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Molecular Identification of the Yeast Strains Associated with Spontaneous Wine Fermentation of Kalecik Karasi and Emir Grapes

Seda Karasu-Yalcin[1], Sule Senses-Ergul[2] and Z. Yesim Ozbas[3]*

[1]Abant Izzet Baysal University, Faculty of Engineering and Architecture, Food Engineering Department, Golkoy 14280, Bolu, Turkey.
[2]Public Health Institution of Turkey, Department of Consumer Safety Laboratories, Refik Saydam Campus, Sihhiye 06100, Ankara, Turkey.
[3]Hacettepe University, Faculty of Engineering, Food Engineering Department, Beytepe 06532, Ankara, Turkey.

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In this study, 49 yeast isolates, which had been isolated during spontaneous wine fermentation of Kalecik Karasi and Emir grapes, were identified by molecular methods. Nineteen of the 24 Kalecik Karasi isolates and 22 of the 25 Emir isolates could be identified to species level by Restriction Fragment Length Polymorphism (RFLP) of the ITS-18S rRNA gene. The identified Kalecik Karasi isolates were found to be strains of *Candida pulcherrima*, *Candida robusta*, *Cryptococcus albidus*, *Kloeckera apiculata*, *Kloeckera apis*, *Saccharomyces cerevisiae* and *Torulaspora delbrueckii*. The Emir isolates belonged to *Candida krusei*, *C. pulcherrima*, *K. apiculata* and *S. cerevisiae*. All of the *S. cerevisiae* strains and most of the non-*Saccharomyces* strains could be differentiated by randomly amplified polymorphic DNA (RAPD-PCR) analysis. Although satisfactory results were obtained by M13 primer, $(GTG)_3$ primer was much more effective in the discrimination of *K. apiculata*, *Cry. albidus* and *C. krusei* strains.

Key words: Yeast, Wine, Spontaneous fermentation, Molecular identification, PCR-RFLP, RAPD-PCR.

The transformation of grape juice into wine by spontaneous alcoholic fermentation is the result of the sequential development and activity of various species of yeasts originating from grapes and the winery equipment[1]. Wine quality is a consequence of the dynamics and composition of the microorganisms involved in its production. The diversity and composition of the yeast microbiota in wine must can vary with the grape variety[2]. It is reported that the fermentation of must is often initiated by indigenous yeasts, mostly non-*Saccharomyces* genera such as *Hanseniaspora/Kloeckera, Rhodotorula, Candida, Debaryomyces, Pichia, Metschnikowia, Issatchenkia* and *Kluyveromyces*[3,4]. Growth of these yeasts is influenced by various factors such as temperature, pH, SO_2, harvest technique, treatments, age of the vineyards, grape variety, grape maturity, climate and geographical location, practical winemaking process and the type of the wine produced[3,4]. At favourable temperatures and with SO_2 present, strains of *Saccharomyces cerevisiae* grow rapidly and increase the alcohol content, leading to the supression of non-*Saccharomyces* yeasts.

* To whom all correspondence should be addressed.
Tel.: +90 3122977112; Fax: +90 3122992123;
E-mail: yesim@hacettepe.edu.tr

Although wine has traditionally been manufactured by spontaneous fermentation, most modern wine-makers prefer to inoculate the musts with selected yeast strains either as mono- or mixed-cultures. This allows a better vinification control and assures a faster fermentation[5,6]. Recently, several studies have focused mainly on autochthonous strains and their potential application in winemaking has been explored. These yeasts produce various enzymes e.g. pectinase, β-glycosidases, proteases, esterases or lipase interacting with grape-derived precursors compounds thus contributing reveal the varietal aroma and improving the winemaking process[4,7]. The number of wine yeast strains available in the world market has been reported to increase as the result of studies that show the strong influence of indigenous yeasts for the wine quality of the given world region. Therefore, oenological, physiological and molecular characterization of native wine yeasts is of great importance to assure wine quality of a specific region.

As most of the wine yeast strains belong to the genus *Saccharomyces*, they are not easily differentiated and/or identified on the basis of classical methods. It is known that the traditional methods for identifying yeasts rely on morphological, physiological and biochemical criteria. These techniques are generally laborious and time consuming, thus they are not appropriate for routine identification[5]. Moreover, they sometimes provide doubtful identification, because of the influence of culture conditions on yeast physiological characteristics[1]. The application of molecular techniques to wine yeast identification has proved to be very powerful for resolving recent and classical issues concerning taxonomy[8,9]. Several genetic methods for yeast strain identification, such as analysis of mithocondrial DNA, DNA fingerprinting by RAPD-PCR and pulsed field gel electrophoresis have been reported to demonstrate a great genetic diversity in oenological strains of *S. cerevisiae*, which is affected by geographical location and by the technology used in vinification[10].

The aim of this study was to determine yeast microbiota during spontaneous wine fermentation of Emir and Kalecik Karasi grape varieties, grown in Turkey. The study also focused on molecular confirmation and discrimination of the yeast strains which were pre-identified by cultural methods.

MATERIALS AND METHODS

Yeast strains

In this research, 49 yeast strains belonging to 11 different species, which had been isolated from spontaneous wine fermentations of Kalecik Karasi and Emir grapes, were used. Twenty four of the isolates were obtained during Kalecik Karasi wine production, while 25 of them were originating from Emir wine production. Those yeast strains had been isolated from grapes and different stages of the natural fermentation process. Since originating from native sources, they were called as "endogenic yeast strains" in this paper. The origin of the endogenic yeast strains were represented in Table 1. The yeast strains used in this study were obtained from our previous study (unpublished data). Identification of the strains according to their morphological and physiological characteristics were also accomplished previously (unpublished data). A total of 15 yeast strains belonging to 9 different species were also used as control (type or reference) strains in the experiments (Table 2).

The yeast strains were stocked at -70°C in Yeast Extract Peptone Dextrose broth (YEPD) containing 20% (v/v) glycerol. The cultures were activated in Yeast Extract Malt Extract (YM) broth when needed. The strains were kept at 4°C on Yeast Extract Malt Extract (YM) agar until use and consecutive transfers were maintained in the same medium.

DNA isolation

The yeast isolates were cultured on Tryptone Glucose Yeast extract (TGY) agar for 24 h at 30°C. The cells were suspended and washed in 1 mL of sterile distilled water and then subjected to DNA extraction according to the method described by Vasdinyei & Deak[10].

The spectrophotometric method was used for determination of the quantity and purity of the isolated DNA[11,12]. The purity of the isolated DNA was checked and it was used in the experiments only if its ratio of A_{260}/A_{280} was in the range between 1.8-2.0.

PCR-RFLP assay

PCR-RFLP method was used for

identification of the yeasts at species level[13]. For amplification of 18S rDNA with the neighbouring ITS1 region, NS1/ITS2 primer pair was used in PCR assay. The sequences of primers are given below:

NS1: 5´GTAGTCATATGCTGTCTC 3´
ITS2: 5´GCTGCGTTCTTCATCGATGC 3´

30 µL reaction volume was used containing 2 µL target DNA solution in the amplification process. The reaction medium included: 0.034 U/µL DNA polymerase (DyNAzyme™ II, Finnzymes, Finland), 0.1 mM deoxynucleoside triphosphate mix (dNTP mix, Finnzymes), 1X PCR reaction buffer [Mg^{+2}-free DyNAzyme™ buffer; 10 mM Tris-HCl, (pH 8.8, 25°C), 50 mM KCl, %0.1 Triton® X-100, Finnzymes], 2.5 mM $MgCl_2$ ($MgCl_2$ solution, Finnzymes), 1 µM NS1 primer (Alpha DNA, Canada), 1 µM ITS2 primer (Alpha DNA).

For the amplification reaction, Eppendorf (Mastercycler® Gradient, USA) and BioRad (MyCycler Thermal Cycler System, USA) thermal cyclers were used. The PCR programme included: an initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 2 min and a final extension at 72°C for 7 min.

For digestion of the amplification products; *Msp*I, *Hae*III, *Alu*I, *Rsa*I and *Scr*FI restriction endonucleases (Roche, Germany) were used separately. In the method, 3 µL of PCR products were digested in 15 µL of reaction mixture consisting of 0.14 U/µL restriction enzyme, 1.5 µL buffer specific to the restriction enzyme (Roche) and 0.1 mg/mL bovine serum albumine (Roche). The mixture was incubated at 37°C for 16-18 hours.

RAPD-PCR assay

Strain discrimination was performed by RAPD-PCR analysis[10,14]. For differentiation of the isolates, two different primers; $(GTG)_3$ (5' GTGGTGGTG 3') and M13 (5' GAGGGTGGCGGTTCT 3') were used separately. For this purpose, 2 µL of target DNA was amplified in a 30 µL of reaction medium in the thermal cycle. The components of the reaction medium were: 0.034 U/µL DNA polymerase (DyNAzyme™ II, Finnzymes), 0.067 mM deoxynucleotide triphosphate mix (dNTP mix; Finnzymes), 1X PCR reaction buffer [Mg^{+2}-free DyNAzyme™ buffer; 10 mM Tris-HCl (pH 8.8, 25°C), 50 mM KCl, %0.1 Triton® X-100, Finnzymes], 2.5 mM $MgCl_2$ ($MgCl_2$ solution, Finnzymes), 0.17 µM primer (Alpha DNA).

The amplification conditions used, were as follows: an initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 1 min, annealing at 36°C for 1 min, extension at 72°C for 2 min and a final extension at 72°C for 7 min.

