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ORIGINAL ARTICLE

Mapping of wine yeast and fungal diversity in the Small Carpathian wine-producing region (Slovakia): evaluation of phenotypic, genotypic and culture-independent approaches

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Abstract The aim of this study was to investigate the yeast and fungal microflora diversity in wine-related samples of the cultivars Veltlín zelený (Grüner Veltliner) and Frankovka modrá (Blaufränkisch) produced in the Small Carpathian region (Slovakia) using different eukaryotic identification approaches. For phenotypic identification we performed auxotrophy profiling using the API 20C AUX kit (bioMérieux, Marcy l'Étoile, France) and culture on selective lysine medium. Genotypic identification was done by real-time PCR assays specific for different wine yeasts and by sequencing of the internal transcribed spaced (ITS) region (ITS1–5.8S rRNA–ITS2 region). An effective culture-independent approach was also employed in the form of analysis of the ITS2 region using fluorescence–ITS PCR (f-ITS PCR), cloning and sequencing. The ITS sequencing results were concordant with the results of the selective culture and real-time PCR assay, but for several strains they did not agree with the results of auxotrophy profiling. In the culture-independent approach, sequences of clones included 100 % matches to *Hanseniaspora uvarum*, *Candida zemplinina*, *Metschnikowia pulcherrima*, *Aureobasidium pullulans*, *Saccharomyces cerevisiae* and *Issatchenkia terricola*, as well as two novel sequences with a 5-bp difference to *Sporobolomyces marcillae*. The data from this study, and particularly the data from the culture-independent investigation, contribute to the picture of the wine microbial community of two cultivars important in wine production in Slovakia and Central Europe.

Keywords Eukaryotic microflora · Internal transcribed spacer · f-ITS polymerase chain reaction · Auxotrophy profiling · Wine

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Introduction

The diversity of *Saccharmoyces cerevisiae* and non-*Saccharomyces* wine yeasts has been analysed both on grapes and during wine fermentation by several research groups in various wine-producing areas of the world. Studies on yeast diversity are generally based on an intention to describe the possible differences in their communities, which may contribute to differences in the quality of specific wine varieties produced in different wine-producing regions. Studies on the identification and discrimination of wine-related yeast strains have employed various methods, including karyotyping by pulsed-field gel electrophoresis (Blondin and Vezinhet 1988), restriction fragment length polymorphism analysis of mitochondrial DNA (Querol et al. 1992a) and fingerprinting based on repetitive delta sequences (Ness et al. 1993). A comparative study of yeast characterization methods has been published (Querol et al. 1992b). The traditional approach to wine yeast characterization is auxotrophy profiling, which is based on studying the ability of cultures to grow on different saccharides as a sole carbon source (Degré et al. 1989). Useful information in terms of distinguishing *S. cerevisiae* from non-*Saccharomyces* yeasts can be obtained

by determining the ability of individual strains to utilize lysine as a sole nitrogen source (Heard and Fleet 1986; Tofalo et al. 2011).

Molecular methodologies are a well-established approach in the field of wine yeast ecology. Modern genotypic identification methods include delta sequence typing, analysis of chromosomal polymorphism, mitochondrial DNA restriction profiling, microsatellite amplification and 5.8S internal transcribed spacer (ITS) rDNA sequence analysis (Nisiotou and Gibson 2005; Chovanová et al. 2011; Fernández-González and Briones 2013). A real-time PCR-based method for species or genus identification has been developed as a rapid approach (Zott et al. 2010).

Culture-independent methods have become widely accepted as a means to obtain a more complete view of microbial communities in food products, including yeasts and fungi in wine-related samples (Francesca et al. 2010; Cocolin et al. 2011). Another reason for a rise in the popularity of culture-independent methods is the fact that DNA sequencing has become increasingly more affordable. These methods are now applied in several areas of microbiological research with an intention to detect unculturable microbes. Culturable microbes, whose relative population proportion is not traditionally reflected on culture media, are another focus of interest. Oenology has been also influenced by culture-independent trends, and the adaptation of established methods as well as the development of new approaches have taken place in recent years (Cocolin et al. 2011; Ivey and Phister 2011).

In 2010 our laboratory performed the first winemaking-related culture-independent study in Slovakia (Brežná et al. 2010). In that study we used the fluorescence–ITS PCR (f-ITS PCR) method, among others, to analyse the fungal microflora. This method is based on amplification of the spacer region between ribosomal RNA genes, one of the amplification primers being fluorescently labelled. ITS regions vary in lengths between species; the resulting labelled amplicons can be analysed by capillary electrophoresis with fluorescence detection, allowing precise determination of the amplicon size. The f-ITS technique has been used in the past mostly in environmental studies (Torzilli et al. 2006; Fechner et al. 2010); its two main advantages is that it is less expensive and less equipment-demanding than temperature gradient gel electrophoresis or denaturing gradient gel electrophoresis (Brežná et al. 2010). In our 2010 study, in addition to using f-ITS PCR to profile community diversity, we also cloned and sequenced the ITS amplicons (Brežná et al. 2010).

