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Molecular typing of the yeast species *Dekkera bruxellensis* and *Pichia guilliermondii* recovered from wine related sources

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Abstract

A total of 63 strains of *Dekkera bruxellensis* and 32 strains of *Pichia guilliermondii* isolated from wine related environments were identified by restriction analysis of the 5.8S-ITS region of the rDNA. These strains were subjected to intraspecific discrimination using mtDNA restriction and RAPD-PCR analysis.

The isolates identified as *D. bruxellensis* yielded 3 different molecular patterns of mtDNA restriction using the endonuclease *Hin*fI. The pattern A was the most frequent (58 strains) among strains from different sources, regions and countries. Pattern B (4 strains) and C (one strain) were determined in isolates from Portuguese wines. The discrimination among the pattern A strains was achieved by a RAPD-PCR assay with 3 primers (OPA-2, OPA-3 and OPA-9). A total of 12 haplotypes were obtained with the combination of the patterns provided by the 3 OPAs. The pattern 2 was the most frequent and extensively distributed being found in strains from different countries and from different sources like wine, barrique wood and insects.

The strains of *P. guilliermondii* were characterized with restriction of mtDNA using the endonuclease *Hin*fI yielding 7 different restriction patterns. These patterns were associated with different efficiencies of 4-ethylphenol production. Patterns A to D corresponded to 19 strains producing low levels of 4-ethylphenol (<1 mg/l) while patterns F and G grouped 13 strains producing high levels of 4-ethylphenol (>50 mg/l), when grown in synthetic media supplemented with 100 mg/l of *p*-coumaric acid.

The high degree of polymorphism observed shows that intraspecific typing is essential for accurate yeast dissemination studies in wine related environments.

Keywords: *Dekkera bruxellensis*; *Pichia guilliermondii*; Molecular typing; 4-Ethylphenol; Wine

1. Introduction

In wine industry, although lactic and acid bacteria have been described as important spoilers (Sponholz, 1992; Ribéreau-Gayon et al., 2000), yeasts are now regarded as the most feared contaminants in wine. Their spoilage effects are film formation in stored wine, cloudiness, sediments and gas production in bottled wines, and off-odors and off-tastes during wine production and bottled wines (Loureiro and Malfeito-Ferreira, 2003).

The yeasts of the genus *Dekkera* (*Brettanomyces*, imperfect form) are described as the most serious spoilage yeasts in red wines, because of their ability to produce high amounts of volatile phenols (4-ethylphenol and 4-ethylguaiacol) imparting off-flavors to red wines (Chatonnet et al., 1992, 1995, 1997). Among the species of this genus, *Dekkera bruxellensis* is the most representative in wines (Mitrakul et al., 1999; Rodrigues et al., 2001). In addition, it has been found that other species are capable of producing volatile phenols (Dias et al., 2003b). Among these, *Pichia guilliermondii* has the ability to produce 4-ethylphenol with efficiencies as high as those observed in *D. bruxellensis* (Dias et al., 2003b). The risk of wine contamination by these yeasts thus justifies the effort to develop rapid identification techniques. Several molecular-based methodologies have been described for a rapid detection and identification of *Dekkera/Brettanomyces*, such as nested PCR, AFLP, RT-PCR, PCR-RFLP, and fluorescence in situ hybridization

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(Ibeas et al., 1996; Barros-Lopes et al., 1999; Egli and Henick-Kling, 2001; Stender et al., 2001; Dias et al., 2003b; Cocolin et al., 2004). For *P. guilliermondii* the restriction analysis of 5.8S-ITS region has been used a rapid method for its identification (Esteve-Zarzoso et al., 1999; Dias et al., 2003b).

Added to species identification, there is a need for differentiating yeast isolates at intraspecific level, which could be very helpful to establish the origin of wine spoilage yeasts, their routes of contamination and the critical points of yeast infection (Loureiro and Malfeito-Ferreira, 2003). Several techniques have been described to type *Dekkera/Brettanomyces* at intraspecific level. A RAPD-PCR technique was applied for strain discrimination in *D. bruxellensis* (Mitrakul et al., 1999). In this work the author found a low level of variability among the strains from culture collections and from only one winery by revealing two different patterns. Mitochondrial DNA (mtDNA) polymorphisms have been extensively used to detect genetic variability in yeast populations of *Saccharomyces* (Querol et al., 1992; Guillamón et al., 1994) including the genus *Brettanomyces* (Ibeas et al., 1996). These authors found variability using RFLP's of mtDNA restriction analysis (RFLP's of mtDNA) but with a lower degree of polymorphism when compared with *S. cerevisiae*. Furthermore, the characterization at intraspecific level in *P. guilliermondii* has not been described.

