

# IDENTIFICATION OF WINE RELATED YEAST SPECIES BY CAPILLARY ELECTROPHORESIS SINGLE- STRAND CONFORMATION POLYMORPHISM

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## Introduction

The yeast species *Dekkera bruxellensis* is the major cause of red wine spoilage worldwide due to the production of high amounts of volatile phenols (4-ethylphenol and 4-ethylguaiacol), [1]. Predominating spoilage yeasts of high-alcohol beverages are also *Saccharomyces* spp., *Zygosaccharomyces bailii* and *Saccharomyces ludwigii* [2].

In recent years, rapid and very sensitive identification techniques, mostly PCR-based, have been developed and applied for spoilage yeast detection in wine, such as nested PCR, AFLP, RT-PCR, FISH, PCR-RFLP and TGGE [3]. Capillary Electrophoresis Single Strand Conformation Polymorphism (CE-SSCP) is a powerful analysis technique that separates heat denaturated DNA fragments of the same length according to their sequence. This technique presents very high sensitivity, detecting differences up to one base pair [4]. CE-SSCP has been applied in studies such as profiling of bacteria and yeast communities in raw milk Salers cheeses [5], but has not been used so far for wine yeast analysis. The aim of the present study was to develop and validate a new identification method, capable to distinguish between various wine yeasts, including the most relevant spoilage yeast species.

## 2. Materials and Methods

The 66 yeast strains (24 species) used in the present study are shown in Table 1. DNA isolation was performed using a previously described method [6]. From multiple sequence alignments of the D1/D2 domains of the 24 species a 164 bp region was chosen and amplified by primer pair NL5 (5'CGAGTTGTAATTTGGAGA-3')-NL6 (5'TACCACCCACTTAGAGCT3'). The 5' ends of NL5 and NL6 were labeled with the fluorescent dye HEX and 6-FAM, respectively. PCR reactions contained 20 ng

template DNA, 0.4 mM of each primer, 2 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP, 1x Taq polymerase buffer (MBI Fermentas) and 0.5 U Taq polymerase (MBI Fermentas). PCR conditions were as follows: initial denaturation at 94 °C for 4 min, followed by 36 cycles of 30 sec at 94 °C, 30 sec at 50°C and 30 sec at 72 °C, with a final extension at 72 °C for 10 min. PCR products were diluted (1:40) and 1 µl was combined with 11.25 µl deionized formamide, 0.25 µl DNA molecular weight standard Genescan-500 (PE, Applied Biosystems) and 0.5 µl 0.3 N NaOH. Samples denaturated at 95°C for 5 min and CE-SSCP analysis was performed at 25°C on an ABI Prism 310 genetic analyzer [7] using Gene Scan Analysis Software.

### **3. Results and Discussion**

CE-SSCP analyses were applied to a collection of 21 mostly wine-related yeast species, including also taxonomically close species. As summarized in Table 1, it was possible to separate 19 of 21 species based on their combined mobility values. Sequencing of the 164 bp fragment revealed between one and 67 base pair differences among clearly distinguishable species (data not shown). CE-SSCP analyses clearly distinguished the closely related *Saccharomyces sensu stricto* species *S. bayanus*, *S. cerevisiae* and *S. paradoxus*, that showed mobility values of 163/195, 168/194 and 167/195 in the HEX/6-FAM labelled strands, respectively. Two pairs of species (*D. anomala* and *Candida vini*; *Hanseniaspora uvarum* and *Saccharomyces ludwigii*) presented very similar/identical mobility values (161/194 and 161/193; 184/193 and 184/193 respectively, in the HEX/6-FAM labelled strands) and cannot be distinguished by CE-SSCP. With the exception of one *Z. bailii* strain, no base pair differences were found among multiple strains of a species. *Candida famata* and *Candida stellata* showed a more complex pattern consisting of multiple peaks, possibly due to the formation of several stable single strand conformations for both strands. With the exception of

*Metschnikowia pulcherrima*, the HEX labelled strand showed lower mobility values than the 6-FAM labelled strand. The mobility values of the spoilage yeasts *D. bruxellensis* and *Z. bailii* can be clearly distinguished from *S. cerevisiae* (162/188, 162/182, and 168/194, in the HEX/6-FAM labelled strands, respectively).

Intraspecific variation showed standard deviations of less than one mobility value between multiple strains of the same species. Standard deviation decreased with the temperature and showed values of 0.05 – 0.30 at 25 °C, compared to 0.15 – 1.14 at 35°C, which is in agreement with data from literature [7]. Variations derived from PCR amplifications, CE-SSCP sample preparations and repeated injections were between 0.1 and 0.7 mobility values (data not shown), corresponding to the values determined for inter-strain variation and indicating the high reproducibility of the method. Our results show that CE-SSCP can be successfully applied to analyze wine related yeast species, leading to highly reproducible results.