The aim of this study reported here was to describe yeast and fungal diversity in wine-related samples from the Small Carpathian wine-producing region represented by Veltlín zelený (Grüner Veltliner) and Frankovka Modrá (Blaufränkisch). An additional aim was to evaluate the effectiveness of selected phenotypic and genotypic methods for the identification of yeast strains—in particular the applicability of f-ITS PCR.

Materials and Methods

Yeast isolates

Yeast strains were isolated from grapes of cvs. Frankovka modrá and Veltlínske zelené from the vintage of 2010 in Modra, Small Carpathian wine-producing region. For the intended analysis of grape surface microflora, we suspended about 20 grape berries in physiological saline solution (0.9 % NaCl) and incubated the suspension at room temperature overnight with vigorous shaking; we then analysed, the resulting grape-rinse solution (G). Samples of musts were prepared as described by Kraková et al. (2012) and included the initial phase must (M1), 7- to 10-day fermented must ("middle fermenting must", M2) and must in the end-fermentation phase (almost wine, M3). Decimal dilutions of the samples were inoculated on YPD agar (g/l: yeast extract, 10; peptone, 20; dextrose, 20; agar, 15; Merck, Darmstadt, Germany) supplemented with 100 μg/ml of the antibiotic chloramphenicol (Sigma, Steinheim, Germany), which inhibits the growth of bacteria. The plates were incubated at 28 °C for 3–4 days, following which 30 colonies were randomly picked from each plated sample and transferred to YPD agar. Additional isolates originating in wine-related samples from Strekov in the South-Slovakian wine-producing region (Kraková et al. 2012) were used for reference purposes. Strains were stored for long term at −85 °C on ceramic beads using the Protect system (Kairosafe, Duino Aurisina, Italy). For short-term storage, the strains were stored on YPD agar plates at 4 °C for a maximum of 4 weeks.

Auxotrophy profiling

Yeast strains were identified using the API 20C AUX microorganism identification test kit (bioMérieux, Marcy l'Étoile, France). A colony of individual yeast strains from YPD agar was suspended in API AUX medium, which was a component of the kit. The test was then run in accordance with the manufacturer's instructions. The results were processed using Apiweb software (bioMérieux).

Testing the ability to utilize lysine as a sole nitrogen source

A colony of each yeast strain, grown on YPD agar at 30 °C for 2 days, was suspended in distilled water and washed three times by centrifugation. A volume of 100 μl of the suspension was streaked on a plate of Lysine medium CM0191 (Oxoid, Basingstoke, UK) and incubated at 30 °C for 48 h. The non-*Saccharomyces* strains should well grow on this medium, and the *Saccharomyces* strains present a slight background film.

Real-time PCR

Each pure culture was grown in YPD medium overnight at 30 °C with shaking (2 Hz). DNA was extracted from yeast cells sedimented by centrifugation at 10,000*g* for 5 min using InstaGene matrix (Bio-Rad, Hercules, CA) by the procedure recommended by the manufacturer. A volume of 2 μl from the obtained DNA solution (containing 100–200 ng DNA) was added to the PCR mixture composed of 12.5 μl FastStart SybrGreen Master Mix (Roche, Mannheim, Germany), 5 pmol of the forward primer, 5 pmol of the reverse primer (Table 1; synthesized by MWG, Ebersberg, Germany) and 7 μl water. Real-time PCR was performed in an ABI 7900HT cycler (Applied Biosystems, Foster City, CA) using a programme consisting of an initial denaturation for 3 min at 95 °C, and 50 cycles of denaturation for 15 s at 95 °C and annealing with polymerization for 60 s at 60 °C. Samples for which a rising amplification curve was obtained were taken as positive. Real-time PCR with a universal eukaryotic system was performed with all DNA samples, and positive amplification by this system was a prerequisite for the sample to be included in subsequent tests with yeast-specific systems.