In the present study, we have applied RFLP's of mtDNA analysis and RAPD-PCR in order to type strains belonging to *D. bruxellensis* and *P. guilliermondii*, isolated from a wide variety of sources related with wine production and from different geographic areas. In addition, a relationship was established between the efficiency of 4-ethylphenol production by *P. guilliermondii* and the polymorphisms of mtDNA.

2. Materials and methods

2.1. Yeast strains and maintenance

A total of 95 strains of *D. bruxellensis* and *P. guilliermondii*, obtained by us or supplied by Évora University culture collection, isolated from different sources and wineries, were included in this study (Table 1). The strains were maintained in GYP medium (20 g/l glucose, 10 g/l peptone, 5 g/l yeast extract and 20 g/l agar, pH 6.0) and in the case of *D. bruxellensis* strains, 5 g/l of calcium carbonate was added to GYP medium. All components were from Sigma (St. Louis, MO, USA). Growth and release of phenolic smell was assessed for all strains using DBDM agar as described by Rodrigues et al. (2001).

2.2. Production of volatile phenols

A loopful of fresh culture (24–48 h) was suspended in Ringer solution and used to inoculate the YNB medium (Difco) (6.7 g/l) supplemented with glucose (20 g/l) and *p*-coumaric acid (100 mg/l) (Sigma Chemical Co., St. Louis, USA), adjusted to pH 5.4 and filter sterilized. Incubation was carried out at 25 °C, with orbital shaking, for a maximum of 7 days. Volatile phenols were measured according to a protocol described by Rodrigues et al. (2001). Briefly, the volatile phenols were extracted by ether-hexan from a 50-ml sample with pH adjusted to 8 with NaOH. The volatile phenols were separated by collecting the organic phase of the mixture. The quantitation was achieved by gas chromatography using a DB-Wax capillary column (J&W Scientific, Folsom, California, USA). Results are the average of at least two independent experiments.

Table 1
Origin of analysed strains

Species[a]	Strains	Source[b]
Dekkera bruxellensis	400, 401, 402, 405, 411, 416, 418, 419, 423, 531, 532, 533, 534, 536, 537, 538, 539, 540, 541, 542, 543, 544, 545, 546, 548	Barrique wood, winery 6
	2101, 2102	Red wines, winery 6
	NP22, NP24, NP26, NP27	*Drosophila*, winery 6
	2172	Red wine (Spain)
	2173, 2174	Bottled red wine (California, USA)
	2288, 2287	Red wine (Utiel-Requena, Spain)
	1327, 1328, 1601, 1600	Sparkling white wine (Ribatejo, Portugal), isolated in 1991 and 1994
	1792, 1794	Red wines (ESB, Portugal)[c]
	1700, 1701, 1702, 1703, 1704, 1791, 2127, 2128, 2129, 2130	Red wines from different wineries (Dão, Portugal)
	1717	White wine (Estremadura, Portugal)
	2113	Bottled red wine, winery 11
	2132, 2133	Red wine in barrel, winery 11
	2104	Red wine, winery 3
	2114, 2115, 2117, 2118, 2120, 2121	Red wines from different wineries (Ribatejo, Portugal)
Pichia guilliermondii	407, 408, 409, 410, 412, 413, 414, 421, 422, 420, 430b	Barrique wood, winery 6
	2106, 2119, 2134, 2135, 2136	Grapes, winery 6
	2131	Red wine, winery 6
	2137, 2138, 2139	Stems, winery 6
	2141	*Drosophila*, winery 10
	2142	Grapes, winery 10
	2143	Stems, winery 10
	2145	*Drosophila*, winery 5
	2105, 2122	Grapes, winery 5
	2125	Pump outlet, winery 5
	2126	Press roll, winery 5
	2107, 2108, 2109	*Drosophila*, winery 1
	2110	Red wine, winery 4

[a] Identification by 5.8S-ITS restriction.
[b] Wineries 1, 3, 4, 5, 6, 10 and 11 from region Alentejo (Portugal), strains supplied by Universidade de Évora, Portugal.
[c] Supplied by Escola Superior de Biotecnologia, Portugal.