Gel electrophoresis

The restriction fragments of ITS-PCR and the amplicons of RAPD-PCR were separated by electrophoresis on respectively, 1.2% and 1.5% (w/v) agarose gels (Promega) in 0.5X TBE buffer with DNA molecular weight marker XIV (Roche). A horizontal electrophoresis system (Bio Rad, USA) was used with Mini-Sub Cell GT and Sub-Cell GT boxes and power supply (PowerPac™ Basic). Gels were stained with ethidium bromide.

Gel Visualization and Statistical Analysis

The obtained DNA fragments in agarose gels were visualized under UV light by using Syngene-InGenius (UK) visualization system. For evaluation of the gel images, GeneSnap, GeneTools and GeneDirectory package programs were used in the same system. The size of DNA fragments were detected by comparing with DNA molecular weight marker XIV (Roche). Restriction patterns of the endogenic yeast strains obtained by PCR-RFLP were compared to those of the control strains by using the same package programs.

The DNA patterns obtained by RAPD-PCR were evaluated by using the package programs in gel visualization system. Cluster analysis was performed for detection of the similarities/differences of the strains. Dendograms were obtained by means of the Unweighted Pair Group Method using the Arithmetic Average (UPGMA) clustering algorithm. Calculation of similarities of band profiles was based on the Dice similarity coefficient.

RESULTS

Identification of the yeast strains by PCR-RFLP

The 18S rDNA-ITS1 region of all of the 49 endogenic yeast strains associated with spontaneous wine fermentation, was successfully amplified. In the application of PCR-RFLP, five different restriction enzymes (*Msp*I, *Hae*III, *Scr*FI,

*Rsa*I and *Alu*I) were used for the digestion of the amplicons. For each enzyme, restriction patterns obtained for endogenic yeast strains were compared to those obtained for the control strains. The restriction patterns obtained for the yeast strains originating from Emir and Kalecik Karasi grapes or from different stages of spontaneous fermentation are represented in Fig. 1-5. Size (bp) of restriction fragments of the type and reference strains are also given on Table 3.

Restriction patterns of the endogenic yeast strains obtained with *Msp*I restriction enzyme were given in Fig.1. Among the isolates obtained from Kalecik Karasi grapes and their natural fermentation media, restriction profiles of *K. apis* (K/C6) and *K. apiculata* (K/C8, K/U6, K/U5) were found as similar to each other. Two DNA fragments (348 and 2042 bp) were obtained in the restriction profiles of these strains. This profile was similar to the patterns of the control strains; *K. apis* NBRC 10831, *K. apiculata* NBRC 0865 and *K. apiculata* IFO 0865 (Table 3). It was found that RFLP pattern of *C. pulcherrima* (K/M3) isolated from must was similar to that of its control strain. For *Cry. albidus* (K/U16, K/U21) originated from grape, four DNA fragments between 280-700 bp were obtained, which was similar to the restriction pattern of *Cry. albidus* NBRC 0939. The digestion patterns of the endogenic strains belonging to *Tp. delbrueckii* (K/C9, K/FS2) and its anamorph form *Candida colliculosa* (K/C4) were similar to each other and fit the pattern of *Tp. delbrueckii* reference strains NBRC 0469 and NBRC 1180. However, the restriction patterns of the strains previously identified as *Candida holmii* (K/S15) and *C. krusei* (K/M5, K/S4 and K/T1) obtained with *Msp*I did not fit those of their control strains. For the *C. holmii* K/S15, four DNA fragments between 265 and 1361 bp were obtained. Five DNA fragments between 258 and 867 bp were obtained for *C. krusei* K/M5, K/S4 and K/T1 strains, originated from must, young wine and sludge, respectively. The same digestion profile was obtained for *C. robusta* (K/FO8, K/FO11) and its teleomorph form *S. cerevisiae* (K/M9, K/C2, K/FB8, K/FO3, K/FO7, K/FB2, K/FS3, K/S13) which has four DNA fragments

Table 1. Endogenic yeast strains isolated during spontaneous fermentation of Emir and Kalecik Karasi grapes

Yeast species[1]	Name of the grape/Isolation step	Number of the strains
Candida colliculosa	K/C4	1
Candida holmii	K/S15	1
Candida krusei	K/M5, K/S4, K/T1, E/S3, E/T6, E/FB7, E/FS5, E/FO2	8
Candida pulcherrima	K/M3, E/U9, E/SO5, E/U7	4
Candida robusta	K/FO8, K/FO11	2
Cryptococcus albidus	K/U16, K/U21	2
Kloeckera apiculata	K/C8, K/U6, K/U5, E/U17, E/M4, E/U3	6
Kloeckera apis	K/C6	1
Pichia anomala	E/FB5	1
Saccharomyces cerevisiae	K/M9, K/C2, K/FB8, K/FO3, K/FO7, K/FB2, K/FS3, K/S13, E/S10, E/S7, E/S8, E/T9, E/T11, E/FS8, E/FO10, E/FO11, E/FO8, E/SO7, E/FB6, E/FB13, E/FB11	21
Torulaspora delbrueckii	K/C9, K/FS2	2

[1]These names are those identified based on morphological and physiological characteristics in our previous study (unpublished data)

Coding; K: Kalecik Karasi, E: Emir, U: grape, M: must, C: Kalecik Karasi must sample taken after SO_2 treatment, SO: Emir must sample taken after SO_2 treatment, FB: beginning of the fermentation, FO: middle of the fermentation, FS: end of the fermentation, S: young wine, T: sludge

between 258-1374 bp. This profile was similar to the digestion profiles of the *S. cerevisiae* reference strains used (Table 3). Digestion patterns of the endogenic strains isolated during wine production from Emir grapes are also represented in Fig. 1. For the strains *K. apiculata* (E/U17, E/M4, E/U3) and *C. pulcherrima* (E/U9, E/SO5, E/U7), two DNA fragments of 357 and 2001 bp, and three DNA fragments between 146-977 bp were obtained respectively, which fit the digestion patterns of the control strains. *Pichia anomala* (E/FB5) gave three DNA fragments between 271 and 1065 bp, similar to the digestion pattern of *P. anomala* IFO 0140. For *C. krusei* (E/S3, E/T6, E/FB7, E/FS5, E/FO2), four DNA fragments between 254 and 838 were obtained. This profile was similar to that of the control strain. Similar digestion patterns were obtained for all of the *S. cerevisiae* isolates obtained from spontaneous fermentation of Emir grapes having four DNA fragments between 258 and 1332 bp, which were also similar to restriction patterns of the reference *S. cerevisiae* strains used.

Restriction patterns of the endogenic yeast strains obtained with *Hae*III restriction enzyme were given in Fig. 2. Among Kalecik Karasi endogenic strains, *K. apis* (K/C6) and *K. apiculata* (K/FB1, K/C8, K/U6) gave similar patterns having three DNA fragments between 309 and 745 bp which fit the digestion profiles of their control strains. Restriction patterns of *C. pulcherrima* (K/M3) and *Cry. albidus* (K/U16, K/U21) originated from grape, were similar to those of the type and reference strains of the related species. *Tp. delbrueckii* (K/C9, K/FS2) isolated from must and at the end of the fermentation, and *C. colliculosa* (K/C4) isolated from must also gave similar restriction profiles with the reference strains *Tp. delbrueckii* NBRC 0469 and NBRC 1180. For *C. holmii* (K/S15) which was a young wine isolate, three DNA fragments between 196 and 637 bp were obtained with *Hae*III enzyme. This profile was different from the digestion pattern of *C. holmii* IFO 1629. For the endogenic strains previously identified as *C. krusei* (K/M5, KS4, KT1), a distinct restriction profile was obtained when compared to the pattern of *C. krusei* IFO 0841 by the use of *Hae*III. It was found that all of the Kalecik Karasi isolates belonging to *C. robusta* and *S. cerevisiae* had the same digestion pattern with this restriction enzyme. DNA patterns of these strains were containing five fragments between 147 and 645 bp, which were also similar to the those of used four reference *S. cerevisiae* strains (Fig. 2 and Table 3).

Restriction patterns of Emir isolates obtained with *Hae*III enzyme were also represented in Figure 2. Among these isolates, *K. apiculata* strains (E/U17, E/M4, E/U3) had similar digestion patterns with that of the reference strain, consisting of three DNA fragments between 302 and 737 bp. In the same gel tested, *C. pulcherrima* strains (E/U9, E/SO5, E/U7) exhibited similar profile (four DNA fragments between 136 and 543 bp) with the type *M. pulcherrima* strain. Restriction pattern of endogenic *P. anomala* (E/FB5) also fit those of the related type strains (Table 3). Digestion profiles of *C. krusei* strains (E/S3, E/T6, E/FS5, E/FO2) had five DNA fragments between 140 and 504 bp, which were the same with that of *C. krusei* IFO 0841. The same restriction profiles were obtained for all endogenic and reference *S. cerevisiae* strains, consisting of five DNA fragments between 131 and 600 bp.