ITS sequencing

DNA was isolated from pure cultures in YPD medium using the extraction method described above for real-time PCR analysis, and PCR was carried out using primers ITS1 and ITS4 (Table 1). The PCR mixture contained 20 pmol of each primer, 200 μmol/l dNTP, 1 U HotStarTaq plus DNA polymerase (Qiagen, Hilden, Germany), 1× PCR buffer and 6 μl of template DNA solution in a total reaction volume of 25 μl. The PCR assay was performed in a Veriti cycler (Applied Biosystems) using a programme consisting of an initial denaturation at 95 °C for 5 min and 40 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and polymerization at 72 °C for 60 s. The PCR products were purified using ExoSAP-IT (Affymetrix, Cleveland, OH) and sequenced for both strands by Macrogen (Seoul, South Korea). The yeast sequences were compared directly with those in GenBank (National Center for Biotechnology Information, Bethesda, MD) using the FASTA computer programme (European Bioinformatics Institute, Hinxton, UK).

Culture-independent analysis of wine-related samples

DNA isolation from grape rinses and must samples was performed using glass bead-mediated cell disruption and a modified DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) protocol for Gram-positive bacteria as described by Brežná et al. (2010). The obtained DNA was amplified using the ITS3 primer and a 1:50 mixture of FAM-labeled versus FAM-unlabeled ITS4 primer. The primer sequences (White et al. 1990) are listed in Table 1. The PCR products—usually mixtures of multiple amplicons—were analysed by capillary gel electrophoresis with fluorescence detection. The same PCR products were also separated by standard electrophoresis in 2.5 % agarose gel, and the major bands were excised from the gel, eluted, purified using QIAquick Gel Extraction Kit (Qiagen) and cloned. Since the blue–white colony screening

Table 1 PCR primers used in the various culture-dependent and culture-independent approaches

Method	Designation	Sequence	Target	Reference
Real-time PCR	Hauf 2 L Hauf 2R	CCCTTTGCCTAAGGTACG CGCTGTTCTCGCTGTGATG	*Hanseniaspora* sp.	Zott et al. 2010
	MPL3 MPR3	CTCTCAAACCTCCGGTTTG GATATGCTTAAGTTCAGCGGG	*Metschnikowia pulcherrima*	Zott et al. 2010
	SC1 SC2	GAAAACTCCACAGTGTGTTG GCTTAAGTGCGCGGTCTTG	*Saccharomyces* sp.	Zott et al. 2010
	Tods L2 Tods R2	CAAAGTCATCCAAGCCAGC TTCTCAAACAATCATGTTTGGTAG	*Torulaspora delbrueckii*	Zott et al. 2010
	TR03 TR04	TCTGCCCTATCAACTTTCGATGGA AATTTGCGCGCCTGCTGCCTTCCTT	Universal eukaryotic	Allmann et al. 1993
Culture-independent approach and ITS sequencing	ITS1	TCCGTAGGTGAACCTGCGG	18S rRNA	White et al. 1990
	ITS3	GCATCGATGAAGAACGCAGC	5.8S rRNA	White et al. 1990
	ITS4	TCCTCCGCTTATTGATATGC	26S rRNA	White et al. 1990
	ITS4-FAM	FAM-TCCTCCGCTTATTGATATGC	26S rRNA	Brežná et al. 2010
	M13F-40 M13R	GTTTTCCCAGTCACGAC AACAGCTATGACCATG	pDrive vector	Qiagen[a]

ITS, Internal transcribed spacer

[a] Sequences of these primers were obtained from the Qiagen PCR cloning handbook (Qiagen, Hilden, Germany)

was not functional for unknown reasons, the correct clones were determined by the size of the PCR product obtained with the M13F-40 and M13R primers, which aligned to the plasmid vector near the cloning site. The diversity of the clones was assessed by amplifying the insert by ITS3 and ITS4 primers and by subsequent *Alu*I and/or *Hha*I restriction analysis. DNA from diverse clones was amplified with M13F-40 and M13R and purified for sequencing. The representative sequenced clones were also characterized by capillary gel electrophoresis with fluorescent detection of FAM-labeled ITS3–ITS4-bordered amplicons.

Setting the thresholds for electrophoregram data

In order to obtain a numerical equivalent of visual impression from electrophoregrams, the data were processed as follows. The baseline threshold provided by the PeakScanner software (Applied Biosystems) was set to a peak height of >10 points. Among the detected peaks, those with an area of >100 points were considered in a following analysis. Finally, relative peak areas were calculated for each sample—i.e. each peak area was divided by the value of the highest peak. The peaks with a relative area of ≥5 % were marked as significant.

Results

Auxotrophy profiling, growth on lysine agar, real-time PCR and ITS sequencing

In this study, we used specific methods that had been found in previous studies to be useful and effective for the identification of yeasts isolated from Slovakian wine-related samples. Auxotrophy profiling and selective culture were used as simple, classical methods. Real-time PCR was used as a relatively simple and rapid molecular method. Finally, ITS sequencing was used as a well-defined molecular method with a potential for high discrimination. The results are summarized in Table 2.