2.3. Identification by 5.8S-ITS analysis

The 5.8S-ITS rDNA region was amplified using the primers ITS1 and ITS4 (Esteve-Zarzoso et al., 1999). Cells were

collected from a fresh colony and resuspended in the PCR mixture. The suspension was heated in a Progene (Techne, Cambridge, UK) termocycler at 95 °C for 15 min. Then, one unit of DyNAzyme™ II DNA Polymerase (Finnzymes OY, Espoo, Finland) was added to each tube. PCR conditions were 40 cycles of denaturation at 94 °C for 1 min, annealing at 55.5°C for 2 min and extension at 72°C for 2 min. Finally, an extension cycle at 72 °C for 10 min. was added. The amplified DNA (10 µl or 0.5–10 µg of DNA) was digested with three restriction endonucleases, *Hin*fI, *Cfo*I and *Hae*III (Roche Molecular Biochemicals, Mannheim, Germany), according to the supplier's instructions. The amplified PCR product and their corresponding restriction fragments were separated on 1.4% and 3% agarose gels, respectively. Sizes of the fragments were estimated by comparing their mobility with a 100-bp DNA length standard (GIBCO-BRL, Gaithersburg, MD, USA).

2.4. DNA extraction and mtDNA restriction analysis

DNA extraction and determination of mtDNA restriction patterns were carried out according to Querol et al. (1992) with some modifications as described in López et al. (2001). Yeast cells were grown in an overnight culture of 5 ml of GPY at 28 °C. After centrifugation, cells were suspended in 1 M sorbitol–0.1 M EDTA. A solution of Zymolyase 20T (1 mg/ml) and Novozym (1 mg/ml) was added to obtain the spheroplasts. DNA samples were digested using the restriction endonuclease *Hin*fI (Roche Molecular Biochemicals, Mannheim, Germany). Mitochondrial restriction fragments were separated on 1% agarose gels in 1× TAE buffer, stained with ethidium bromide and visualized under UV light.

2.5. RAPD-PCR assay

RAPD profiles were obtained using 3 decamer primers (OPA 2, OPA 3, and OPA 9) from Operon Technologies. PCR reactions were performed in 25 µl reactions containing 100 ng of DNA, 3.5 mM $MgCl_2$, 200 ng of primer, 25 ìM of each dNTP and 1 U of Biotools DNA polymerase (Biotools B&M Labs S.A., Madrid, Spain). The amplification reactions were carried out in a Progene (Techne) thermocycler under the following conditions: initial denaturation at 94 °C for 5 min, followed by 45 cycles of 92 °C for 1 min, 36 °C for 1 min, 72 °C for 2 min, and a final extension at 72 °C for 10 min. Reaction products were analyzed by electrophoresis through 2% agarose gels in 1× TAE and stained with ethium bromide. The molecular sizes of DNA fragments were obtained with comparison with 100 pb molecular marker.

3. Results

3.1. Identification of yeasts producing 4-ethylphenol

All the tested strains were able to grow in DBDM medium and to release a phenolic smell as a qualitative indicator of 4-ethylphenol production according to Rodrigues et al. (2001) and Dias et al. (2003b). These strains were identified by restriction analysis of 5.8S-ITS region. A total of 63 strains were identified as *D. bruxellensis* and 32 strains as *P. guilliermondii* (results not shown).

The production of 4-ethylphenol by *D. bruxellensis* and *P. guilliermondii* strains was quantitatively determined by gas chromatography. The strains belonging to *D. bruxellensis* were all able to yield high levels of 4-ethylphenol (more than 50 mg/l, data not shown). However, in *P. guilliermondii*, different levels of this compound were obtained among the strains tested (see Table 3). It was possible to distinguish 2 different groups of strains depending on the level of 4-ethyphenol production. One group showed a range from 0.1 to 1 mg/l and included strains isolated from different sources like barrique wood, grapes, press roll, insects, pump outlet and wine. On the other hand, a group of strains showed the ability to produce high amounts of 4-ethylphenol, ranging from 51.0 to 68.9 mg/l. These strains were isolated from barrique wood, wine, grapes, stems and insects, in different wineries.