18S rDNA-ITS1 restriction patterns of the

Table 2. Type and reference strains used in this study

Yeast strain
Candida holmii IFO 1629[a]
Candida krusei IFO 0841[b]
Metschnikowia pulcherrima NCAIM Y 01466[a]
Cryptococcus albidus NBRC 0939[b]
Kloeckera apis NBRC 10831[a]
Kloeckera apiculata NBRC 0865[b]
K. apiculata IFO 0865[b]
Pichia anomala IFO 0140[b]
P. anomala NCAIM Y 01109[a]
Saccharomyces cerevisiae IFO 2359[b]
S. cerevisae NCYC 232[b]
S. cerevisiae NBRC 0221[b]
S. cerevisiae STV 85[b*]
Torulaspora delbrueckii NBRC 0469[b]
Tp. delbrueckii NBRC 1180[b]

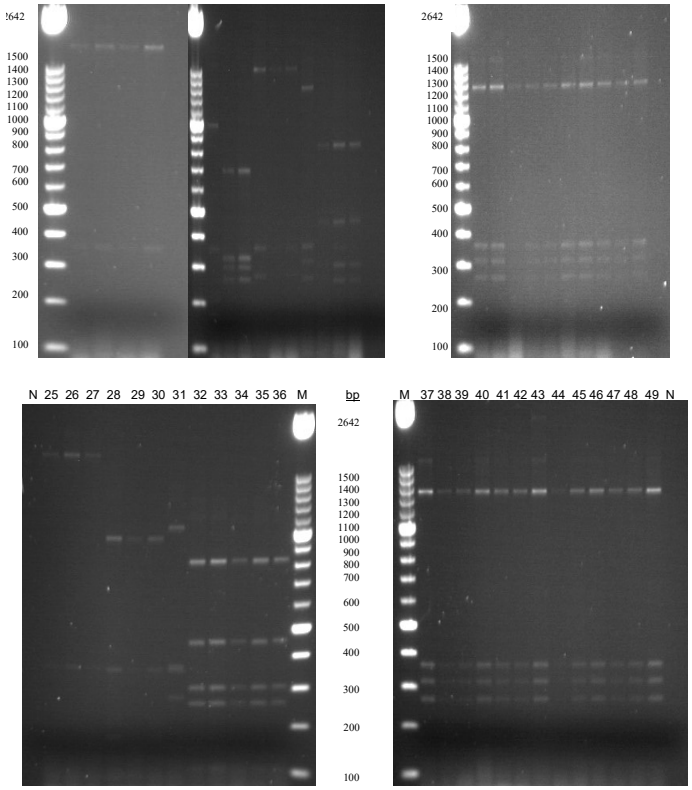
NBRC: Culture collection of National Institute of Technology and Evaluation (NITE) Biological Resource Center, Japan; IFO: Culture collection of Institute of Fermentation, Osaka, Japan; NCAIM Y: National Collection of Agricultural and Industrial Microorganisms, Hungary ; *Obtained from the culture collection of Department of Food Engineering, University of Ankara, Turkey; [a]: type strain; [b]: reference strain

Table 3. Restriction pattern analysis of PCR-amplified 18S rDNA- ITS1 of type and reference yeast strains

Yeast strain	Restriction fragments (bp)				
	*Msp*I	*Hae*III	*Scr*FI	*Rsa*I	*Alu*I
Candida holmii IFO 1629	1577, 354, 276	645, 412, 258	878, 720, 417	1076, 538, 366, 163, 121	654, 505, 447, 383
Candida krusei IFO 0841	939, 551, 409, 212	508, 249, 221, 181, 159	611, 431, 409, 292	1174, 514, 331	1024, 371, 323
Metschnikowia pulcherrima NCAIM Y 01466	1017, 366, 183	552, 242, 217, 150	1000, 337, 140	643, 425, 400, 324, 179, 124	640, 447, 350, 306
Cryptococcus albidus NBRC 0939	791, 429, 400, 355	536, 368, 261, 239, 152	820, 681, 413	1108, 417, 163, 125	659, 447, 243, 208
Kloeckera apis NBRC 10831	1912, 355	774, 730, 341	926, 651, 404	1035, 519	689, 595, 458, 289
Kloeckera apiculata NBRC 0865	1911, 351	713, 681, 313	917, 651, 409	1026, 514	678, 595,467,296
K. apiculata IFO 0865	1919, 355	713, 681, 313	917, 651, 409	1026, 514	678, 595, 467, 296
Pichia anomala IFO 0140	1076, 354, 275	545, 509, 443, 319, 282, 167, 141	707, 675, 409	1024, 404, 157, 123	643, 363, 330, 267
P. anomala NCAIM Y 01109	1149, 556, 468	565, 527, 472, 341, 309, 202, 178	713, 682, 409	1100, 443, 179, 134	656, 380, 343, 303
Saccharomyces cerevisiae IFO 2359	1347, 450, 404, 359	591, 253, 221, 157, 123	676, 616, 412, 303	1017, 573, 509, 143	645, 439, 375, 278
S. cerevisae NCYC 232	1337, 365, 316, 263	600, 259, 237, 173, 143	706, 643, 420, 313	1008, 573, 509, 139	640, 431, 363, 261
S. cerevisiae NBRC 0221	1424, 445, 419, 371	624, 268, 237, 185, 160	676, 621, 412, 296	1026, 573, 514, 152	672, 460, 396, 292
S. cerevisiae STV 85	1287, 351, 319, 270	609, 259, 237, 173, 150	700, 643, 420, 313	1008, 573, 509, 150	640, 435, 375, 268
Torulaspora delbrueckii NBRC 0469	1714, 376, 280	595, 504, 278, 186, 152	917, 681, 426	1066,519, 158	645, 573, 438, 372
Tp. delbrueckii NBRC 1180	1721, 351 266	565, 480, 259,167, 149	981, 643, 426	1074, 528, 155	651, 568, 438, 376

endogenic wine yeast strains obtained with *Scr*FI restriction enzyme were represented in Fig. 3. *K. apis* (K/C6) originated from must and *K. apiculata* (K/C8, K/U6, K/U5) isolated from must and grape had similar digestion patterns after use of *Scr*FI enzyme. Their digestion profiles were also similar to those of the control strains of the same species (Table 3). In the same gel, restriction patterns of the endogenic strains of *C. pulcherrima* (K/M3), *Cry. albidus* (K/U16, K/U21), *C. colliculosa* (K/C4) and *Tp. delbrueckii* (K/C9, K/FS2) fit the patterns of their control strains. For *C. holmii* (K/S15) which was isolated from young wine, four DNA fragments between 337 and 735 bp were obtained. This profile was different from the pattern of *C. holmii* IFO 1629 which had three DNA fragments between 417-878 bp. Number and size of DNA fragments obtained for the endogenic strains of *C. krusei* (K/M5, K/S4, K/T1) digested with *Scr*FI enzyme were similar to those of the reference strain of the same species. *C. robusta* and *S. cerevisiae* endogenic strains had the same restriction profiles with each other and also with the tested reference *S. cerevisiae* strains. Restriction profiles of those strains had four DNA fragments between 300 and 723 bp.

Fig. 3 also represents the restriction patterns of Emir isolates obtained by the use of *Scr*FI enzyme. Among the yeast strains isolated from different stages during natural wine

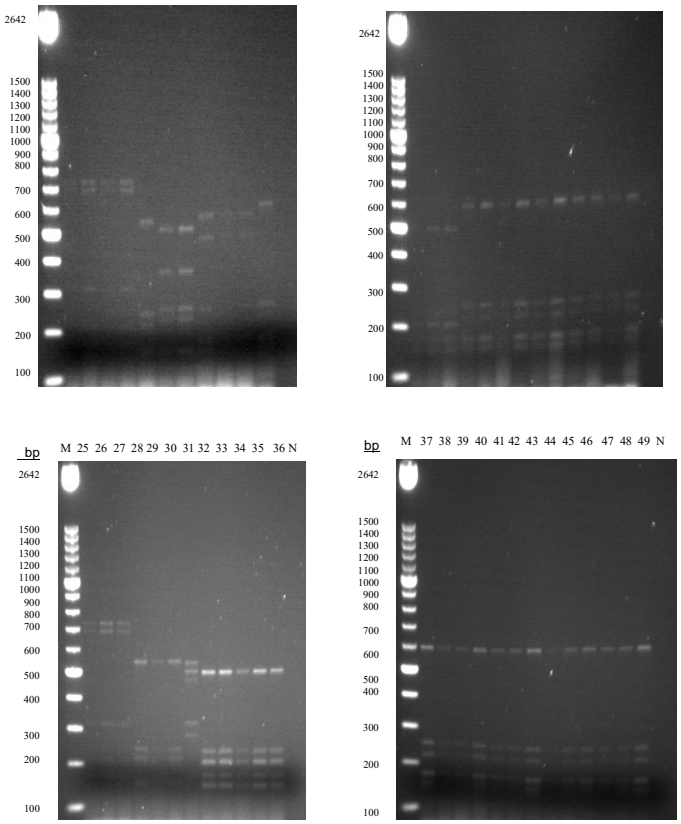


M: 100 bp DNA marker, N: negative control, 1: *K. apis* (K/C6), 2-4: *K. apiculata* (K/C8, K/U6, K/U5), 5: *C. pulcherrima* (K/M3), 6-7: *Cry. albidus* (K/U16, K/U21), 8: *Tp. delbrueckii* (K/C9), 9: *C. colliculosa* (K/C4), 10: *Tp. delbrueckii* (K/FS2), 11: *C. holmii* (K/S15), 12-14: *C. krusei* (K/M5, K/S4, K/T1), 15-16: *C. robusta* (K/FO8, K/FO11), 17-24: *S. cerevisiae* (K/M9, K/C2, K/FB8, K/FO3, K/FO7, K/FB2, K/FS3, K/S13), 25-27: *K. apiculata* (E/U17, E/M4, E/U3), 28-30: *C. pulcherrima* (E/U9, E/SO5, E/U7), 31: *P. anomala* (E/FB5), 32-36: *C. krusei* (E/S3, E/T6, E/FB7, E/FS5, E/FO2), 37-49: *S. cerevisiae* (E/S10, E/S8, E/SO7, E/T9, E/T11, E/FS8, E/S7, E/FO10, E/FO11, E/FO8, E/FB6, E/FB13, E/FB11)

Fig. 1. 18S rDNA-ITS1 restriction patterns of the endogenic wine yeast strains with *Msp*I

fermentation of Emir grapes, *K. apiculata* (E/U17, E/M4, E/U3) and *C. pulcherrima* (E/U9, E/SO5, E/U7) had the same digestion profiles with those of the related control strains. For the endogenic strain *P. anomala* E/FB5, isolated at the beginning of the wine fermentation, three DNA fragments between 439 and 872 bp were obtained which gave a different pattern from those of the type strains (Table 3). Restriction patterns of the endogenic *C. krusei* strains (E/S3, E/T6, E/FB7, E/FS5, E/FO2) isolated during natural fermentation process of Emir grapes were all similar to each other and also to the reference strain of *C. krusei* IFO 0841. By the use of *Scr*FI enzyme, endogenic *S. cerevisiae* strains, most of which were isolated from different stages of fermentation, gave the same digestion patterns with each other and also with the related reference strains (Fig. 3 and Table 3).