The identification of the *S. cerevisiae* isolates by the API 20C auxotrophy profiling system corresponded with the ITS sequencing results. However, for the other isolates, several discrepancies were observed between the results of auxotrophy profiling and ITS sequencing. Real-time PCR results were overall in agreement with those of ITS sequencing, but failed to identify *Metschnikowia* sp. isolates. All non-*Saccharomyces* strains grew very well on lysine medium, while the isolates identified as *S. cerevisiae* grew only slightly on lysine medium. It is possible to conclude that the results of ITS sequencing were always in agreement with those of the selective culture, but not with those of auxotrophy profiling and real-time PCR for *Metschnikowia* sp. strains (Table 2).

ITS culture-independent approaches

The ITS DNA of the fungal microflora was amplified by PCR in a culture-independent manner, then cloned and sequenced. The sequenced clones are listed in Table 3, and they provided evidence of the presence of different yeasts, such as *Hanseniaspora uvarum*, *Metschnikowia pulcherrima*, *Saccharomyces cerevisiae*, *Aureobasidium pullulans*, *Torulaspora delbrueckii*, *Candida zemplinina* and *Issatchenkia terricola.* Additionally, there were four clones with sequences differing slightly from those available in the database, and their sequences were submitted to GenBank under accession numbers JX986833–JX986836. Namely, clone D12 (accession number JX986836) from the surface of red wine grapes originating from Modra exhibited a 2-bp difference from its closest published counterpart *Hanseniaspora uvarum*, clone B-4 (accession number JX986835) from white wine must from Strekov differed from *Uncinula necator* by 1 bp and the B18 (accession number JX986833) and C8 (accession number JX986834) clones from the same vintage differed from *Sporobolomyces marcillae* by 5 bp.

The amplification profiles of the ITS DNA were used as a fast and simple diversity indication of the fungal community. The results of capillary gel electrophoresis with fluorescence detection are depicted in Figs. 1 and 2. In two of the samples, namely, Veltlin M1 Modra and Frankovka M1 Strekov, the PCR amplification failed for unknown reasons, but this happened reproducibly. The sample Veltlin M3 from Strekov was unavailable.

Discussion

In this investigation, we attempted to evaluate the effectiveness of different identification methods to rapidly identify wine-related yeast isolates. Unfortunately, the API 20C system did not produce satisfactory results. Firstly, all of the *Hanseniaspora uvarum* strains were identified as *Kloeckera* sp. because *Kloeckera apiculata* is the anamorph name of *Hanseniaspora uvarum*. Secondly, the isolates identified by ITS sequencing as *Metschnikowia* sp., *Torulaspora delbrueckii*, *Saccharomycopsis vini* and *Pichia* were misidentified by the API 20C system as *Saccharomyces cerevisiae*/*Candida famata*, *Candida mognoliae*, *Rhodotorula minuta* and *Candida krusei*/*incospicua*, respectively. Such misidentifications regarding *Torulaspora delbrueckii*/*Candida magnoliae* and *Pichia*/*Candida* have also been documented by other authors (Arias et al. 2002; Taqarort et al. 2008). As mitigating factor, no member of the genera *Metschnikowia*, *Torulaspora* and *Saccharomycopsis* are included in the API 20C database and therefore these kinds of yeasts cannot be identified using this system.

Real-time PCR performed well, but one ambiguous result was obtained with one of the three *Metschnikowia* sp. isolates

Table 2 Comparison of the different of approaches in terms of identification efficiency

No. of isolates analysed	Strain identity based on different methods						
	ITS sequencing[a]	Real-time PCR[b]				Growth on lysine medium	Auxotrophy profiling
		A	B	C	D		
3	DQ367881 *Metschnikowia* sp.: 99 %	+ 1 strain of the 3 tested was positive	-	-	-	Non-*Saccharomyces*	*Saccharomyces cerevisiae* 2 *Candida famata*
1	AB469378 *Saccharomycopsis vini* :99 %	-	-	-	-	Non-*Saccharomyces*	*Rhodotorula minuta*
2	FN376418, AY027508 *Pichia fermentans*; FM864201 *Pichia kluyveri* : 99 %	-	-	-	-	Non-*Saccharomyces*	*Candida krusei* / *incospicua*
6	FJ515178, AM160628 *Hanseniaspora uvarum*: 100 %	-	+ All 6 strains were positive	-	-	Non-*Saccharomyces*	*Kloeckera* sp.
2	AB469378 *Torulaspora delbrueckii* :99 %	-	-	-	+ All 2 strains were positive	Non-*Saccharomyces*	*Candida magnoliae*
7	AM262829, AM262828, AM262827, AY525600 *Saccharomyces cerevisiae*: 100 %	+ All 7 strains were positive	-	-	-	*Saccharomyces*	*Saccharomyces cerevisiae* 1
5	FN393995, GQ376091, FJ793809 *Saccharomyces cerevisiae*, AY428861 *Saccharomyces boulardii*: 100 %	+ All 5 strains were positive	-	-	-	*Saccharomyces*	*Saccharomyces cerevisiae* 2
5	EU145764 *Saccharomyces cerevisiae*: 100 %	+ All 5 strains were positive	-	-	-	*Saccharomyces*	*Saccharomyces cerevisiae* 2

