

MATERIALS AND METHODS

YEAST STRAINS AND CULTIVATION

All strains used in this study are listed in Table 1. There are a total of 75 strains, from 15 different suppliers of the genera *Saccharomyces cerevisiae* (var. *cerevisiae*) (SC), *S. eubayanus* or *S. cerevisiae var. eubayanus* (SB) and *S. uvarum*, *S. eubayanus* var. *uvarum* (SU) according to the manufacturer. To avoid contamination with wild type yeasts, all samples were first rehydrated and plated on Wallerstein Nutrient Agar (Thermo Scientific™ Oxoid™, Basingstoke, UK). Single colonies from Saccharomyces yeasts were chosen and cultivated on PDA-Agar (Carl Roth, Karlsruhe, Germany).

Table 1a: List of the commercial yeast strains

Name	Strain	Origin	Producer/Brand
Fermol Bayanus Lipari	SB	Italy	AEB
Fermol Complet Killer	SB	France	AEB
Fermol Elegance	SCxSB	hybrid	AEB
Fermol Lime	SC	hybrid	AEB
Fermol Red Fruit	SC	Italy	AEB
Fermol Tropical	SC	hybrid	AEB
Zymasil	SC	France	AEB
Glutaferm One	SC	Italy	AEB
Andante	SC	hybrid	Coccitech
Brio	SC	hybrid	Coccitech
Maestoso	SC	hybrid	Coccitech
Ossia Organic	SC	hybrid	Coccitech
Vivace	SB	hybrid	Coccitech
Siha Aktivhefe 2	SC	Germany	Eaton
Siha Aktivhefe 3	SC	Germany	Eaton
Siha Aktivhefe 4	SB	France	Eaton
Siha Aktivhefe 5	SB	France	Eaton
Siha Aktivhefe 7	SC	Germany	Eaton
Siha Aktivhefe 8	SC	Germany	Eaton
SIHA Cryarome	SC	France	Eaton
Sihaferm Element	SC	Germany	Eaton
Q-Citrus	SC	unknown	Enartis
Selection	SC	unknown	Enartis
VintageRed	SC	unknown	Enartis
Oenoferm Freddo	SB	Australia	Erbslöh
Oenoferm Bio	SB	Germany	Erbslöh
Oenoferm Klosterneuburg	SC	Austria	Erbslöh
Oenoferm PinoType	SC	Austria	Erbslöh
Oenoferm Rouge	SC	Austria	Erbslöh
Oenoferm X-treme	SCxSB	hybrid	Erbslöh
Vinoferm Aroma	unknown	unknown	Essedielle
Vinoferm Crio	unknown	hybrid	Essedielle
Filtraferm Bayanus	SB	unknown	Filtra
Zymaflore VL2	SC	France	Laffort
Zymaflore VL3	SC	France	Laffort
Actiflore RMS2	SB	unknown	Laffort
Bio 118 Champagne	SB	France	LaFood
Ribes	SB	unknown	LaFood
Passionsfruit	SB	unknown	LaFood

Table 1b: List of the commercial yeast strains

Name	Strain	Origin	Producer/Brand
Cross Evolution	SC	hybrid	Lallemand
Enoferm M1	SB	Australia	Lallemand
ICV Opale	SC	unknown	Lallemand
Lalvin EC1118	SB	France	Lallemand
Lalvin EGH2 "Pannonia"	SC	Austria	Lallemand
Lalvin ICV-K1	SC	France	Lallemand
Lalvin QA23	SB	Portugal	Lallemand
Lalvin R2	SB	France	Lallemand
Lalvin R-HST	SC	Austria	Lallemand
Uvaferm 43	SB	France	Lallemand
Uvaferm CEG	SC	France	Lallemand
Uvaferm PM	SB	France	Lallemand
Uvaferm WAM	SC	Spain	Lallemand
Excellence TXL	SCxSB	hybrid	Lamothe-abiet
L.A.L13	SC	unknown	Lamothe-abiet
CC.Cabernet Sauvignon	SC	France	Oenobrands
CC.Merlot	SC	France	Oenobrands
CC.Sauvignon	SC	France	Oenobrands
Fermiblanc Arom	SC	France	Oenobrands
Fermicru AR2	SC	France	Oenobrands
Fermicru LS2	SB	France	Oenobrands
Fermicru LVCB	SC	Chile	Oenobrands
Fermicru XL	SC	Chile	Oenobrands
Fermiflor	SC	Korsika	Oenobrands
Fermirouge	SC	France	Oenobrands
La Persane	SCxSU	hybrid	Oenofrance
Weiß+Fruchtig	SC	unknown	Preziso
Rot+Fruchtig	SC	unknown	Preziso
Oenofrance l`Elegante	SC	France	Sofralab
Oenofrance La Fruitée	SC	unknown	Sofralab
VitiLevure Quartz	SB	France	Sofralab
Combotiol	unknown	unknown	Vason
Evy Bayanus	SB	unknown	Vason
Premium Protiol	SC	unknown	Vason
SO.Delight	SC	France	Vialatte
SO.Fruity	SC	France	Vialatte

DNA ISOLATION

Genomic DNA was isolated from liquid yeast cultures applying the commercial kit MasterPure™ Yeast DNA Purification Kit (Epicentre, Madison, USA) according to the manufacturer's protocol. The DNA isolation of samples, which could not be cultivated on plates due to their expired shelf-life, was performed according to the protocol of Legras and Karst (2003).

