Identification of Wine Related Yeast Species by Capillary Electrophoresis Single Strand Conformation Polymorphism Analysis (CE-SSCP) of the 26S rRNA Gene

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Introduction

In wine industry yeast spoilage presents a severe problem related to great economic loss. Dekkera bruxellensis is described as the most serious spoilage yeast, due to its ability to produce high amounts of volatile phenols which cause off-flavours [2]. Pichia guilliermondii has the ability to produce the same phenols with efficiencies as high as those found in D. bruxellensis [3]. Besides its beneficial effect in wine fermentation Saccharomyces cerevisiae is also able to cause spoilage after fermentation as it resists high ethanol concentrations [4]. Yeasts of the genus Zygosaccharomyces also cause spoilage in wines and among them Zygosaccharomyces bailii is considered one of the most dangerous and frequently found yeasts in spoiled food and beverages [].

It is important to detect spoilage yeasts quickly to allow wineries to intervene rapidly and effectively. In recent years there has been a great effort to develop rapid identification techniques. In comparison to traditional culture dependent methods, these new PCR-based methods allow faster detection and identification. CE-SSCP presents a powerful analysis technique that separates DNA fragments of the same length according to their sequence [6]. It is based on the heat denaturation of PCR amplified DNA where single stranded fragments are formed. These fragments are subjected to capillary electrophoresis under non denaturing conditions, where they form folded conformations due to their sequence. In this study, a CE-SSCP assay was developed based on a 164 bp fragment of the D1/D2 domain of the 26S rRNA gene in order to distinguish between various wine yeasts.

Materials and Methods

Yeast strains

Genomic DNA of 22 wine related yeast species was used. The strains were obtained from the Instituto Superior de Agronomia, Portugal (ISA), the Portuguese Yeast Culture Collection, Portugal (PYCC), the American Type Culture Collection, USA (ATCC), the Centraalbureau voor Schimmelcultures, The Netherlands (CBS) and the Colección Española de Cultivos Tipo, Spain (CECT).

DNA isolation

Total yeast genomic DNA was isolated from cultures grown in 1 ml YPD medium for 48 hours at 30 ºC. Cells were harvested by centrifugation and DNA isolation was performed as previously described Yeast cells were cultivated in 5 ml of YPD medium (24 h, 28ºC, 160 rpm) and DNA isolation was performed using a previously described method [1]. DNA was quantified, diluted to 20 ng/µl using 10 mM TrisHCl 1 mM EDTA pH 8,0 and stored at -80ºC until use.

Primers and PCR amplification

Based on sequence polymorphisms of the D1/D2 domain of the 26 S rDNA primers were designed for the amplification of a 164 bp fragment (SSCPF and SSCPR). The 5' ends of the forward and reverse primer were labelled with the fluorescent dyes HEX and 6-FAM, respectively.

The PCR reactions contained 20 ng template DNA, 0,4 µM of each primer, 2 mM $MgCl_2$, 0,2 mM of each deoxynucleoside triphosphate, 1x Taq polymerase buffer (MBI Fermentas) and 0,5 U Taq polymerase (MBI Fermentas), all adjusted to a final volume of 6 µl with MilliQ water. PCR was carried out in a icycler thermal cycler (Biorad). An initial denaturation step of 94 ºC for 4 min was followed by 36 cycles of 30 sec at 94 ºC, 30 sec at 50 ºC and 30 sec at 72 ºC, with a final extension at 72ºC for 10 min.

Correct amplification was checked by agarose gel electrophoresis. Samples were stored at -20 ºC until CE-SSCP analysis.

Primer	SSCPF	SSCPR
Position in D1/D2 domain (S. cerevisiae) [bp]	78 - 95	225 – 242
Sequence (5' – 3')	CGAGTTGTAATTTGGAGA	TACCACCCACTTAGAGGCT
5' - fluorochorme	HEX	6 –FAM

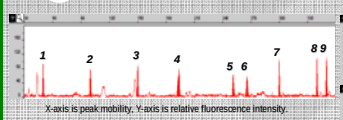
CE-SSCP data acquisition

For CE-SSCP data acquisition PCR products were diluted 1:40 in ultrapure sterile water. 1 µl diluted PCR product was combined with 11,25 µl deionized formamide, 0,25 µl internal DNA molecular weight standard Genescan-500 TAMRA (PE, Applied Biosystems) and 0.5 µl 0.3 N NaOH. Samples were mixed thoroughly and incubated at 95 ºC for 5 min, followed by incubation on ice for denaturation and subsequent formation of single stranded conformations.