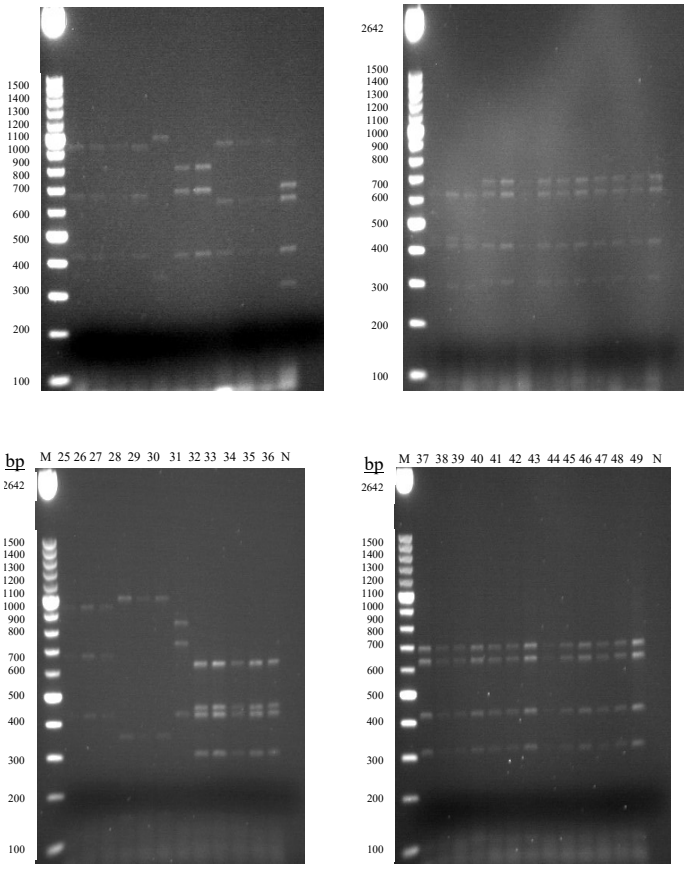
Digestion profiles of the yeast isolates obtained with the use of *Rsa*I restriction endonucleases were given in Figure 4. Among the strains isolated during natural fermentation of Kalecik Karasi wine, restriction patterns of *K. apis*, *K. apiculata*, *C. pulcherrima*, *Tp. delbrueckii* and *Cry. albidus* fit those of the related control strains. 18S rDNA-ITS1 restriction pattern of *C. colliculosa* K/C4 had three DNA fragments between 395 and 852 bp, different from the pattern



M: 100 bp DNA marker, N: negative control, 1: *K. apis* (K/C6), 2-4: *K. apiculata* (K/C8, K/U6, K/U5), 5: *C. pulcherrima* (K/M3), 6-7: *Cry. albidus* (K/U16, K/U21), 8: *Tp. delbrueckii* (K/C9), 9: *C. colliculosa* (K/C4), 10: *Tp. delbrueckii* (K/FS2), 11: *C. holmii* (K/S15), 12-14: *C. krusei* (K/M5, K/S4, K/T1), 15-16: *C. robusta:* (K/KO8, K/FO11), 17-24: *S. cerevisiae* (K/M9, K/C2, K/FB8, K/FO3, K/FO7, K/FB2, K/FS3, K/S13), 25-27: *K. apiculata* (E/U17, E/M4, E/U3), 28-30: *C. pulcherrima* (E/U9, E/SO5, E/U7), 31: *P. anomala* (E/FB5), 32-36: *C. krusei* (E/S3, E/T6, E/FB7, E/FS5, E/FO2), 37-49: *S. cerevisiae* (E/S10, E/S8, E/SO7, E/T9, E/T11, E/FS8, E/S7, E/FO10, E/FO11, E/FO8, E/FB6, E/FB13, E/FB11)

Fig. 2. 18S rDNA-ITS1 restriction patterns of the endogenic wine yeast strains with *Hae*III

of reference *Tp. delbrueckii* strains (NBRC 0469 and 1180). The endogenic *C. holmii* strain (K/S15), originated from Kalecik Karasi young wine had a distinct DNA profile when compared to the type strain. Additionally, three *C. krusei* strains (K/M5, K/S4, K/T1) isolated from different stages of natural wine fermentation process, had different digestion patterns from *C. krusei* IFO 0841. Similar to the results obtained with the other restriction enzymes, the same DNA patterns were obtained for all *S. cerevisiae* and also *C. robusta* endogenic Kalecik strains, as well as for the reference *S. cerevisiae* strains with the use of *Rsa*I enzyme. Among the strains isolated during natural fermentation process of the Emir grapes, *K. apiculata* (E/U17, E/M4, E/U3) strains gave similar profiles with the reference strain, having two DNA fragments of 495 and 1022 bp with the restriction enzyme of *Rsa*I (Figure 4 and Table 3). In the same gel, molecular confirmations of *C. pulcherrima* (E/U9, E/SO5, E/U7) and *P. anomala* (E/FB5) were also successful with this enzyme when their digestion profiles were compared with those of the related type strains. In the restriction patterns of *C. pulcherrima* strains,

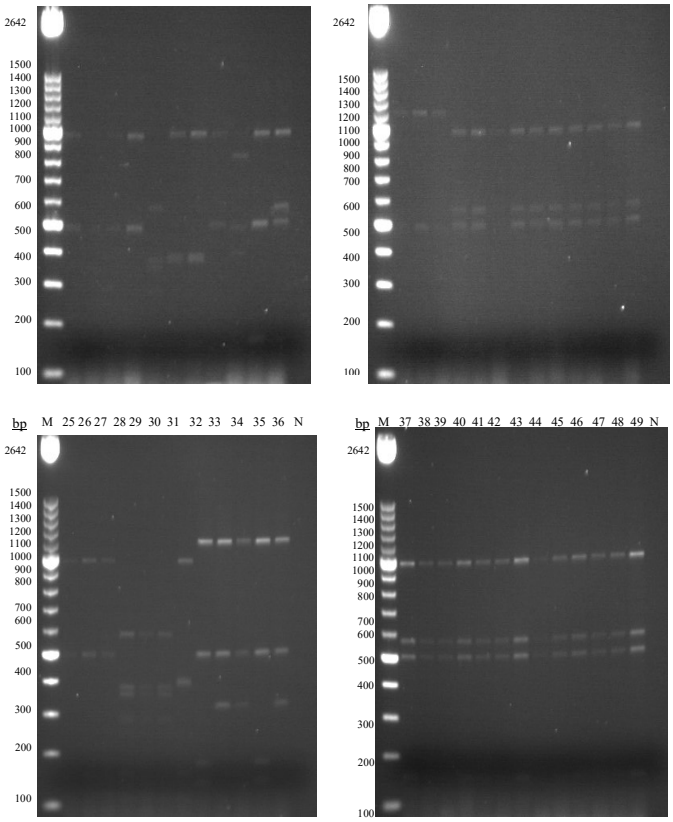


M: 100 bp DNA marker, N: negative control, 1: *K. apis* (K/C6), 2-4: *K. apiculata* (K/C8, K/U6, K/U5), 5: *C. pulcherrima* (K/M3), 6-7: *Cry. albidus* (K/U16, K/U21), 8: *Tp. delbrueckii* (K/C9), 9: *C. colliculosa* (K/C4), 10: *Tp. delbrueckii* (K/FS2), 11: *C. holmii* (K/S15), 12-14: *C. krusei* (K/M5, K/S4, K/T1), 15-16: *C. robusta:* (K/KO8, K/FO11), 17-24: *S. cerevisiae* (K/M9, K/C2, K/FB8, K/FO3, K/FO7, K/FB2, K/FS3, K/S13), 25-27: *K. apiculata* (E/U17, E/M4, E/U3), 28-30: *C. pulcherrima* (E/U9, E/SO5, E/U7), 31: *P. anomala* (E/FB5), 32-36: *C. krusei* (E/S3, E/T6, E/FB7, E/FS5, E/FO2), 37-49: *S. cerevisiae* (E/S10, E/S8, E/SO7, E/T9, E/T11, E/FS8, E/S7, E/FO10, E/FO11, E/FO8, E/FB6, E/FB13, E/FB11)

Fig. 3. 18S rDNA-ITS1 restriction patterns of the endogenic wine yeast strains with *Scr*FI

six DNA fragments were observed between 110-589 bp, while four DNA fragments were obtained between 112 and 1022 bp for *P. anomala* E/FB5. Digestion profiles of five *C. krusei* strains isolated from Emir young wine (E/S3), sludge (E/T6) or different stages of fermentation (E/FB7, E/FS5, E/FO2) were different from each other. DNA patterns of the strains E/T6, E/FB7 and E/FO2 had three DNA fragments between 326 and 1174 bp, which fit the pattern of the reference strain. For E/S3 and E/FS5 strains, four DNA fragments between 133-1165 bp were obtained, which was different from the pattern of the reference strain. For *S. cerevisiae* strains originated from different fermentation stages of Emir wine production, four DNA fragments between 133-1091 bp were obtained, similar to the profiles obtained for the reference *S. cerevisiae* strains.