PCR AMPLIFICATION

All PCR amplifications were carried out in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany).

ITS-PCR

Application of ITS1/4-PCR ensured that the selected yeast colonies belonged to the *Saccharomyes* sensu stricto complex (Valente et al., 1996) (data not shown). For amplification of the Internal Transcribed Spacers (ITS1 and ITS2) and 5.8S rDNA gene regions, the primers ITS1 (5'- TCC GTA GGT GAA CCT GCG G - 3') and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC - 3') (White et al., 1990) were used. PCR reactions were carried out in a final volume of 15 µl containing 1,5 µl of template DNA, 2X GoTaq® Green Master Mix (Promega, USA) and 4,5 µM of each primer. The amplification conditions were: 5 min at 95 °C, 35 cycles of (1 min at 95 °C, 1 min at 55 °C, 1 min at 72 °C) and 8 min at 72 °C.

MICROSATELLITE ANALYSIS

Microsatellite analysis was performed with primers C5, C11, SCYOR267C and SC8132X (Table 2) as single PCR. As fragment analysis was done with the sequencer (Licor, 4300 DNA analyzer), all forward primers were labelled on the 5' end with different fluorescent dyes: IRDye 700 (phosphoramidite) and IRDye 800 (phosphoramidite). The 15 µl PCR reaction consisted of 1,5 µl of template DNA, 2X GoTaq® Master Mix (Promega, Madison, USA) and 5 µM of forward and reverse primer. For primer C5, C11 and SCYOR267C amplification conditions were: 4 min at 94 °C, 35 cycles of (30 s at 94 °C, 30s at 55 °C, 1 min at 72 °C) and 7 min at 72 °C; for primer SC8132X the annealing temperature was changed to 64,4 °C.

Table 2: List of microsatellite primers (SC8132X is primer ORF3 by Field and Wills, 1998)

Primer	Sequence	Publication
C5 fw	5'- TGA CAC AAT AGC AAT GGC CTT CA - 3'	Legras et al, 2005
C5 rev	5'- GCA AGC GAC TAG AAC AAC AAT CAC A - 3'	González Techera et al, 2001
C11 fw	5'- TTC CAT CAT AAC CGT CGT GGA TT – 3'	Legras et al, 2005
C11 rev	5'- TGC CTT TTT CTT AGA TGG GCT TTC – 3'	Legras et al, 2005
SCYOR267C fw	5'- TAC TAA CGT CAA CAC TGC TGC CAA – 3'	Legras et al, 2005
SCYOR267C rev	5'- GGA TCT ACT TGC AGT ATA CGG G – 3'	Legras et al, 2005
SC8132X fw	5'- CGT CTC AAC TTG TGA TGG GTT TTG G – 3'	Field & Wills, 1998
SC8132X rev	5'- CCT CGT TAC TAT CGT CTT CAT CTT GC – 3'	Field & Wills, 1998

INTERDELTA PCR TYPING AND MINISATELLITE ANALYSIS

For a better discrimination of the yeast strains, PCRs with interdelta primer and minisatellite marker M13 were done. PCR reactions were carried out in a 10 µl reaction volume consisting of 1,5 µl of template DNA, 2X GoTaq® Master Mix (Promega, Madison, USA) and 5 µM of forward and reverse primer, in the case of M13 10 µM primer. The used primers were δ1 (5'- CAA AAT TCA CCT ATA TCT CA – 3'), δ2 (5'- GTG GAT TTT TAT TCC AAC A – 3') described by Ness et al. (1993) and δ12 (5'- TCA ACG ATG GAA TCC CAA – 3') designed by Legras and Karst (2003). PCR combinations δ1-2 and δ12-2 were described by Legras and Karst (2003). PCR conditions were 4 min at 94 °C, 30 cycles of (30 s at 94 °C, 1 min at 55 °C, 2 min at 72°C) and 10 min at 72°C.

The M13 primer with the sequence 5'- GAG GGT GGC GGT TCT – 3' was established by Huey and Hall (1989). Two different temperature regimen were used, with adaptions of those described by Suranská et al (2016). Amplification conditions for temperature program M13/37 °C were 4 min at 94 °C, 38 cycles of (40 s at 94 °C, 80 s at 37 °C, 45 s at 72 °C) and 5 min at 72 °C and for M13/50 °C: 4 min at 94 °C, 38 cycles of (1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C) and 5 min at 72 °C.

ELECTROPHORESIS AND FRAGMENT LENGTH ANALYSIS (FLA)

For the verification of the yeasts as *Saccharomyces* the ITS1/4-PCR products were loaded onto 1,5 % agarose gels (1 × TAE) and run for 1 h at 5 V/cm. The same conditions were used for the microsatellite PCRs to estimate the DNA concentration of the amplicons.