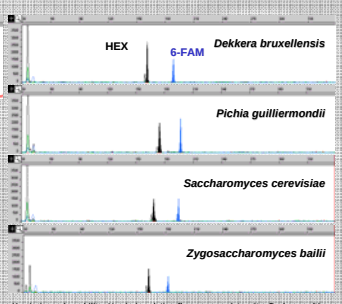
CE-SSCP analysis was performed using an ABI Prism 310 genetic analyzer (PE Applied Biosystems) with a 47 cm length, 50 µm inner diameter capillary. The non denaturing polymer used consisted of 3 % (w/w) Gene Scan Polymer (PE Applied Biosystems) and 10 % (w/w) glycerol. As electrophoresis buffer 1x TBE containing 10 % glycerol was used. The injection time and voltage were set to 5 sec and 15 kV respectively, while electrophoresis voltage was 12 kV. The syringe pump time was 300 sec. and data collection was performed during 25 min. As the electrophoresis temperature has a great influence on the mobility values, analysis was carried out at three different constant temperatures (35, 30 and 25 ºC), in order to compare the results. A matrix file was created following the manufacturer's instructions to account for spectral overlap of the various fluorescent molecules. To obtain comparable results, Gene Scan Analysis Software 3.5 (PE Applied Biosystems) was used.

RESULTS

1 CE-SSCP data analysis



X-axis is peak mobility, Y-axis is relative fluorescence intensity.



X-axis is peak mobility, Y-axis is relative fluorescence intensity. Peaks resulting from PCR amplification with the labeled primers SSCPF and SSCPR, showed as filled peaks, were assigned mobility values relative to the internal standard (not visible at this scale).

- Mobility values of the internal standard Genescan – 500 TAMRA (red dye) at 25ºC, were assigned to peaks 1-9 of the internal standard, corresponding to 50, 100, 150, 194, 251, 266, 300, 340 and 350.
- The software then aligned the sample peaks in reference to the internal standard peaks (red peaks resulting from TAMRA labeled standard) using the Global Southern Alignment method.
- The four species analyzed in this preliminary study can be clearly distinguished by their peak patterns.

2 Intraspecific variation and influence of electrophoresis temperature on the mobility values

Species	Strain	35ºC HEX	35ºC 6-FAM	30ºC HEX	30ºC 6-FAM	25ºC HEX	25ºC 6-FAM
Dekkera bruxellensis	ISA 1600	155,44	190,60	158,60	189,79	161,48	188,70
	ISA 1649	155,25	190,91	158,74	189,80	161,36	188,98
	ISA 1791	155,10	190,91	158,61	189,95	161,43	188,72
	ISA 2104	155,05	190,74	158,54	189,81	161,58	188,58
Intraspecific variation	average	155,21	190,79	158,62	189,84	161,46	188,75
	SD (+/-)	0,18	0,15	0,08	0,08	0,09	0,17
Pichia guilliermondii	ISA 2105	187,07	197,26	180,83	197,70	174,99	197,28
	ISA 2126	187,04	197,69	180,83	197,72	174,89	197,38
	ISA 2131	186,69	197,42	181,19	198,20	174,62	197,28
	ISA 2145	186,36	197,30	180,72	197,57	174,98	197,27
Intraspecific variation	average	186,79	197,42	180,89	197,80	174,87	197,30
	SD (+/-)	0,33	0,19	0,20	0,28	0,17	0,05
Saccharomyces cerevisiae	L169	176,20	193,36	174,92	193,88	168,74	194,74
	L170	175,98	193,22	174,96	194,06	168,68	194,60
	L 196	177,78	192,66	174,66	193,60	168,49	194,60
	PYCC 4455T	176,87	193,00	174,85	194,06	168,72	194,73
Intraspecific variation	average	176,71	193,06	174,85	193,90	168,66	194,67
	SD (+/-)	0,81	0,31	0,13	0,22	0,11	0,08
Zygosaccharomyces bailii	ISA 1149	161,10	189,26	159,18	185,88	162,28	182,69
	ISA 1214	160,11	191,26	158,67	188,41	161,68	184,16
	ISA 1265	161,48	188,73	159,14	185,74	162,19	182,81
	PYCC 5167T	160,87	189,06	159,25	186,15	162,33	182,80
Intraspecific variation	average	160,89	189,58	159,06	186,55	162,12	183,12
	SD (+/-)	0,58	1,14	0,26	1,25	0,30	0,70