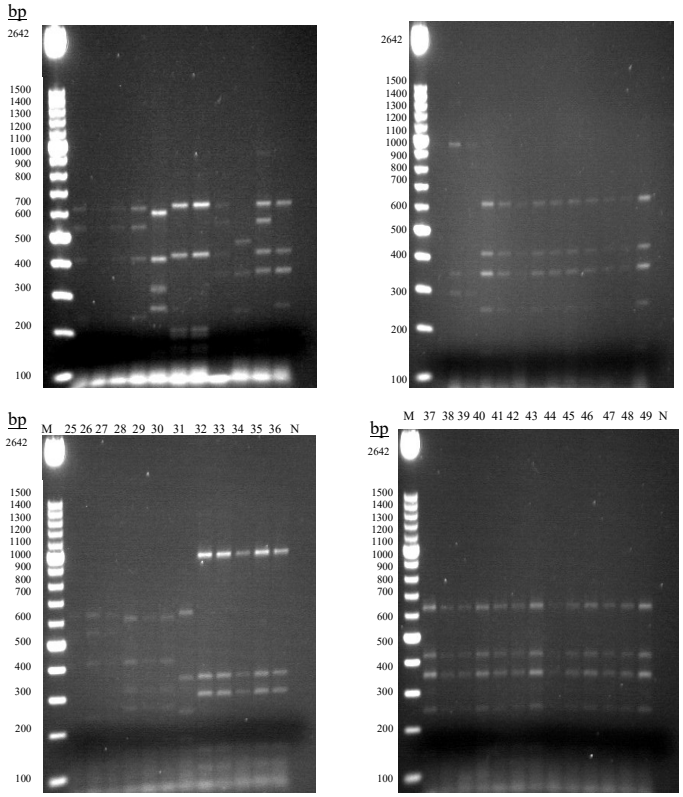
Restriction profiles of the yeast isolates obtained with the use of *Alu*I restriction enzyme were given in Figure 5. According to restriction patterns of the endogenic strains isolated during Kalecik Karasi wine production, *K. apis* (K/C6) and *K. apiculata* (K/C8, K/U6 and K/U5) strains gave similar profiles, having four DNA fragments between 214-626 and 228-626 bp, respectively. These profiles were also similar to those of the related control strains. Molecular confirmation of



M: 100 bp DNA marker, N: negative control, 1: *K. apis* (K/C6), 2-4: *K. apiculata* (K/C8, K/U6, K/U5), 5: *C. pulcherrima* (K/M3), 6-7: *Cry. albidus* (K/U16, K/U21), 8: *Tp. delbrueckii* (K/C9), 9: *C. colliculosa* (K/C4), 10: *Tp. delbrueckii* (K/FS2), 11: *C. holmii* (K/S15), 12-14: *C. krusei* (K/M5, K/S4, K/T1), 15-16: *C. robusta:* (K/KO8, K/FO11), 17-24: *S. cerevisiae* (K/M9, K/C2, K/FB8, K/FO3, K/FO7, K/FB2, K/FS3, K/S13), 25-27: *K. apiculata* (E/U17, E/M4, E/U3), 28-30: *C. pulcherrima* (E/U9, E/SO5, E/U7), 31: *P. anomala* (E/FB5), 32-36: *C. krusei* (E/S3, E/T6, E/FB7, E/FS5, E/FO2), 37-49: *S. cerevisiae* (E/S10, E/S8, E/SO7, E/T9, E/T11, E/FS8, E/S7, E/FO10, E/FO11, E/FO8, E/FB6, E/FB13, E/FB11)

Fig. 4. 18S rDNA-ITS1 restriction patterns of the endogenic wine yeast strains with *Rsa*I

identification was also achieved for *C. pulcherrima* K/M3, isolated from must, and *Cry. albidus* (K/U16, K/U21) originated from Kalecik Karasi grape. When *Alu*I enzyme was used, *C. colliculosa* (K/C4) and its telemorph form *Tp. delbrueckii* (K/C9, K/FS2) strains differed in patterns. For *C. colliculosa* K/C4, three DNA fragments between 250 and 481 bp were obtained which was a different profile from those of the reference strains. Restriction patterns of *Tp. delbrueckii* K/C9 and K/FS2, isolated from must and at the end of the wine fermentation fit the restriction pattern of the reference strain (Fig. 5 and Table 3). *C. holmii* K/S15 originated from Kalecik Karasi young wine and its type strain differed in patterns. DNA patterns of *C. krusei, C. robusta* and *S. cerevisiae* endogenic strains fit the patterns of the related reference strains with the use of *Alu*I enzyme. After resticton analysis, three DNA fragments between 284-955 bp for *C. krusei*, and four DNA fragments between 239 and 643 bp for *S. cerevisiae* strains were obtained, respectively. Results of PCR-RFLP analysis with *Alu*I restriction endonucleases for the strains isolated during Emir wine production can also be seen in Fig. 5. Of these isolates, restriction patterns of endogenic *K. apiculata*, *C. pulcherrima*, *C. krusei* and *P. anomala* strains were similar to those of the control strains. For *S. cerevisiae* isolates, four DNA fragments were obtained between 250 and 661 bp with the use of

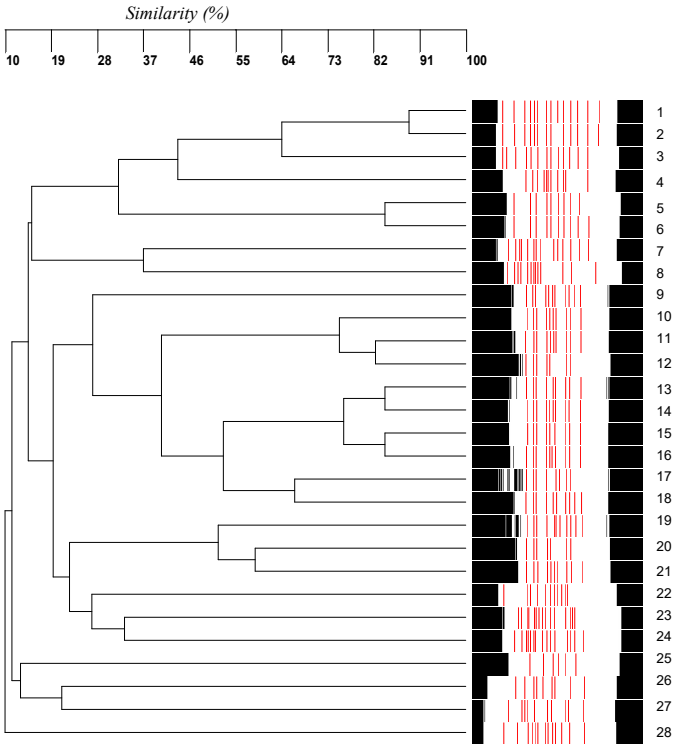


M: 100 bp DNA marker, N: negative control, 1: *K. apis* (K/C6), 2-4: *K. apiculata* (K/C8, K/U6, K/U5), 5: *C. pulcherrima* (K/M3), 6-7: *Cry. albidus* (K/U16, K/U21), 8: *Tp. delbrueckii* (K/C9), 9: *C. colliculosa* (K/C4), 10: *Tp. delbrueckii* (K/FS2), 11: *C. holmii* (K/S15), 12-14: *C. krusei* (K/M5, K/S4, K/T1), 15-16: *C. robusta:* (K/KO8, K/FO11), 17-24: *S. cerevisiae* (K/M9, K/C2, K/FB8, K/FO3, K/FO7, K/FB2, K/FS3, K/S13), 25-27: *K. apiculata* (E/U17, E/M4, E/U3), 28-30: *C. pulcherrima* (E/U9, E/SO5, E/U7), 31: *P. anomala* (E/FB5), 32-36: *C. krusei* (E/S3, E/T6, E/FB7, E/FS5, E/FO2), 37-49: *S. cerevisiae* (E/S10, E/S8, E/SO7, E/T9, E/T11, E/FS8, E/S7, E/FO10, E/FO11, E/FO8, E/FB6, E/FB13, E/FB11)

Fig. 5. 18S rDNA-ITS1 restriction patterns of the endogenic wine yeast strains with *Alu*I

*Alu*I enzyme. This profile fit the pattern of the control strains, in agreement with the results obtained by the use of other tested restriction enzymes.