The sizes of the amplified fragments of the four microsatellite loci were measured on a LI-COR 4300 DNA Analyzer (LI-COR Biosciences, Lincoln, USA). Concentrated Size Standard IRDye700 and IRDye800 50-350bp (Li-COR Biosciences, Lincoln, USA) were used as internal size standard. Data analysis was managed with the software SAGA GT/MX Server Edition.

The separation of the amplification products from delta and M13 analysis was done on 1,8 % agarose gels (0,5 × TBE) for 3 to 4 h at 3 Vcm. The documentation of the resulting PCR band pattern was done with the gel image system E-Box (Peqlab, UK) and further analysis with the software GelAnalyzer 2010 developed by Istvan Lazar (www.gelanalyzer.com). The statistical analysis of the data was done with the software PAST 3 (Hammer et al., 2001). For all dendrograms presented, the distances between the yeast strains were calculated by Euclidean distance, and the phylogenetic tree was constructed using the Unified Pair Method with Arithmetic Mean (UPGMA), a hierarchical clustering method.

RESULTS AND DISCUSSION

For the creation of genetic fingerprints of the selected commercial yeasts, four microsatellite markers were used, which have already been shown to be effective in a previous study (Silhavy et al., 2006). The marker C5 and C11 are located in the inter-genic regions of chromosome IV respectively X, SCYOR267C on ORF 2 (YOR267C) and SC8132X on ORF 3 (YPL009c) (Field and Wills, 1998; Legras et al., 2005). The microsatellite analysis data are shown as dendrogram in Figure 1.

It was found that with the use of the four SSR markers not all strains could be differentiated. This is not surprising for the yeasts Oenoferm Klosterneuburg and Oenoferm Rouge, since the second is a clone selection of the first (cf. Erbslöh yeast data sheet). The different characteristics which occur during fermentation could be detected by gene expression analysis or Hxt-analysis (Köllner et al., 2010). Microsatellite analysis is a very robust method that is not affected by physiological parameters. The used markers in this study are highly polymorph. Generally SSRs show a higher mutation rate than other neutral regions of the DNA. This circumstance, which results in high levels of allelic diversity makes them so useful for genetic studies (Sweet et al., 2012).

The large cluster of non-differentiable yeasts in the upper part of the dendrogram marked with a rectangle consists on the one hand of yeasts suspected to be related to the yeast strain EC 1118 (Prise de mousse, selected by the Institute Oenologique de Champagne) like Uvaferm PM or Bio 118 Champagne, on the other hand yeasts that also are either derivatives of this yeast or just coincidentally have the same allele profile at the four investigated loci. Martinez et al. (2007) showed that LVCB and EC1118 had the same genetic profile with two different methods, RAPD and PFGE (pulsed field gel electrophoresis). Salinas et al. (2010) suggest that the yeast LVCB, which is an *S. cerevisiae* strain