- The influence of electrophoresis temperature on the mobility values CE-SSCP analysis was evaluated at (35, 30 and 25 ºC). A single DNA isolation, PCR amplification and CE-SSCP analysis was performed for each strain of the 4 species.
- The standard deviation decreased with the temperature and showed values of 0.05 – 0.30 at 25 ºC, compared to 0.15 – 1.14 at 30ºC. The intraspecific variation showed standard deviations of less than 1 mobility value between strains belonging to the same species. An exception is Z. bailii, that presented higher variation in the 6-FAM labeled strand at 35 and 30 ºC. This was due to strain ISA1214, that differed in mobility values from the other strains. Sequencing of the D1/D2 domain of these strains revealed that strain ISA1214 has one base pair difference in comparison to the other strains.
- Subsequent analysis were always performed at 25 ºC.

3 Reproducibility - Determination of run to run variation

Species	Strain	Average (SD) HEX	Average (SD) 6-FAM
D. bruxellensis	ISA 1600	162.08 (0.23)	187.60 (0.42)
	ISA 1649	162.00 (0.32)	187.96 (0.32)
P. guilliermondii	ISA 2105	174.55 (0.67)	196.73 (0.27)
	ISA 2126	174.54 (0.43)	196.89 (0.15)
S. cerevisiae	L 169	167.86 (0.49)	194.39 (0.14)
	L 170	168.07 (0.46)	194.28 (0.12)
Z. bailii	ISA 1265	162.38 (0.21)	182.39 (0.41)
	PYCC 4531	162.36 (0.29)	182.48 (0.30)

- A single DNA extraction and quantification was performed for two strains of each of the four species mentioned in the Table. Duplicate PCR amplifications per strain were carried out and from each PCR product two CE–SSCP samples were prepared and analyzed in three CE-SSCP runs at 25 ºC. Average and standard deviation were calculated between the results of the resulting 12 runs for each strain.
- Standard deviations were less than 1 mobility value.

4 Analysis of other wine related yeast species

Species	Strain	HEX	6-FAM
Candida cantarelli	PYCC 3073	166,21	190,06
Candida famata	PYCC 3056	161,00	189,34
		170,59	
Candida stellata	CBS 157	161,45	192,63
		177,52	197,55
Candida vanderwaltii	PYCC 3671	161,39	193,72
Candida veronae	PYCC 3664	160,81	189,15
Candida vini	ISA 1007	161,42	192,61
	PYCC 2597	161,56	192,61
Dekkera anomala	ISA 1652 Type	161,16	193,86
	IGC 5133	161,22	193,59
Dekkera bruxellensis	ISA 1600	161,48	188,70
	ISA 1700	162,13	187,82
	ISA 1703	162,08	187,70
	ISA 2117	162,09	187,71
Hanseniaspora uvarum	ISA 1189/CBS 276	184,40	193,32
	MT1/BG/10	184,40	193,32
Kluyveromyces marxianus	IGC 3886 Type/CBS 712	169,40	186,37
	ATCC 10022/CBS 6432	169,47	186,63
	IGC 3286	169,41	186,65
	IGC 2902	169,35	186,53
Lodderomyces elongisporus	ISA 1421	186,37	202,23
	ISA 1308	185,26	202,09
Metschnikowia pulcherrima	PYCC 5625	160,95	158,72
	IGC 4384	160,88	157,57
Pichia anomala	IGC 4121 Type/CBS 5759	156,05	190,69
	IGC 2495	156,02	190,51
	IGC 3294	155,91	190,49
	IGC 4380	156,04	190,64