3.2. Molecular characterization of D. bruxellensis isolates

The isolates which grew in DBDM medium and were identified as *D. bruxellensis* by restriction analysis of 5.8S-ITS, were subsequently analyzed at strain level. A mtDNA restriction analysis using the endonuclease *Hin*fI was applied to the total of the isolates in order to characterize them. Three different molecular patterns were obtained (Fig. 1a). The pattern A was the most frequent (Table 2), and was determined in a total of 58 strains. These strains were isolated from different sources (barrique wood, red, white and sparkling wine, insects), different geographic areas within a country (Alentejo, Dão, Estremadura and Ribatejo, in Portugal) and different countries (Portugal, Spain and USA). Pattern B was

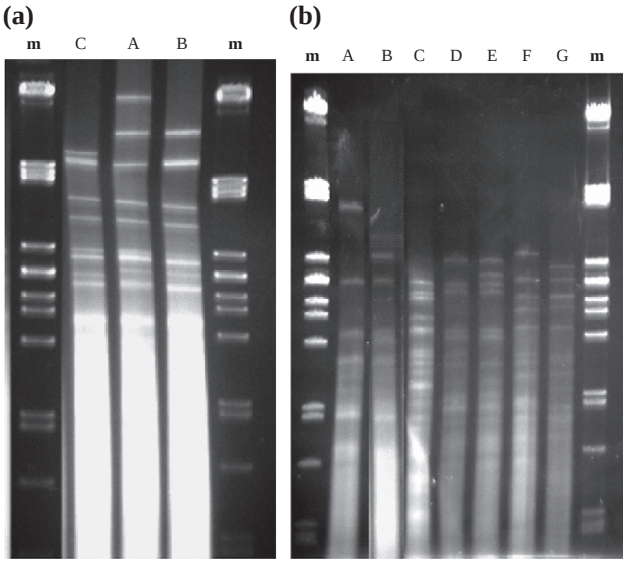

Fig. 1. MtDNA restriction patterns obtained with *Hin*fI in (a) *D. bruxellensis* and (b) *P. guilliermondii* strains. Molecular marker: Lambda DNA digested with *Pst*I.

Table 2
Pattern types obtained by RFLP mtDNA and RAPD composite patterns for the *Dekkera bruxellensis* strains analysed in the present study

Strain	MtDNA/*Hin*fI	RAPD			Haplotype (RAPDs)
		OPA 2	OPA 3	OPA 9	
1327, 1328, 1601	A	1	1	1	1
1792, 2102, 2114, 2117, 2118, 2128, 2129, 2130, 2173, 2174, 544, 545, 546, 548, NP26, NP27	A	2	1	2	2
1700, 1704	A	2	2	3	3
1701, 1702, 1703, 2127, 2288, 2287, 531, 532, 533, 534, 536, 537	A	2	2	4	4
1717	A	3	2	5	5
2113, 538, 539, 540, 541, 542, 543, NP22, NP24	A	2	1	6	6
2132, 2133	A	4	3	4	7
2101	A	1	1	4	8
2115	A	4	1	4	9
2120	A	4	4	4	10
400, 401, 402, 405, 411, 416, 418	A	5	5	4	11
419, 423, 2172	A	5	1	4	12
1600, 1794, 2104, 2121	B	–	–	–	–
1791	C	–	–	–	

determined in 4 strains, isolated from wines in different wineries in Portugal. Finally, only 1 strain, isolated from wine in Dão region, showed the pattern C (see Table 2). Despite the low variability obtained with this technique, in some cases it was possible to discriminate between strains taken from different samples, wineries or vintage years. Thus, in Dão region was determined a strain with pattern C isolated from wine, while the rest of strains isolated within this region showed the pattern A. In Alentejo, the pattern A was found in wineries 6 and 7, while in winery 3 the unique strain included in this study showed the pattern B. Finally, in Ribatejo, patterns A and B were also determined in strains isolated from wines. In this region, the sparkling wine was spoiled by cloudiness, and the pattern A was recovered in samples from 1991 (strains ISA 1327 and 1328) and 1994 vintages (strain ISA 1601).