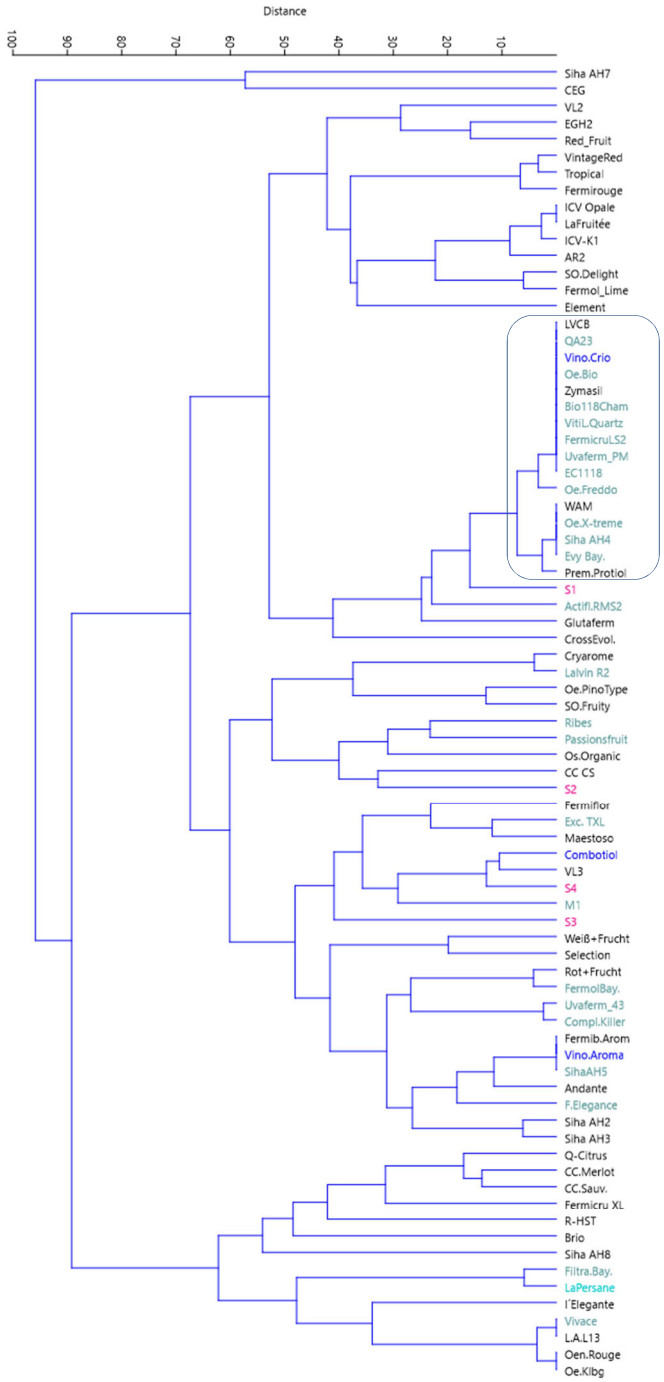

Fig. 1: Hierarchical clustering of the microsatellite analysis (black: *S. cerevisiae*, green-gray: *S. eubayanus*, turquoise: *S. uvarum*, blue: unknown, pink: autochthonous yeast strains)

from Chile, is derived from EC 1118. The yeast strain QA23 could be a derivate. In the study by Bradbury et al. (2006) only at two loci differences to EC 1118 revealed. This strain is marketed by different manufacturers both as *S. cerevisiae* and as *S. bayanus*, whereas the Institute Français de la Vigne et du Vin describes it on their homepage (www.vignevin.com) as *Saccharomyces cerevisiae* var. *bayanus*. In the studies of Legras et al. (2007) and Borneman et al. (2016) it was also clustered with EC 1118. This picture changes with interdelta and M13 analysis (Fig. 2 and 4).

It is a known problem that yeasts are labelled as *S. bayanus* and they genetically resemble *S. cerevisiae* (Fernández-Espinar et al., 2001). Dunn et al. (2005) concluded that this differentiation should be abolished, if there is not an overwhelming evidence of phenotypic or genetic differences. They showed in their study that the genome of strain EC 1118 has a high similarity to the strain SC288C, which is a standard laboratory *S. cerevisiae* strain. Borneman et al. (2016) found in their research that there is very limited genetically variation between the commercial wine yeast strains and furthermore showed that nearly all of the tested yeast strains were *S. cerevisiae* and not interspecific hybrids or other strains from the *Saccharomyces* sensu stricto complex. Coi et al. (2017) have come to conclusion that the yeast EC1118 is a cross between a flor yeast and *S. cerevisiae*.

In wine fermentation yeasts which belong to the species of *S. bayanus* (*S. uvarum* × *S. eubayanus*)/*S. uvarum* are usually related to the ability to ferment at lower temperatures and greater production of aroma-active higher alcohols than *S. cerevisiae* strains (Castellari et al., 1994; Massoutier et al., 1998; Stribny et al., 2015). Nevertheless in the descriptions of the producers it is possible to find some yeasts which are declared *S. cerevisiae* strains, but are announced with the ability of low temperature fermentation. In the microsatellite analysis two of these yeasts could be found in the second part of the previously mentioned cluster, namely Uvaferm WAM (Lallemand) and Premium Protiol (Vason). These strains could be *S. uvarum* or alternatively hybrids of *S. cerevisiae* × *S. uvarum*, or *S. cerevisiae* × *S. kudriavzevii* (Borneman et al., 2012). Regardless to other facts in this article the species names of the yeasts were left as stated by the manufacturers. Four putative autochthonous yeast strains were as well included, so that the usefulness of the new database could be verified. The strains S2 and S3 were sampled at the same manufacturer.

When looking at the dendrogram of delta analysis (Fig. 2), there are also individual yeasts that have very similar profiles. Nevertheless, this method attains a distinct discrimination, which was also shown by Stadlwieser et al. (2006). Compared to the results of the microsatellite analysis those yeasts, which showed similar band patterns are now more separated. The data shown are the assembly of the two delta profiles achieved with primer combination δ1/δ2 and δ12/δ2. It is pointed out that the disadvantage of the method is that, like the subsequent M13-PCR, it does not always give reproducible results (Fernández-Espinar et al., 2001). However, the use of a higher annealing temperature leads to the formation of more stable products (Ciani et al., 2004). Ayoub et al. (2006) also showed in their study that the discrimination with interdelta analysis is possible, when the same genetic pattern occurs with microsatellite analysis and vice versa. Schuller et al. (2004) showed with interdelta analysis using primer combination δ12/δ2 from Legras (2003) a better discrimination than the primer combination δ1/δ2 by Ness et al. (1993). This result could not be confirmed in the present study, the highest resolution could be achieved with the combination of both analyses, that was also the case in the study of Pfliegler and Sipiczki (2016). The reason could be the use of a higher annealing temperature, which resulted in the generation of fewer bands (Ciani et al., 2004) and furthermore in a lower resolution. Pfliegler and Sipiczki (2016) found that the primer pairs δ1/δ2 and δ12/δ2 have to amplify different genomic interdelta regions, as the results of the two primer combinations were not comparable. This is in agreement with our study, as shown in Figure 3.