+, Indicates positive PCR amplification; -, indicates negative PCR amplification

[a] Percentage accompanying each strain is the percentage similarity

[b] A, *Saccharomyces cerevisiae*; B, *Hanseniaspora* sp.; C, *Metschnikowia pulcherrima*; D, *Torulaspora delbrueckii*

Table 3 Comparison of the results obtained by ITS2 fragment sequencing and capillary electrophoresis separation of the fungal clones

Sample[a]	Clone name	Identity by ITS2 fragment sequencing[b]	Length of the amplicon by sequencing (bp)	Length of the amplicon by electrophoresis (bp)
VG Strekov	A12	*Hanseniaspora uvarum*: 100 %	399	394[c]
VM1 Strekov	E2	*Candida zemplinina*: 100 %	278	274[c]
VM1 Strekov	E4	*Metschnikowia pulcherrima*: 100 %	254	249[c]
VM1 Strekov	B13	*Aureobasidium pullulans*: 100 %	342	337–338[c]
VM1 Strekov	B18	*Sporobolomyces marcillae*: 99 % (350/355)	394	390
VM1 Strekov	B-4	*Uncinula necator*: 99 % (330/331)	371	365[c]
VM2 Strekov	C-6	*Saccharomyces cerevicae*: 100 %	419	413[c]
VM2 Strekov	F1	*Candida zemplinina*: 100 %	278	274[c]
VM2 Strekov	F4	*Candida zemplinina*: 100 %	278	274[c]
VM2 Strekov	C8	*Sporobolomyces marcillae*: 99 % (350/355)	394	390
VG Modra	D-2	*Candida zemplinina*: 100 %	278	274[c]
VG Modra	D-3	*Issatchenkia terricola*: 100 %	281	276[c]
VG Modra	G29	*Candida zemplinina*: 100 %	278	274[c]
FG Strekov	A3	*Hanseniaspora uvarum*: 100 %	399	393
FG Strekov	B2	*Metschnikowia pulcherrima*: 100 %	254	247
FG Strekov	B6	*Metschnikowia pulcherrima*: 100 %	254	247
FM2 Strekov	C1	*Saccharomyces cerevisiae*: 100 %	420	413
FM2 Strekov	C15	*Saccharomyces cerevisiae*: 100 %	420	413
FG Modra	D12	*Hanseniaspora uvarum*: 99 % (357/359)	399	393
FG Modra	D13	*Hanseniaspora uvarum*: 100 %	399	393
FG Modra	E12	*Aureobasidium pullulans*: 100 %	342	336
FM2 Modra	G3	*Torulaspora delbrueckii*: 100 %	445	438
FM2 Modra	G5	*Saccharomyces cerevisiae*: 100 %	420	413

[a] FG, cv. Frankovka modrá ripe grape rinse; FM1, Frankovka initial must; FM2, Frankovka middle-fermentation phase; FM3, Frankovka end-fermentation phase after filtration; VG, cv. Veltlínske zelené grape rinse; VM1, Veltlínske zelené initial must; VM2, Veltlínske zelené middle-fermentation phase; VM3, Veltlínske zelené end-fermentation phase after filtration

[b] The nearest GenBank match (identify); percentage accompanying each strain is the percentage homology of sequences without primers. When not 100%, the number of samples showing homology relative to the total sample is given in parenthesis

[c] Data from Brežná et al. 2010.

which was positive to *Saccharomyces cerevisiae* PCR. None of our *Metschnikowia* sp. isolates were successfully amplified by the *Metschnikowia pulcherrima* PCR assay. It would therefore appear that this assay is excessively specific, as previously reported by Zott et al. (2010) who developed the assay.