As shown before, we obtained a low variability in population of *D. bruxellensis* isolates when the mtDNA analysis was applied. Consequently, in order to discriminate among the 58 strains that showed the pattern A, we applied a RAPD-PCR assay with 3 different primers: OPA-2, OPA-3 AND OPA-9. According to Mitrakul et al. (1999), OPA-9 was the best test to discriminate strain from culture collections. With this technique, we obtained higher variability than with mtDNA restriction analysis. All the oligonucleotides tested were useful to strain discrimination, and we could determinate 5 different patterns with OPA-2 and OPA-3, and 6 patterns with OPA-9. However, better resolution was obtained when the haplotypes were determined. A total of 12 haplotypes were obtained with the combination of the patterns provided by the three OPAs. The pattern 2 was the most frequent and extensively distributed (16 strains). Thus, it was found in strains from different regions in Portugal and USA, and from different sources like wine, barrique wood and insects. Twelve strains isolated from Portuguese and Spanish wines, and from barrique wood, showed the pattern 4. Patterns 6 and 11 were also quite frequent, determined in 9 and 7 strains, respectively, but limited to wineries 6 (15 strains, including 13 from barrique wood) and 11 (one strain) in Alentejo (Portugal). The other patterns were not so frequent and some of them were only represented by: (i) one strain (patterns 5, 8, 9 and 10); (ii) 2 (pattern 7) or 3 strains (pattern 1) from the same winery; (iii) 2 strains from 2 different wines of the same region (pattern 3); (iv) 3 strains from two different countries (pattern 12).

Strain discrimination with RAPD-PCR, was useful to establish differences within a winery. Concerning winery 6 because of its higher diversity of sources, we detected pattern 2 in wine, insect and barrique wood, pattern 6 in insect and barrique wood. In particular, strains recovered from barrique wood showed 5 different haplotypes (2, 4, 6, 11, 12). Pattern 11 was present exclusively in barrique wood strains while patterns 2, 4, 6 and 12 were also determined in strains from Portuguese, Spanish and American wines.

Table 3
Production of 4-ethylphenol (4-EP) and pattern types obtained by RFLP mtDNA for the *Pichia guilliermondii* strains analysed in the present study

Strain	4-EP (mg/l)[a,b]	MtDNA/*Hin*fI	Origin
407	0.9	A	Barrique wood, winery 6
408	0.9	B	Barrique wood, winery 6
409	0.7	A	Barrique wood, winery 6
410	0.6	C	Barrique wood, winery 6
412	0.3	A	Barrique wood, winery 6
413	0.6	D	Barrique wood, winery 6
414	1.0	A	Barrique wood, winery 6
421	0.7	B	Barrique wood, winery 6
422	0.1	B	Barrique wood, winery 6
420	0.7	A	Barrique wood, winery 6
430b	51.0	G	Barrique wood, winery 6
2105	61.5	F	Grapes, winery 5
2106	0.5	E	Grapes, winery 6
2107	0.5	A	*Drosophila*, winery 1
2108	0.4	A	*Drosophila*, winery 1
2109	0.4	A	*Drosophila*, winery 1
2110	0.5	A	Red wine, winery 4
2119	0.3	E	Grapes, winery 6
2122	52.4	F	Grapes, winery 5
2125	0.4	B	Pump outlet, winery 5
2126	53.2	F	Press roll, winery 5
2131	63.9	F	Wine, winery 6
2134	65.3	F	Grapes, winery 6
2135	60.3	G	Grapes, winery 6
2136	64.4	F	Grapes, winery 6
2137	68.7	G	Stems, winery 6
2138	61.7	F	Stems, winery 6
2139	57.5	F	Stems, winery 6
2141	55.4	G	*Drosophila*, winery 10
2142	68.9	F	Grapes, winery 10
2143	65.3	F	*Drosophila*, winery 5
2145	56.4	G	Stems, winery 10

[a] Maximum production of 4-ethylphenol (mg/l) in synthetic medium added of 100 mg/l of *p*-coumaric acid.
[b] Data for strains 2105–2110 and 2122–2126 were obtained from Dias et al. (2003b).

3.3. Characterization of *P. guilliermondii* isolates

A total of 32 strains of *P. guilliermondii* were characterized with restriction of mtDNA using the endonuclease *Hin*fI. The isolates were obtained from different wineries only in Alentejo (Portugal) and showed a high variability of mtDNA, being possible to determinate 7 restriction patterns (Fig. 1b). This high level of variability was also determined in isolates within the same winery (Table 3). This is the case of the strains from barrique wood (winery 6), with 5 different patterns (A, B, C, D and G). Three patterns (E, F and G) were also determined in other sources from winery 6, and two patterns in winery 5 (F and B) and 10 (F and G). The patterns A, F and G were the most common, distributed in 4 wineries included in this study.