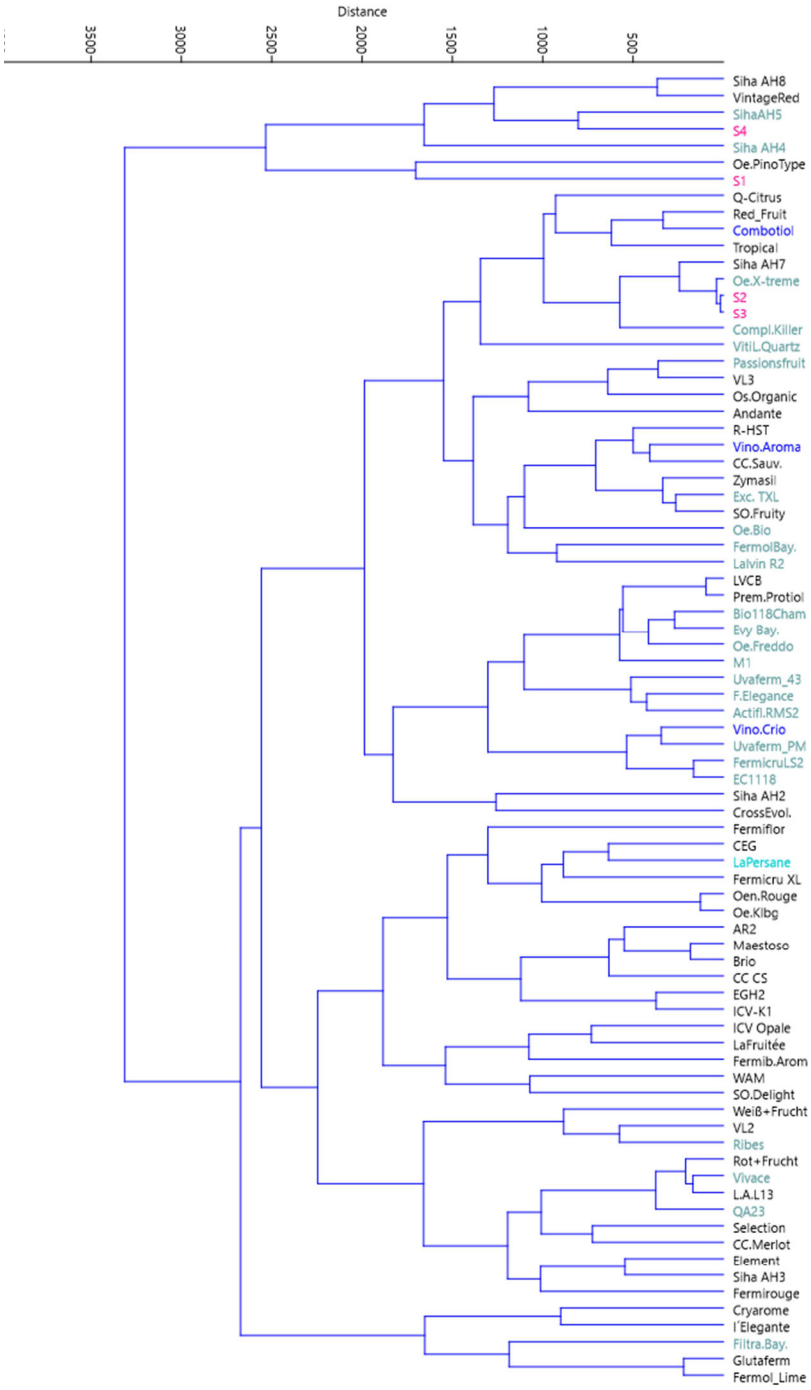

Fig. 2: Hierarchical clustering of the combined delta analysis (black: S. cerevisiae, green-gray: S. eubayanus, turquoise: S. uvarum, blue: unknown, pink: autochthonous yeast strains)