Species	Strain	HEX	6-FAM
Pichia guilliermondii	ISA 2105	174,63	197,09
	ISA 2126	174,64	197,08
	ISA 2145	174,64	197,07
	ISA 2286	174,65	197,09
Rhodotorula mucilaginosa	IGC 5166	163,46	193,48
	IGC 4456 Type/CBS 380	163,26	195,48
Saccharomyces bayanus	IGC 4565/CBS 378	163,46	195,63
	IGC 4569/CBS 425	163,37	195,48
	IGC 4568/CBS 424	163,55	195,94
Saccharomyces cerevisiae	IGC 4455 Type/CBS 1171	168,07	194,29
	IGC 2608/CBS 1782	167,80	193,86
	IGC 3983	167,95	194,00
	IGC 3931	168,09	194,00
Saccharomyces ludwigii	ISA 1089	183,92	193,05
	ISA 1088	184,10	193,18
Saccharomyces paradoxus	IGC 4570 Type	166,63	194,88
	IGC 4576/CBS 406	166,54	195,04
	IGC 4578/CBS 5829	166,59	194,88
	IGC 4656	166,63	194,89
Saccharomycodes ludwigii	ISA 1083	155,84	190,44
Zygosaccharomyces bailii	IGC 5167 Type/ISA 1149 T	162,16	182,71
	ISA 1022/IGC 4267	162,66	182,19
	IGC 4806	162,35	182,90
	CBS 2856	162,79	182,32
Zygosaccharomyces bisporus	IGC 5335 Type	162,26	182,75
	IGC 5336	162,38	182,79
	IGC 5337	162,21	182,78
	IGC 5381	162,37	182,78
Zygosaccharomyces rouxii	PYCC 5276 Type	163,28	189,15
	IGC 3693/CBS 5714	163,32	189,09
	IGC 3694/CBS 5717	163,39	189,17

- Distinct strains of 22 wine related yeast species were analyzed. Due to the high reproducibility of the method a single DNA isolation, PCR amplification and CE-SSCP run were performed.
- It was possible to separate yeast species based on their mobility values of both strands. However, some species showed very similar mobility values: Dekkera anomala and Candida vini; Sacchromyces cerevisiae and Saccharomyces paradoxus; Zygosaccharomyces bailii and Zygosaccharomyces bisporus.
- Within the Saccharomyces sensu stricto complex it was possible to distinguish S. bayanus from S. cerevisiae and S. paradoxus, showing a difference of 3 mobility values in the HEX labeled strain.
- Candida famata, Candida stellata, Issatchenkia orientalis and Schizosaccharomyces pombe strains showed a more complex peak pattern consisting of several peaks. This might be due to several stable single strand conformations for both strands.
- Metschnikowia pulcherrima was the only species where the HEX labeled strand showed higher mobility values than the 6-FAM labeled strand.

Conclusions

We identified a 164 bp fragment inside the D1/D2 domain of the 26S rDNA, that shows sufficient nucleotide divergence among species and can be used for the distinction of wine related yeast species based on their CE-SSCP mobility values.

The range of mobility values (156 – 187 for primer SSCPF and 158 - 202 for primer SSCPR) creates a sufficiently high number of combinations for the unequivocal distinction of 16 from 22 wine related yeast species. However, three pairs of species (Dekkera anomala and Candida vini; Sacchromyces cerevisiae and Saccharomyces paradoxus; Zygosaccharomyces bailii and Zygosaccharomyces bisporus) were not distinguishable.

Intraspecific standard deviations increased with the capillary electrophoresis temperature. At 25ºC, standard deviations associated to run-to-run variations, evaluated for 8 strains, were less than 0.7 mobility values, showing that CE-SSCP is a reproducible and portable method for wine yeast identification.

References

[1] López V., Querol A., Ramón D., and Fernández-Espinar M.T. 2001. Int J Food Microbiol 68:75-81

[2] P. Chatonnet, D. Dubourdieu, J. Boidron, M. Pons. 1992. J Science Food Agriculture 60: 165-178

[3] L. Dias, S. Dias, T. Sancho, H. Stender, A. Querol, M. Malfeito-Ferreira, V. Loureiro. 2003. Int J Food Microbiol 20: 567-574

[4] P. Martorell, A. Querol, M. T. Fernández-Espinar. 2005. Appl Environ Microbiol 71: 6823-6830

[5] B. Esteve-Zardoso, T. Zorman, C. Belloch, A. Querol. 2003. Systematic and Applied Microbiology 26: 404 - 411.

[6] C. C. Tebbe, A. Schmalenberger, S. Peters, F. Schweiger. 2001. Environmental Molecular Microbiology, UK, 161-175

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