Analysis of the ITS2 region appeared to be a promising DNA marker for identifying wine-related yeast strains by a culture-independent approach. The majority of clones, i.e. 19 of 23, were 100 % identical to published sequences of *Hanseniaspora uvarum*, *Metschnikowia pulcherrima*, *Saccharomyces cerevisiae*, *Aureobasidium pullulans*, *Torulaspora delbrueckii*, *Candida zemplinina* and *Issatchenkia terricola*, and so they could be identified to the species level. However, within the limited scope of the studies performed in our laboratory, *Torulaspora delbrueckii* appears to be characteristic for Modra red wine samples.

Overall, the species composition of the communities determined by the cloning and sequencing analyses resembles that reported in from other wine fermentation studies (Mills et al. 2002; Di Maro et al. 2007), including the recently studied regions geographically close to Slovakia, such as the Czech Republic (Šuranská et al. 2012) or Austria (Lopandic et al. 2008).

The capillary gel electrophoresis with fluorescence detection of ITS amplicons method provided a simple indication of each sample's biodiversity. The length of the ITS amplicon is characteristic for each fungal species. To keep the annotation of peaks simple and compendious, we assigned species names based only on our own cloning data from Brežná et al. (2010) and Table 3 of the present study. We considered the cloned PCR amplicons to be the most probable indicator of any other PCR amplicons eventually to be obtained under the same conditions. The peak of *Pichia membranifaciens* overlaps with unidentified *Pichia* sp. similar to *Pichia fermentans* or *Pichia kluyveri* which had been cultivated from Strekov samples (Brežná et al. 2010; Chovanová et al. 2011; Kraková et al.