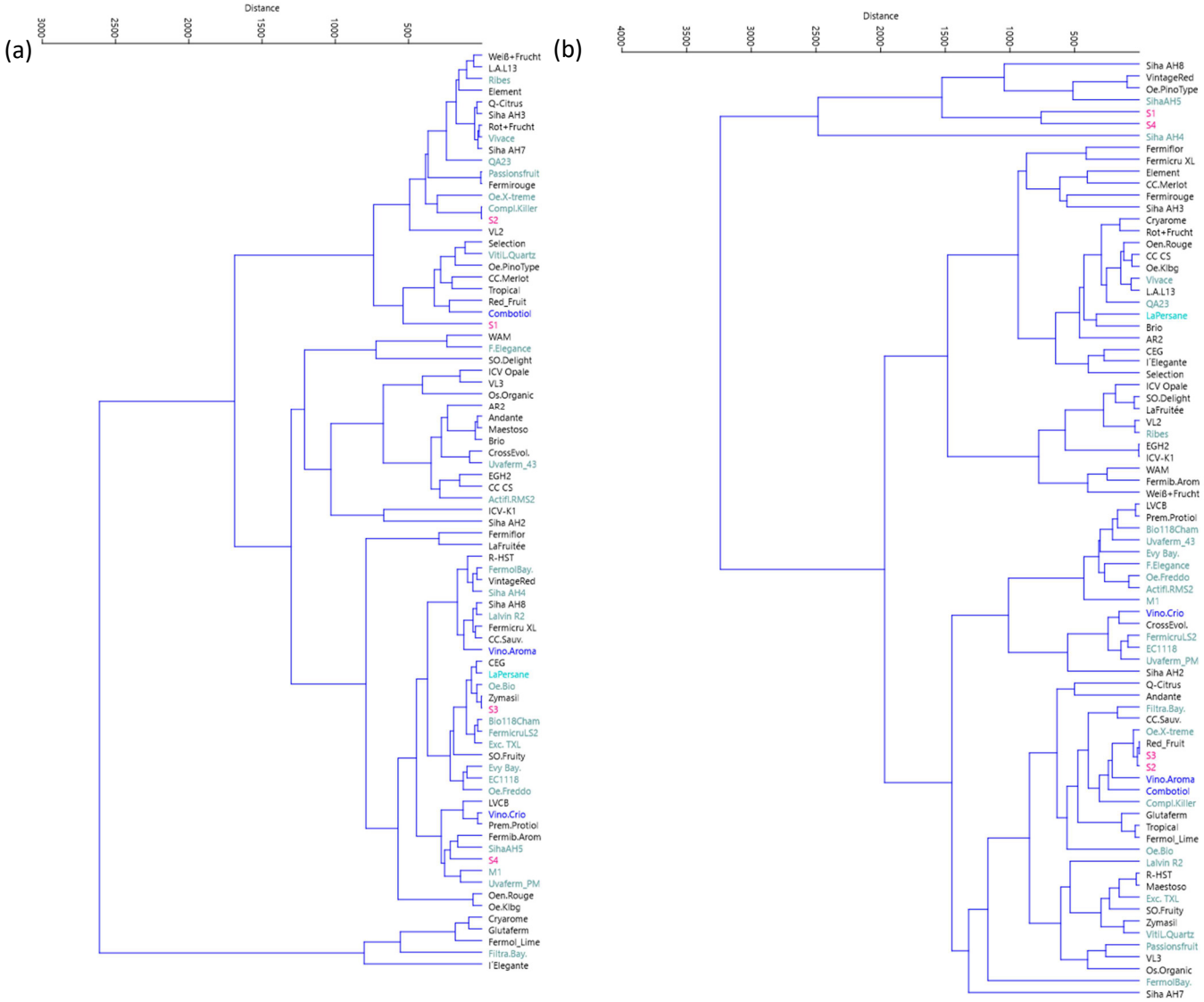

Fig. 3: Comparison of hierarchical clustering of the (a) δ1/δ2 and (b) δ12/δ2 analyses (black: S. *cerevisiae*, green-gray: S. *eubayanus,* turquoise: S. *uvarum*, blue: unknown, pink: autochthonous yeast strains)

PCR fingerprinting with the M13 primer was also able to differentiate all yeasts well (Fig. 4). The downside of this method is that M13 produces many bands (Fig. 5) and these are not always reproducible. Like delta analysis, this method relies on clean DNA and DNA concentration to provide reliable results (Fernández-Espinar et al., 2001; Vaudano and Garcia-Moruno, 2008; Pfliegler et al., 2014). Higher DNA quality is associated with higher costs for purification. Microsatellite PCR, on the other hand, is a very robust method that gives distinct results even without or only with a very preliminary purification of the DNA (Howell et al., 2004). The only disadvantage is that if an exact determination of the band length is necessary, then here too the costs become higher due to the use of a capillary sequencer. However, for rapid assessment, the use of agarose gels and reference yeast strains is sufficient in most cases. This is especially important if the question has to be clarified, whether it is an autochthonous yeast or not, that could be used as terroir yeast from a winemaker or must producer. Simple SSR analysis is as well a very useful tool for the analysis of yeast fermentations to see which yeast strain completes it (Pavelescu et al., 2005).

