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 *Saccharomycodes 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 $MgCl_2$, 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 ºC for 4 min, followed by 36 cycles of 30 sec at 94 ºC, 30 sec at 50ºC and 30 sec at 72 ºC, with a final extension at 72 ºC 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 95ºC for 5 min and CE-SSCP analysis was performed at 25ºC 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 ºC, compared to 0.15 – 1.14 at 35ºC, 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

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