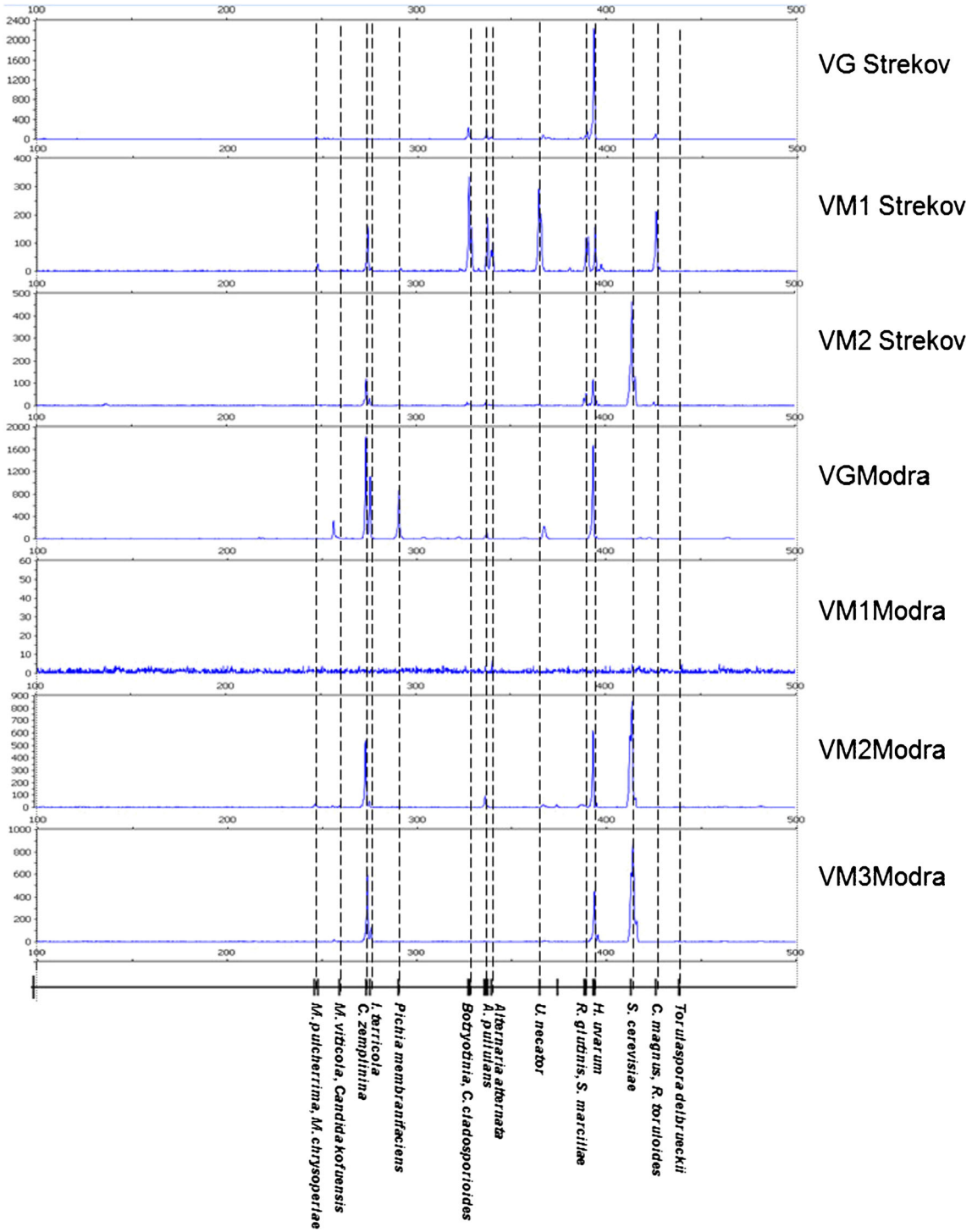

Fig. 1 Eukaryotic microbial PCR profiles of cv. Veltlínske zelený (Grüner Veltliner) samples based on internal transcribed spacer (ITS) polymorphism and analysed by fluorescent capillary gel electrophoresis. Following the *dotted lines* it is possible to distinguish the presence of a specific peak for a yeast species in the analysed samples. Positions on the *x-axis* indicate the lengths in basepairs. The *y-axis* represents the intensity of the fluorescent signal (relative fluorescence units). See footnote to Table 3 for designations of samples

2012). *Saccharomycopsis vini* isolated from Modra white wine (Kraková et al. 2012) could contribute to the peak of *Hanseniaspora uvarum*.

Several patterns can be found in the amplification profiles of ITS2 region (Figs. 1, Fig. 2). As has been observed in earlier studies, *Saccharomyces cerevisiae* is not present in significant amounts on healthy grapes (Fleet et al. 2002; Mannazzu et al. 2002) and, consequently, was not detected in our grape samples. *S. cerevisiae* propagates during the fermentation. In our case, it became detectable either in musts