In addition, the isolates belonging to *P. guilliermondii* were tested in order to know their ability to produce 4-ethylphenol. A group of isolates yielded low levels of 4-ethylphenol (<1 mg/l), while 13 strains produced high levels of 4-ethylphenol (>50 mg/l). In the case of *P. guilliermondii*, it was possible to find a relation between the mtDNA restriction pattern and the level of 4-ethylphenol production. The strains producing high levels of 4-ethylphenol shared the patterns F and G were while the strains with patterns A, B, C, and D yielded low amounts of this compound. Concerning the strains with higher production of 4-ethylphenol, they were isolated from several sources in 3 wineries. In winery 6, the pattern F was found in 4 strains isolated from grapes and stems and in one strain recovered from wine. In winery 5, the pattern F was determined in 2 strains from grapes, one strain from *Drosophila*, and one strain from the press roll during harvest. In wineries 6 and 10, we determined the presence of 4 strains with the pattern G, in grapes and stems. This pattern was obtained as well in one strain isolated from *Drosophila* in the winery 10.

4. Discussion

In the present study we evidenced the high degree of molecular polymorphism either in *D. bruxellensis* or in *P. guilliermondii.* The use of molecular techniques, mtDNA restriction analysis and RAPD-PCR, was very useful for strain typing in both species. In *D. bruxellensis* strains, we found low variability in mtDNA when the restriction with *Hin*fI was applied, but the use of RAPD-PCR provided higher intraspecific variability. It was possible to evidence the presence of strains with the same haplotype in barrique wood, wine and *Drosophila* isolated from the winery air. These results indicate that the contamination by this yeast may be due to flies contacting wine before barrel filling. A filtration of wine before the ageing of wine and sanitation treatments of winery equipment could be made to avoid the contamination problems. Also we could conclude from our results, the common distribution of some haplotypes (2, 4 and 12) which was found in strains distributed by different regions in Portugal, Spain and USA. Further analysis of higher number of samples would determine if the observed predominance of pattern 2 may be true also for sources from other countries. Mitrakul et al. (1999) found a lower degree of polymorphism (only 2 patterns) that may be explained by the higher number and origin diversity of isolates studied by us. Similarly, the AFLP polymorphism detected by Barros-Lopes et al. (1999) was only evidenced in 7 type strains of the former synonyms of *D. bruxellensis.*

Concerning *P. guilliermondii*, the mtDNA restriction with *Hin*fI provided a high variability among the strains. We observed 7 different patterns, which were present in strains from different sources only in wineries from Alentejo (Portugal). The most relevant patterns (F and G) were determined in strains isolated from grapes, stems, barrique wood, wine and *Drosophila*; showing that the grapes and stems could be the origin of wine contamination.

The assessment of 4-ethylphenol production was carried out in synthetic medium with glucose as sole carbon source eliciting the observation of maximum conversion rates (Dias et al., 2003a,b). All the strains of *D. bruxellensis* tested in this study were able to yield high amounts of 4-ethylphenol and so we could not confirm the existence of non-producing strains as mentioned by Fugelsang (1997). The species *D. anomala* is a known contaminant in beer production and it is also a high 4-ethylphenol producer (Dias et al., 2003b). We have never isolated this species from wines and so, in accordance to others using molecular methods (Mitrakul et al., 1999; Egli and Henick-Kling, 2001; Stender et al., 2001; Cocolin et al., 2004) *D. bruxellensis* appears to be the sole representative of this genus in wines. In fact, some *D. anomala* strains isolated from wines and identified by classical techniques were shown to be *D. bruxellensis* (Mitrakul et al., 1999).

The work of Dias et al. (2003b) showed the different ability of *P. guilliermondii* to produce 4-ethylphenol. Our results confirmed those observations and for the first time identified highly productive strains isolated from wines. Moreover, we found a relation between the mtDNA restriction patterns and the production of 4-ethylphenol by *P. guilliermondii* strains. Only those strains with pattern F or G were high producers of 4-ethylphenol and so these patterns could be used as molecular markers of this physiological behavior among *P. guilliermondii*. The ability to produce 4-ethylphenol in real wine conditions should be further investigated in order to assess the spoilage risk associated with this species.

In conclusion, the results presented in this work show that *D. bruxellensis* and probably *P. guilliermondii* can be considered as very important spoilage yeasts in wine industry, capable to produce high amounts of 4-ethylphenol and, consequently, off flavors in wine. In addition, the use of typing techniques must be based on an intraspecific level in order to detect the presence of the yeasts producing 4-ethylphenol and to establish the dissemination routes in wine-related environments. The knowledge of critical points that are susceptible to be continued during the manufacture of wine could be very useful to establish preventive hygienic measures in the winery.

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