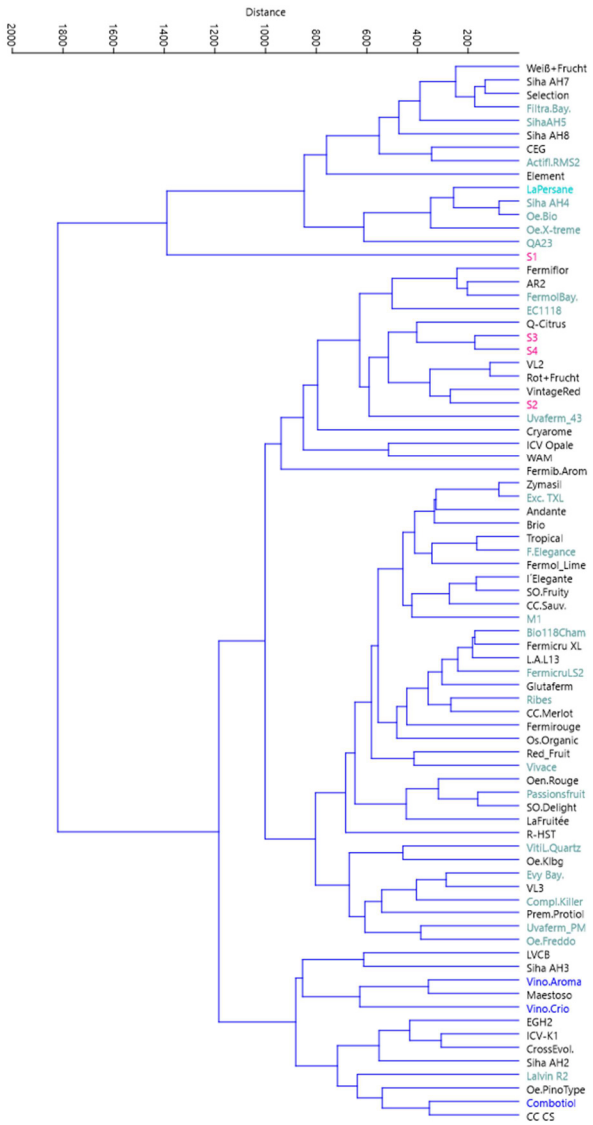

Fig. 4: Hierarchical clustering of the M13 analysis (black: *S. cerevisiae, green-gray: S. eubayanus, turquoise: S. uvarum,* blue: unknown, pink: autochthonous yeast strains)

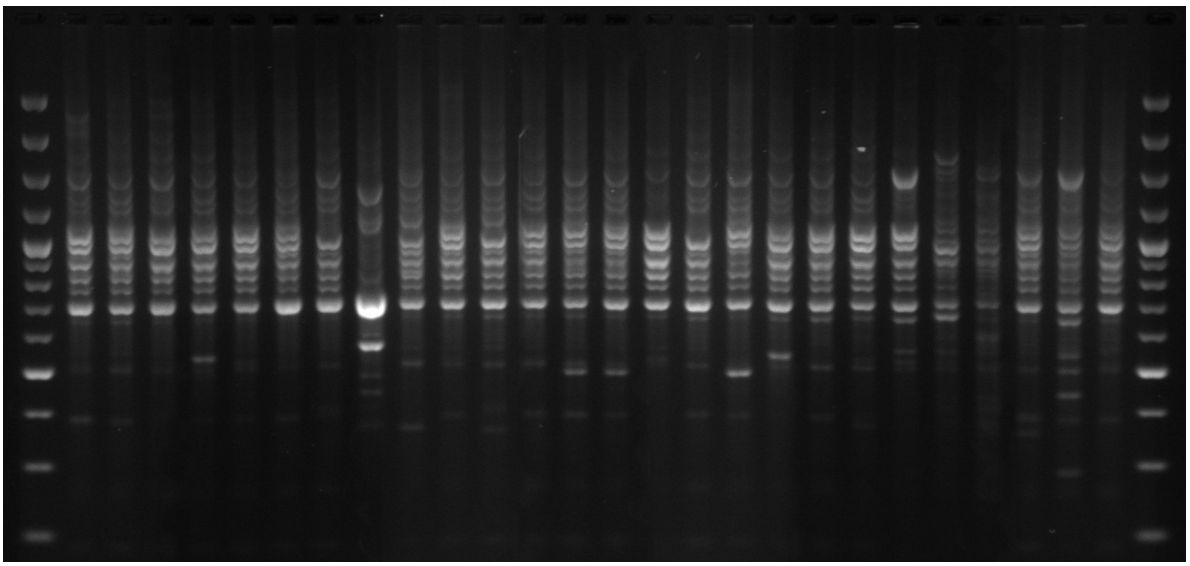

Fig. 5: PCR with Primer M13 (50 °C), first and last lane: molecular mass marker GeneRuler 100 bp plus (Thermofisher, Waltham, USA)

Furthermore, combinations of the three methods were tested. The first combination shown in Fig. 6 (a) is as reported by Suranská et al. (2016) which was used to distinguish autochthonous yeasts in southern Moravia. Also with the commercial yeasts of this study, a distinct differentation could be achieved. The combination of microsatellite and delta analysis also achieved an obvious discrimination of the yeast strains (Fig. 6 (b)), which was expected, since both methods alone ensure clear distinction.