In PCR-RFLP, identification of most of the tested endogenic strains could be confirmed by using five different restriction enzymes. In evaluation of PCR-RFLP results, those endogenic strains, restriction patterns of which could be confirmed by all of the five restriction enzymes were accepted as identified. Therefore, identification of the strains could not be confirmed by molecular methods if a negative result was obtained for even one enzyme. Among 24 Kalecik Karasi isolates, identification results of 19 (79%) of them were in agreement with their previous results, obtained by using API ID 32C (BioMèrieux, France) and some additional identification tests (unpublished data). The molecular identification results revealed that the strains were in the species of *C. pulcherrima* (K/M3), *C. robusta* (K/FO8, K/FO11), *Cry. albidus* (K/U16, K/U21), *K. apiculata* (K/C8, K/U6, K/U5), *K. apis* (K/C6), *S. cerevisiae* (K/M9, K/C2, K/FB8, K/FO3, K/FO7, K/FB2, K/FS3, K/S13), and *Tp. delbrueckii* (K/C9, K/FS2). When PCR-RFLP results of Emir isolates were evaluated, confirmations of 22 (88%) of the 25 strains were successful. These strains were in the species of *C. krusei* (E/T6, E/FB7, E/FO2), *C. pulcherrima* (E/U9, E/SO5, E/U7), *K. apiculata*



1: *K. apiculata* IFO 0865, 2: *K. apiculata* NBRC 0865, 3: *K. apiculata* K/U5, 4: *K. apis* K/C6, 5: *K. apiculata* K/C8, 6: *K. apiculata* K/U6, 7: *Cry. albidus* K/U16, 8: *Cry. albidus* K/U21, 9: *S. cerevisiae* NBRC 0221, 10: *S. cerevisiae* K/FB8, 11: *S. cerevisiae* K/FO3, 12: *S. cerevisiae* K/FO7, 13: *S. cerevisiae* STV85, 14: *C. robusta* K/FO8, 15: *C. robusta* K/FO11, 16: *S. cerevisiae* K/M9, 17: *S. cerevisiae* NCYC 232, 18: *S. cerevisiae* K/C2, 19: *S. cerevisiae* K/FS3, 20: *S. cerevisiae* K/FB2, 21: *S. cerevisiae* K/S13, 22: *K. apis* IFO10831, 23: *Cry. albidus* NBRC 0939, 24: *C. pulcherima* K/MP3, 25: *M. pulcherima* NCAIM Y 01466, 26: *Tp. delbrueckii* K/FS2, 27: *Tp. delbrueckii* NBRC 1180, 28: *Tp. delbrueckii* K/C9.

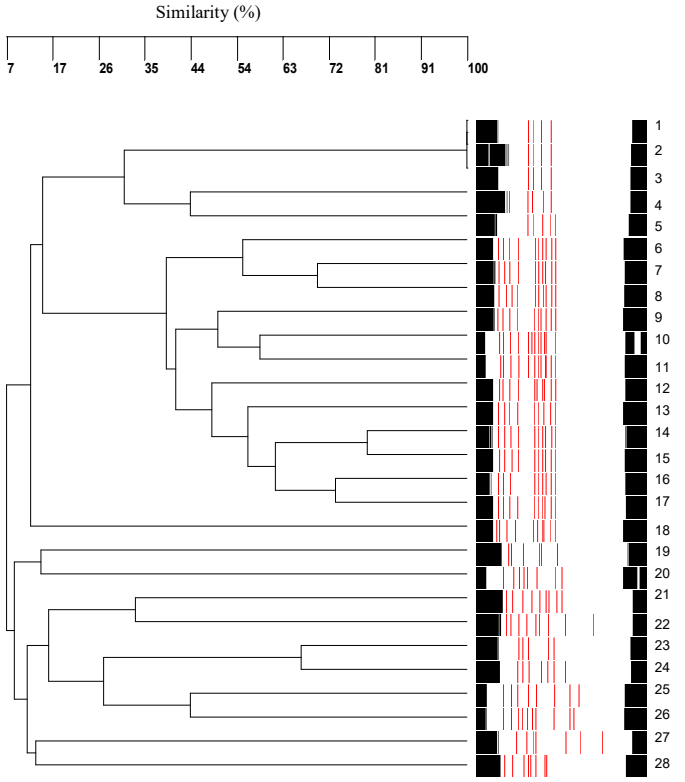
Fig. 6. Cluster analysis of RAPD-PCR fingerprints obtained by using $(GTG)_3$ primer for the Kalecik Karasi isolates and control strains

(E/U17, E/M4, E/U3), and *S. cerevisiae* (E/S10, E/S7, E/S8, E/T9, E/T11, E/FS8, E/FO10, E/FO11, E/FO8, E/SO7, E/FB6, E/FB13, E/FB11).

Strain discrimination by RAPD-PCR

The yeast strains which were identified by PCR-RFLP were discriminated by using RAPD-PCR. DNA fingerprints obtained by both $(GTG)_3$ and M13 primers were evaluated by UPGMA cluster analysis. Figure 6 represents the RAPD-PCR fingerprints and the results of cluster analysis obtained by using $(GTG)_3$ primer for the identified Kalecik Karasi strains and the control strains. Eight of the 28 strains in Fig. 6 were control strains. Generally, strains of the same species clustered together with a few exceptions. The strains *K. apiculata* K/U6 and K/C8 originating from grape and must, respectively, were found as 84% similar. The similarity between the six strains in the first group, including two subgroups, was obtained as 34%. The similarity between *Cry. albidus* K/U16 and K/U21 was 37%, both originating from Kalecik Karasi grapes.

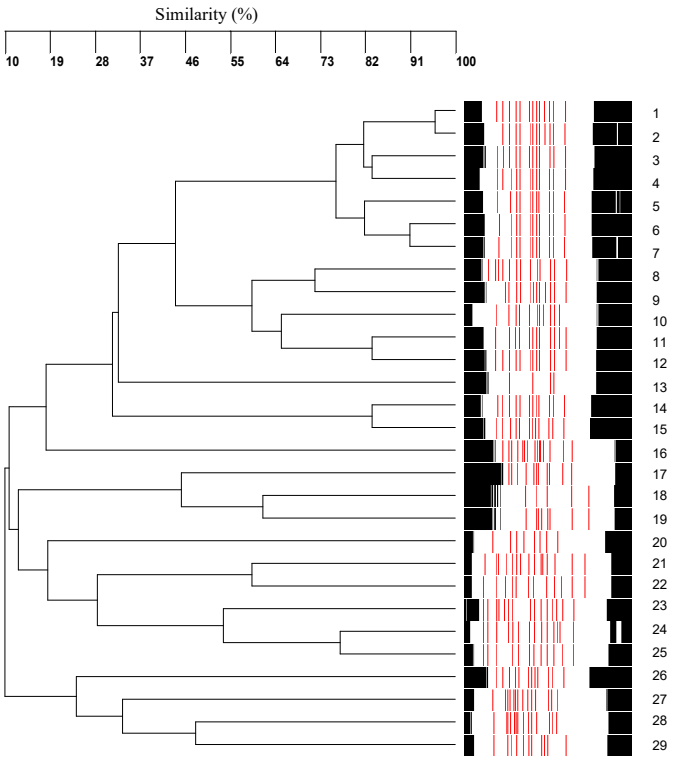
In Fig. 6, thirteen strains (numbers 9-21) including *S. cerevisiae* and *C. robusta* clustered in two groups. In the first group, the similarity between eight *S. cerevisiae* and two *C. robusta*



1: *K. apiculata* K/C8, 2: *K. apiculata* K/U6, 3: *K. apiculata* K/U5, 4: *K. apiculata* IFO 0865, 5: *K. apiculata* NBRC 0865, 6: *S. cerevisiae* K/S13, 7: *S. cerevisiae* K/FB2, 8: *S. cerevisiae* K/FS3, 9: *S. cerevisiae* NCYC 232, 10: *C. robusta* K/FO8, 11: *C. robusta* K/FO11, 12: *S. cerevisiae* K/FO7, 13: *S. cerevisiae* STV85, 14: *S. cerevisiae* K/FB8, 15: *S. cerevisiae* K/FO3, 16: *S. cerevisiae* K/M9, 17: *S. cerevisiae* K/C2, 18: *S. cerevisiae* NBRC 0221, 19: *K. apis* K/C6, 20: *Tp. delbrueckii* K/C9, 21: *Cry. albidus* NBRC 0939, 22: *C. pulcherrima* K/M3, 23: *Cry. albidus* K/U16, 24: *Cry. albidus* K/U21, 25: *Tp. delbrueckii* K/FS2, 26: *Tp. delbrueckii* NBRC1180, 27: *M. pulcherrima* NCAIMY 01466, 28: *K. apis* IFO10831

Fig. 7. Cluster analysis of RAPD-PCR fingerprints obtained by using M13 primer for the Kalecik Karasi isolates and control strains

strains were 28%. In the second group (numbers 19-21) there were 3 *S. cerevisiae* strains which were 53% similar to each other. *C. robusta* K/FO8 which was isolated during fermentation was found to be 84% similar to one of the reference strain used; *S. cerevisiae* STV 85. The same similarity was also observed between *C. robusta* K/FO11 and *S. cerevisiae* K/M9, isolated during fermentation and originated from must, respectively. The strains *S. cerevisiae* K/FO3 and K/FO7 both isolated at the middle stage of the fermentation, were 82% similar to each other. Additionally, 80% similarity was detected between *C. robusta* K/FO8 and *S. cerevisiae* K/M9, as well as between *S. cerevisiae* K/FB8 and K/FO3. Considerable differences were determined between the reference strains and some of the endogenic *S. cerevisiae* isolates. For example, *S. cerevisiae* K/FS3, isolated at the end of the fermentation had no similarity (0%) with the reference *S. cerevisiae* strains NBRC 0221 and STV 85. Besides, *S. cerevisiae* K/S13, originating from young wine was also considerably different from the all other endogenic and reference *S. cerevisiae* strains (Fig. 6). In the same figure, it can be seen that the similarities between *Tp. delbrueckii* strains were very low. *Tp. delbrueckii* K/FS2 and NBRC 1180 were 21% similar to each other, while the strain K/C9 was considerably different from them. *C.*