## References

- [1] Chatonnet P, Viala C, Dubourdieu D (1997) Am J Enol Vitic 48:443-448
- [2] du Toit M, Pretorius IS (2000) S Afr J Enol Viticult 21:74-96
- [3] Fernández-Espinar MT, Martorell P, de Llanos R, Querol A (2006) in: Querol A and Fleet GH (Eds.) Yeasts in Food and Beverages. Springer Verlag Germany, pp. 55-82
- [4] Tebbe CC, Schmalenberger A, Peters S, Schweiger F (2001) in: Rochelle, P. A. (Ed.), Environmental Molecular Microbiology: Products and Applications. Horizon Scientific Press, Wymondham, UK, pp 161-175
- [5] Callon C, Delbes C, Duthoit F, Montel M-C (2006) Syst Appl Microbiol 29:172-180
- [6] Lopez V, Querol A, Ramon D, Fernandez-Espinar MT. 2001. Int J Food Microbiol 68: 75-81
- [7] Duthoit F, Godon JJ, Montel M-C (2003) Appl Environ Microbiol 69:384-3848

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Table 1 - CE-SSCP mobility values for HEX/6-FAM labelled DNA strands of 60 yeast strains belonging to 21 species. Yeast strains were obtained from American Type Culture Collection (ATCC); Portuguese Yeast Culture Collection (PYCC) and Instituto Superior de Agronomia, Portugal (ISA).

Species	Strain	HEX	6-FAM	Species	Strain	HEX	6-FAM
<i>Candida cantarelli</i>	PYCC3073	166.21	190.06		ISA2126	174.64	197.08
	PYCC3056	161.00	189.34	<i>Pichia guilliermondii</i>	ISA2145	174.64	197.07
<i>Candida famata</i>		170.59			ISA2286	174.65	197.09
	CBS157	161.45	192.63		PYCC4456T	163.26	195.48
<i>Candida stellata</i>		177.52	197.55	<i>Saccharomyces bayanus</i>	PYCC4565	163.46	195.63
<i>Candida veronae</i>	PYCC3664	160.81	189.15		PYCC4569	163.37	195.48
	ISA1007	161.42	192.61		PYCC4568	163.55	195.94
<i>Candida vini</i>	PYCC2597	161.56	192.61		PYCC4455T	168.07	194.29
	ISA1652T	161.16	193.86		PYCC2608	167.80	193.86
<i>Dekkera anomala</i>	PYCC5133	161.22	193.59	<i>Saccharomyces</i>	PYCC3983	167.95	194.00
	ISA1600	161.68	188.30	<i>cerevisiae</i>	PYCC3931	168.09	194.00
	ISA1649	162.00	187.96		L169	167.86	194.39
<i>Dekkera bruxellensis</i>	ISA1700	162.13	187.82		L170	168.07	194.28
	ISA1703	162.08	187.70		ISA1089	183.92	193.05
	ISA2117	162.09	187.71	<i>Saccharomyces ludwigii</i>	ISA1088	184.10	193.18
	ISA1189	184.40	193.32		PYCC4570T	166.63	194.88
<i>Hanseniaspora uvarum</i>	MT1/BG/10	184.40	193.32		PYCC4576	166.54	195.04
	PYCC3886T	169.40	186.37	<i>Saccharomyces paradoxus</i>	PYCC4578	166.59	194.88
	CBS6432	169.47	186.63		PYCC4656	166.63	194.89
<i>Kluyveromyces marxianus</i>	PYCC3286	169.41	186.65		ISA1083	155.84	190.44
	PYCC2902	169.35	186.53		PYCC5167T	162.16	182.71
<i>Lodderomyces elongisporus</i>	ISA 1421	186.37	202.23		ISA1022	162.66	182.19
	ISA1308	185.26	202.09	<i>Zygosaccharomyces bailii</i>	PYCC4806	162.35	182.90
<i>Metschnikowia pulcherrima</i>	PYCC5625	160.95	158.72		CBS2856	162.79	182.32
	PYCC4384	160.88	157.57		ISA1265	162.38	182.39
	PYCC4121T	156.05	190.69		PYCC4531	162.36	182.48
	PYCC2495	156.02	190.51		PYCC5335T	162.26	182.75
<i>Pichia anomala</i>	PYCC3294	155.91	190.49	<i>Zygosaccharomyces bisporus</i>	PYCC5336	162.38	182.79
	PYCC4380	156.04	190.64		PYCC5337	162.21	182.78
<i>Pichia guilliermondii</i>	ISA 2105	174.63	197.09		PYCC5381	162.37	182.78