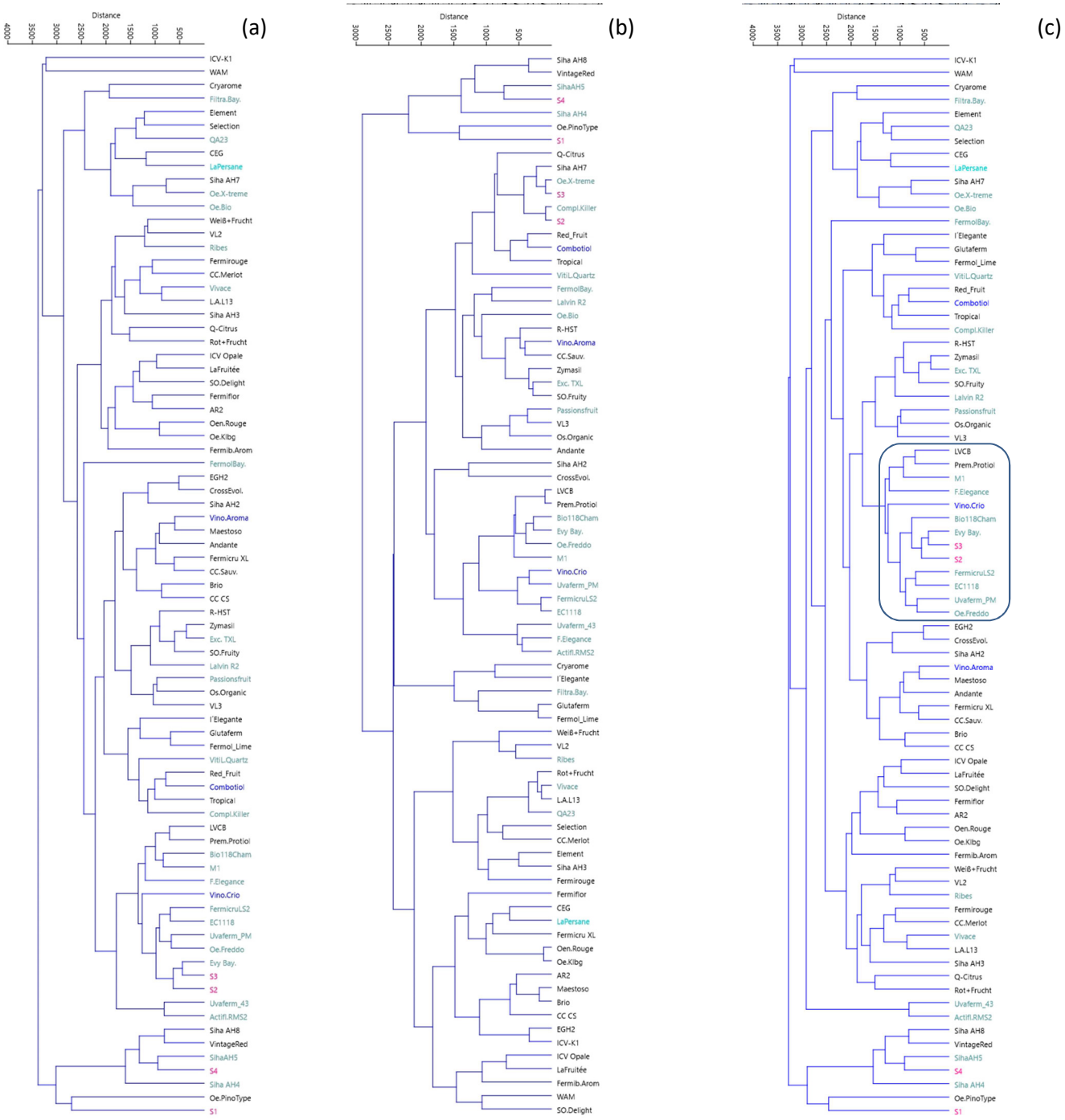

Fig. 6: Hierarchical clustering of the combined analysis (a) delta/M13, (b) Microsatellite/delta and (c) all three methods combined (black: *S. cerevisiae*, green-gray: *S. eubayanus*, turquoise: *S. uvarum*, blue: unknown, pink: autochthonous yeast strains)

In Figure 6 (c) the assembled data of all three methods is shown. This combination resulted in a high distinctness of all yeast strains used. As mentioned before a clear differentiation between *S. cerevisiae* and *S. bayanus/uvarum* is not possible what is notable, is the clustering of the yeasts including EC1118 (marked with a rectangle), which more or less is strongly influenced by the delta analysis. All of these yeasts (except the two autochthonous yeasts) are either declared *S. bayanus* yeast strains or in the case of Premium Protiol and Vino.Crio ideal for fermentation at low temperature, which would be typical for *S. bayanus* yeasts. An explanation could be that the yeast strain Premium Protiol is an unidentified interspecific hybrid of *S. cerevisiae* and *S. bayanus*. As it is not announced by the producer of Vino.Crio to which species the yeast belongs, both explanations are possible.

Summing up: it depends on the task which method will be used for the differentiation. By default, microsatellite PCR could be preferred, as it is the most reliable of the three methods followed by delta analysis. The genetic profiles of the commercial yeasts generated in this study are stored in a database and can thus be used to verify new yeast selections from wine or must samples. Our samples of autochthonous yeast were used for testing the method and could be distinguished clearly from the commercial ones.

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