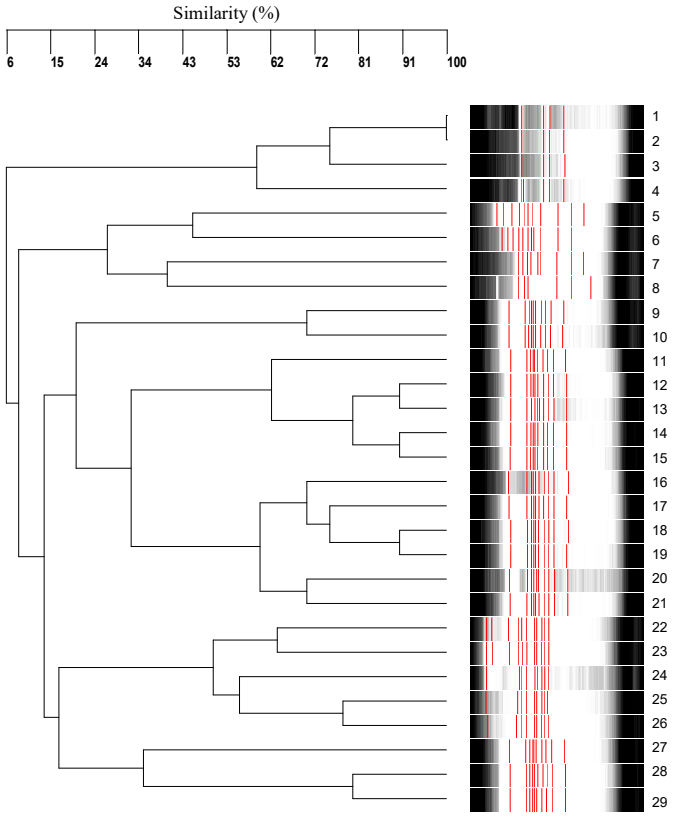


1: *S. cerevisiae* E/T11, 2: *S. cerevisiae* E/FO10, 3: *S. cerevisiae* E/T9, 4: *S. cerevisiae* E/FS8, 5: *S. cerevisiae* E/SO7, 6: *S. cerevisiae* E/FO11, 7: *S. cerevisiae* E/FO8, 8: *S. cerevisiae* STV85, 9: *S. cervisiae* E/S10, 10: *S. cerevisiae* NCYC 232, 11: *S. cerevisiae* NBRC 0221, 12: *S. cerevisiae* E/S7, 13: *S. cerevisiae* E/S8, 14: *S. cerevisiae* E/FB6, 15: *S. cerevisiae* E/FB13, 16: *C. krusei* IFO 0841, 17: *C. krusei* E/T6, 18: *C. krusei* E/FB7, 19: *C. krusei* E/FO2, 20: *M. pulcherrima* NCAIMY 01466, 21: *K. apiculata* IFO 0865, 22: *K. apiculata* NBRC 0865, 23: *K. apiculata* E/M4, 24: *K. apiculata* E/U17, 25: *K. apiculata* E/U3, 26: *S. cerevisiae* E/FB11, 27: *C. pulcherrima* E/U7, 28: *C. pulcherrima* E/U9, 29: *C. pulcherrima* E/SO5

Fig. 8. Cluster analysis of RAPD-PCR fingerprints obtained by using $(GTG)_3$ primer for the Emir isolates and control strains

pulcherrima K/M3 and the type strain; *C. pulcherrima* NCAIM Y 01466 had very different profiles and could be discriminated.

RAPD-PCR fingerprints and the results of cluster analysis obtained by using M13 primer for the identified Kalecik Karasi strains and the control strains were represented in Fig. 7. It was found that three of the five *K. apiculata* strains (numbers 1-3) were the same in patterns, having 100% similarity. Of these strains, K/C8 was isolated from must, while K/U6 and K/U5 were originating from grape. Thirteen strains belonging to *S. cerevisiae* (numbers 6-18) clustered in two groups. The first group was including the numbers 6-16. In the same dendogram, *S. cerevisiae* K/C2 and reference strain NBRC 0221 were in the second group. These two strains were only 20% similar to each other. The highest similarity (80%) was detected between *S. cerevisiae* K/FB8 and K/FO3, isolated at the beginning and at the end of the fermentation, respectively. The similarity of the reference *S. cerevisiae* strain, NBRC 0221 to the strains in the first group was between 0-20%. The strains *S. cerevisiae* K/FB2, K/FB8 and K/FO3 were 70% similar to each other. The similarity was not surprising as these strains were originating from



1: *C. krusei* E/T6, 2: *C. krusei* E/FB7, 3: *C. krusei* E/FO2, 4: *C. krusei* IFO 0841, 5: *C. pulcherrima* E/U9, 6: *C. pulcherrima* E/SO5, 7: *C. pulcherrima* E/U7, 8: *M. pulcherrima* NCAIMY01466, 9*: S. cerevisiae* E/FB13, 10: *S. cerevisiae* E/FB11, 11: *S. cerevisiae* E/FO11, 12: *S. cerevisiae* E/T9, 13: *S. cerevisiae* E/T11, 14: *S. cerevisiae* E/FS8, 15: *S. cerevisiae* E/FO10, 16: *S. cerevisiae* E/S8, 17: *S. cerevisiae* NBRC 0221, 18: *S. cerevisiae* STV85, 19: *S. cerevisiae* E/S10, 20: *S. cerevisiae* NCYC 232, 21: *S. cerevisiae* E/S7, 22: *K. apiculata* IFO 0865, 23: *K. apiculata* NBRC 0865, 24: *K. apiculata* E/U17, 25: *K. apiculata* E/M4, 26: *K. apiculata* E/U3, 27: *S. cerevisiae* E/FB6, 28: *S. cerevisiae* E/FO8, 29: *S. cerevisiae* E/SO7

Fig. 9. Cluster analysis of RAPD-PCR fingerprints obtained by using M13 primer for the Emir isolates and control strains

the same fermentation medium. Additionally, the similarity between *S. cerevisiae* K/M9 and K/C2 was detected as 73%. The isolate K/C6 and the reference strain NBRC 10831 which belong to *K. apis* were found as completely different from each other by the use of M13 primer. It was determined that *Tp. delbrueckii* K/FS2 and control strain NBRC 1180 were 44% similar to each other. However, *Tp. delbrueckii* K/C9 had a distinct profile from these strains. The similarity between *Cry. albidus* K/U16 and K/U21 was found as 66%. These two isolates could be discriminated from *Cry. albidus* NBRC 0939 with very low similarities. The strain *C. pulcherrima* K/M3 were found as 25% similar to the type strain *M. pulcherrima* NCAIM Y 01466.

Results of cluster analysis of RAPD-PCR fingerprints obtained by using $(GTG)_3$ primer for the Emir isolates and control strains were represented in Figure 8. It was found that *S. cerevisiae* strains clustered in three groups. The first cluster was divided into two subgroups, having an intraspecific similarity of 44%. The highest similarity (96%) between *S. cerevisiae* strains was obtained for E/T11 and E/FO10, isolated from sludge and from must at the middle of the fermentation, respectively. The strains E/FO11 and E/FO8, both isolated at the middle of the fermentation were found as 90% similar to each other. The intraspecific similarity between E/FS8 and E/FO11 strains were detected as 86%. The strain E/FO10 was 83% similar to E/T9 and E/FS8. Additionally, E/T9 exhibited a 83% similarity to E/FS8 and E/S7. The same similarity was also detected between a control *S. cerevisiae* strain, NBRC 0221 and a young wine strain, E/S7. In Figure 8, the first seven strain of *S. cerevisiae* clustered together, forming two subgroups which was 74% similar to each other. The strains in this cluster could be discriminated from the control strains owing to very low similarities in fingerprints. *S. cerevisiae* E/S10, originating from young wine, could be effectively differentiated from other *S. cerevisiae* strains, which can be observed from the similarity percentages in the dendogram (Fig. 8). As another group, three control strains, E/S10 and E/S7 clustered together, having intraspecific similarity of approximately 60%. It was found that one of the reference strains *S. cerevisiae* NBRC 0221 and the strain E/S7, originating from young wine were 83% similar to each other. The similarity between the fingerprints of the strains E/S8 and E/S7, both originating from young wine, were detected as 50%. Additionally, E/8 strain had a very distinct profile, discriminating this strain from other *S. cerevisiae* strains. The profile of the strain FB/11, isolated at the beginning of the fermentation, was also considerably different from those of the other *S. cerevisiae* strains. Other two strains, both isolated from must at the beginning of the fermentation (E/FB6 and E/FB13) were found as 83% similar in patterns. Among *C. krusei* strains given in the dendogram in Fig. 8, the highest intraspecific similarity (61%) was detected between E/FB7 and E/FO2. *K. apiculata* E/U17 and E/U3, both originating from Emir grape were 76% similar in patterns. Three endogenic *K. apiculata* strains were discriminated from the control strains with very low similarities. Three *C. pulcherrima* isolates and the type strain *M. pulcherrima* NCAIM Y 01466 differed in fingerprints. The similarity between the endogenic *C. pulcherrima* strains E/U9 and E/SO5 was detected as 48% by the use of $(GTG)_3$ primer.

Figure 9 represents the results of cluster analysis of RAPD-PCR fingerprints obtained by using M13 primer for the Emir isolates and control strains. The strains *C. krusei* E/T6 and E/FB7, isolated from sludge and from the must at the beginning of the fermentation respectively, could not be differentiated by the use of M13 primer as 100% similarity was obtained between them. Both of these strains were 75% similar to the strain E/FO2, isolated from must at the middle of the fermentation. It was found that similarity of the reference strain IFO 0841 with the endogenic *C. krusei* strains changed between 44-66%. In the dendogram, another cluster can be observed including *C. pulcherrima* isolates and the type strain *M. pulcherrima* NCAIM Y 01466. The endogenic strains E/U9 and E/SO5 were 45% similar in fingerprints (Fig. 9). The similarity of NCAIM Y 01466 with *C. pulcherrima* isolates changed between 23-40%. Sixteen *S. cerevisiae* strains clustered in two groups (Fig. 9). In the first group including three reference strains, the isolates E/T9 and E/T11, both originating from sludge, were found as 90% similar to each other. In the same group, 90% similarity was also obtained for E/FS8 and E/FO10, isolated from the must at the end and

at the middle of the fermentation, respectively. Another result of interest was 90% similarity between reference strain STV 85 and E/S10. It was determined that fingerprints of the strains E/FB13 and E/FB11, both isolated from the must at the beginning of the fermentation, were 70% similar to each other. In the other cluster formed by *S. cerevisiae* strains, the similarity between E/FO8 and E/SO7 was detected as 80%. The three strains in this group were completely different from the others or had very low similarities with the *S. cerevisiae* strains in the other group. Most of the similarity levels obtained between *K. apiculata* strains clustered in another group, was above 50%. Of these strains, E/M4 and E/U3 were 77% similar to each other. The strain E/U17 could be differentiated from these two strains with a similarity level of 55%. *K. apiculata* isolates were discriminated from the reference strains with 40-60% similarity.