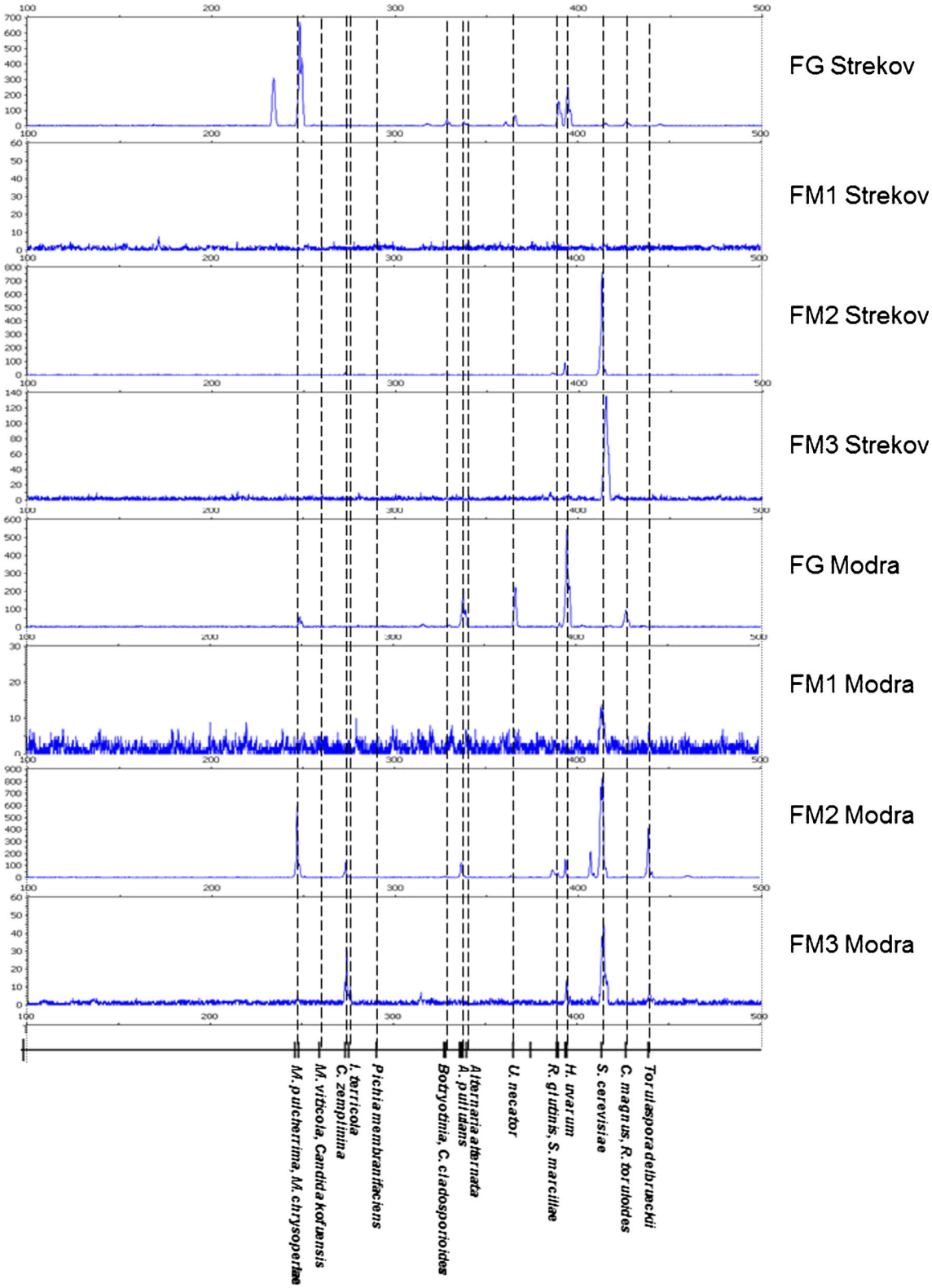

Fig. 2 Eukaryotic microbial PCR profiles of cv. Frankovka modrá (Blaufränkisch) samples based on ITS polymorphism and analysed by fluorescent capillary gel electrophoresis. Following the *dotted lines* it is possible to distinguish the presence of a specific peak for a yeast-species in the analysed samples. Positions on *x-axis* indicate the lengths in basepairs. The *y-axis* represents the intensity of the fluorescent signal (relative fluorescence units). See footnote to Table 3 for designations of samples

at the beginning of fermentation (M1; red wine from Modra) or after 7–10 days of fermentation (M2; other samples). In the must stages of M2 and M3, the peak of *S. cerevisiae* is the highest one in the f-ITS PCR profiles.

Brežná et al. (2010) found that *Hanseniaspora uvarum* had the strongest and most dominant peak in the f-ITS PCR of all samples tested, with the exception of Frankovka grapes from Strekov. We did not observed such an overwhelming dominance of *H. uvarum* in the present study, although this species was still a significant species with a high proportional representation in the fungal community composition. However, other species shared a prominent status in our study. The species represented by the highest peaks at the M2 and M3 must stages in all sample types was *S. cerevisiae*. *Candida zemplinina* also had a very strong signal, in particular in Veltliner samples (VG, VM2 and VM3) from Modra. The peak of *Metschnikowia pulcherrima/M. chrysoperlae* was prominent in two Frankovka samples (FG Strekov and FM2 Modra). The sample VM1 from Strekov had many prominent peaks: *Botryotinia fuckeliana*/*Cladosporium cladosporioides*, *Aureobasidium pullulans*, *Alternaria alternata*, *Uncinula necator*, *Candida zemplinina*, *Rhodotorula glutinis*/*Sporobolomyces marcillae*, *Hanseniaspora uvarum*, *Cryptococcus magnus*, *Rhodosporidium toruloides*.

The best candidates for novel species in this study are the fungi which differ by 5 bp from their closest counterpart *Sporobolomyces marcillae*, which had been cloned from the middle and end fermentation phases of white wine must from Strekov (accession numbers JX986833 and JX986834, respectively). These fungi were not observed in the culture-dependent study run parallel to this one (Kraková et al. 2012) and were also not detected in the Strekov wine-related samples from the previous year (Brežná et al. 2010).

Under the particular conditions of our study, *Candida zemplinina* was a more prominent species in Modra samples than in the Strekov samples based on peak height and occurence (Figs. 1 2). However, the parallel results of the culture-dependent study conducted by Kraková et al. (2012) indicated the opposite, with a total of 32 isolates obtained from Strekov samples and none from Modra samples. These observations are another case of differences obtained in cultivation versus culture-independent studies.

Since culture-independent studies of the wine microbial community in Slovakia and Central Europe are still comparatively rare, our results represent a valuable contribution towards completing the picture. We obtained novel sequences of fungi related to *Sporobolomyces marcillae*. With respect to cv. Frankovka modrá, *Torulaspora delbrueckii* has been hypothesized as a species with different occurrence rates in the Modra and Strekov sampling locations, respectively. The presence of a number of species previously associated with wine production was confirmed. This study confirms the usefulness of f-ITS PCR combined with cloning and sequencing as valid tools for microbial community analysis. In addition, the data collected by this and previous studies using f-ITS enable the construction of a useful database of f-ITS peaks that, in the near future, can be applied to the direct analysis of wine-related samples by performing only f-ITS fingerprinting without sequencing or cloning. This approach will facilitate effective analysis of many samples from different vintages in order to obtain a representative picture of yeast and fungal diversity.

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