DISCUSSION

In this research, identification of most (84%) of the yeast strains associated with spontaneous wine fermentation could be confirmed by PCR-RFLP. Identification results of the rest of the endogenic yeast strains (16%) were not in agreement with the previous ones obtained by API ID 32C combined with some morphological and biochemical methods (unpublished data). It was reported that although these commercial identification systems were widely used, their scope was limited to clinical field. Identifications performed on the basis of physiological features only or in combination with morphological observations were often approximate. It was stated that many species of interest to food industry could not be reliably distinguished on the basis of physiological and morphological features[15]. It is known that the DNA-based identifications are far more reliable than those from phenotypic tests, and much faster[16]. However, there were also reports about identification of the yeast isolates by PCR-RFLP which were completely in agreement with the results obtained by traditional physiological methods[1].

In the present study, it was determined that the confirmed yeast strains by PCR-RFLP were in eight species; *C. pulcherrima*, *C. robusta*, *Cry. albidus*, *K. apiculata*, *K. apis*, *Tp. delbrueckii*, *C. krusei* and *S. cerevisiae*. Additionally, the yeast microbiota associated with Emir and Kalecik Karasi spontaneous wine fermentation were found to be similar, but differed in several species. *S. cerevisiae*, *C. pulcherrima* and *K. apiculata* were common species in both fermentations. *C. krusei* was detected only during spontaneous fermentation of Emir grapes. However, *C. robusta*, *Cry. albidus*, *K. apis* and *Tp. delbrueckii* were included only in Kalecik Karasi isolates. Approximately 50% of the total identified isolates were *S. cerevisiae* strains. Most of the strains isolated at the end of the natural fermentations of both grapes belonged to *S. cerevisiae*, except one *Tp. delbrueckii* (K/FS2) strain. Among Kalecik Karasi strains confirmed, *K. apiculata* (K/C8), *K. apis* (K/C6), and *Tp. delbrueckii* (K/C9) had been isolated from must after addition of SO_2. Only three endogenic *S. cerevisiae* strains (K/S13, E/S7, E/S8) originated from young wine. *C. krusei* E/T6 and *S. cerevisiae* E/T9 strains had been isolated from sludge during the process of Emir wine production. It was reported that the growth of non-*Saccharomyces* species belonging to the genera *Kloeckera* and *Candida* were generally limited to the first few days of fermentation, because of their weak ethanol tolerance. However, studies have shown that *K. apiculata* and *Candida stellata* can survive at significant levels during fermentation, and for longer periods than thought previously[17]. In the present study, *C. krusei* E/T6 which was isolated from sludge, survived longer than most of the strains. *Tp. delbrueckii* K/FS2 was the other species determined at the end of the fermentation. It was reported in a study by Lopandic *et al.*[18], 11 yeast species belonging to genera *Candida, Hanseniaspora, Issatchenkia, Kregervanrija, Lachancea, Metschnikowia, Pichia, Saccharomyces* and *Zygoascus* were identified during spontaneous fermentations of Austrian wines. These genera were quite different from those obtained in our study except *Candida* and *Saccharomyces*. Lopandic *et al.*[18] also reported that *Candida* persisted well into the middle stage of fermentation, in agreement with our results for the species *C. krusei* and *C. robusta*. Another *Candida* species was reported as *Candida zemplinina* occuring in different stages of Picolit wine fermentations[19]. Zott *et al.*[20] reported yeast

dynamics and species identification for Merlot wine during cold maceration and the most aboundant non-*Saccharomyces* species was *Lodderomyces elongisporus*, followed by *M. pulcherrima, Hanseniaspora uvarum, P. anomala, Pichia membranefaciens, Tp. delbrueckii* and *Issatchenkia orientalis*. When compared to our results, *Tp. delbrueckii* was the only common non-*Saccharomyces* species which survived until the end of the fermentation.

According to the RAPD-PCR results, most of the identified endogenic yeast strains could be differentiated. There were common differentiation results obtained with the M13 and $(GTG)_3$ primers. For example, the fingerprint of *S. cerevisiae* E/S8 originated from young wine, was considerably different from those of other *S. cerevisiae* strains, for both of the primers. The strains *K. apis* K/C6 and NBRC 10831, which were found completely different by the use of M13 primer, had very low similarities when $(GTG)_3$ was used. Additionally, no similarities were detected between *Tp. delbrueckii* K/FS2 and K/C9 for both of the primers. When the dendograms obtained with two primers are compared, it can be seen that different results were obtained for some of the strains. Although *K. apiculata* K/C8, K/U6 and K/U5 could not be differentiated from each other with the use of M13 primer, they were discriminated by $(GTG)_3$ primer. Moreover, the similarity of K/U5 to K/C8 and K/U6 was found as 45% and 43%, respectively. Another difference between two primers was observed for *Cry. albidus* K/U16 and K/U21 strains, which had 37% and 66% similarities with the use of $(GTG)_3$ and M13 primers, respectively. *S. cerevisiae* E/T11 was found as 96% similar to *S. cerevisiae* E/FO10 with the use of $(GTG)_3$, while this similarity was 60% when M13 primer was used. The similarity between reference *S. cerevisiae* STV 85 and E/S10 was 90% and 72% when M13 and $(GTG)_3$ were used, respectively. The primer $(GTG)_3$ was also more effective in discrimination of *C. krusei* strains. The strains E/T6 and E/FB7, isolated from sludge and at the beginning stage of fermentation, could not be differentiated by M13 primer, while the smilarity between them was obtained as only 37% with the use of $(GTG)_3$ primer. Although satisfactory results were obtained by M13 primer, $(GTG)_3$ primer was much more effective in discrimination of certain strains. In a study by Nikolaou *et al.*[2], genetic biodiversity between *S. cerevisiae* strains isolated from red and white wine fermentations and type strains were detected by RAPD-PCR with M13 primer. PCR analysis with primer M13 was suggested as an effective method for discrimination at strain level of species belonging to genus *Saccharomyces*. M13 primer was also effectively used by Urso *et al.*[19], which studied characterization of *S. cerevisiae* strains isolated from Picolit wine fermentations. In our study, all of the endogenic *S. cerevisiae* strains could be differentiated by both M13 and $(GTG)_3$ primers although the similarity between certain strains were high. In a study performed by Xufre *et al.*[5], six enological *Saccharomyces* spp. were differentiated by RAPD-PCR. It was reported that 21 of the 27 primers used gave satisfactory results in amplification reactions and only four of them were able to discriminate among the six strains. In another study, inter- and intra-specific differentiation of natural wine strains of *Hanseniaspora* (*Kloeckera*) were performed by RAPD-PCR[3]. It was reported that M13 and RM13 primers were used for species differentiation, while RM13 was found to be applicable in discriminating among strains within species of *H. uvarum*. Walczak *et al.*[21] reported the differentiation of *Candida* genus yeast isolated in brewing by using four primers; $(GTG)_5$, $(GAC)_5$, $(GACA)_4$ and M13. For strains belonging to *C. sake*, the best differentiation was reported to be done with M13, while primer $(GTG)_5$ was applied for the identification of *C. pelliculosa*, *C. lambica* and *S. cerevisiae* and for the differentiation of 16 strains of *S. cerevisiae*[21]. In our study, RAPD-PCR analysis with more than one primer has been demonstrated as a useful method for adequate strain-specific identification.

It is known that the rapid identification of yeast species occuring during grape must fermentations would be highly useful in winemaking because it could furnish quantitative information about the composition and dynamics of the yeast populations that may affect the organoleptic properties of the final product[1]. In recent years, increasing attention has been focused on the study of the biodiversity of natural strains of *S. cerevisiae* with the goals of understanding the ecology of this yeast and the selection of new

wine strains[22]. Additionally, a renewed interest in non-*Saccharomyces* strains appeared with the aim to gain some benefits of the aromatic properties produced by these yeasts[23]. Some non-*Saccharomyces* species associated with wine-making have been suggested as starter cultures due to their specific metabolic characteristics[17]. In this context, to improve the chemical composition and sensory properties of wine, the inclusion of non-*Saccharomyces* wine yeasts, together with *Saccharomyces* strains as a part of multistarter fermentations, has been proposed as a tool to take advantage of spontaneous fermentation[17].

CONCLUSIONS

Kalecik Karasi (red) and Emir (white) are known as the most important wine grape varieties grown in Turkey. Our study revealed the natural yeast microbiota of these grapes by molecular methods for the first time. This is also the first research about molecular characterization of the yeast microbiota during spontaneous fermentation process of these grapes. Molecular identification and genotyping of endogenic *S. cerevisiae* and also non-*Saccharomyces* species of spontaneous wine fermentation may further lead to evaluation of them as potential candidates in mixed wine fermentations after studying their enological and technological properties.

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